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SESSION 9 Panel Discussion: Quality Assurance Chairman: P.H. WHITEHEAD *

Introductory Remarks

The maintenance of a high standard of scientific inquiry into crime in every respect, from visiting the crime scene, if necessary, to giving evidence in Court, is the first obligation of any forensic scientist. It is incumbent on him to ensure that as far as is humanly possible the results of his analysis be it the examination of blood, documents, bullets, glass or other matter, attain the high standards demanded by society through the Courts. No expert witness of any length of experience would ever claim in Court that he had never made a mistake - as indeed would any other professional person - but society rightly expects the expert witness in particular to be especially vigilant in the conduct of his examination and assiduous in his pursuit of all possible causes of error.

The majority of forensic scientists in the United Kingdom are employed by "official" bodies such as the Home Office (the government department responsible for 'home affairs'), or police authorities such as Strathclyde in Scotland, or the Metropolitan Police in London. Relatively few scientists are engaged in forensic science outside these main bodies and, of those who do from time to time appear as expert witnesses, few are engaged full time in the practice of forensic science.

The Home Office has been the driving force behind the development of forensic science in England and Wales ever since in 1933 a Home Office Committee recommended the setting up of a series of small laboratories throughout the country. However, since 1970 a number of large custom-built forensic science laboratories have been established under the Home Office at Chepstow, Chorley, Huntingdon and Wetherby and in London under the Metropolitan Police. In addition, the Home Office maintains laboratories at Aldermaston and Birmingham. Each of these operational laboratories is well equipped. The laboratories each have a staff of about 60-85 scientists whereas the Metropolitan Police Laboratory has about 200 scientists. The Home Office also maintains a research laboratory dedicated to research in forensic science at Aldermaston - the Central Research Establishment, Home Office Forensic Science Service (CRE). The organisation and direction of Forensic Science in the Home Office is the responsibility of the 'Controller', Home Office Forensic Science Service' Miss M. Pereira.

The centrally directed forensic science service lends itself particularly to a system of co-ordinated quality assurance. Over the past 10 years the Home Office has in particular utilised the facilities available at the CRE to develop an extensive programme of "quality assurance trials" in which materials routinely examined by the operational laboratories e.g. bloodstains, glass, fibres, etc., are submitted to those laboratories for analysis, the subsequent results being collected and analysed by the CRE. In this way monitoring procedures have been established to identify any possible technical weaknesses in the analytical procedures. These trials on occasions are carried out in such a manner that the operational laboratories are unaware that the case received at the laboratory is indeed a CRE trial.

A further step implemented by the Home Office to maintain standards has been the appointment within the Service of senior personnel, i.e. Assistant Directors,

* Home Office Forensic Sciec. Lab. Sandbeck Way, Andby Lane
GB-Wetherby, WYSH L22 4DN

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mechanism exists by which one can distinguish between the analysts offering reliable testimony and those whose work is scientifically unacceptable.

Accurate and reliable hemogenetic studies of physiological stain evidence have frequently provided invaluable information for law enforcement and courts of law. However, if the status quo continues, it is likely that such evidence will suffer the same fate as evidence derived from polygraph tests, voice print tests, and hypnosis. These new scientific procedures were ruled inadmissible in many jurisdictions because they could not be established as reliable.

Techniques for validation of methodology have been developed in research laboratories. Techniques for quality control are well-established in many clinical laboratories, blood banks, paternity clinics, and government regulatory laboratories. Guidelines for quality assurance in the testing of physiological evidence have been formulated and proposed for use in United States crime laboratories (4).

The problem is not so much one of establishing what should be done for quality assurance. The problem is one of implementation in the absence of a relevant professional community. Measures for quality assurance must be understood, accepted, and rigorously enforced. Finally, the courts must be provided with proof of reliability.

REFERENCES

1) Frye v. United States, 293 F. 1013 (D.C. Cir. 1923)

2) People v. Young, 418 Mich. 1, 340 N.W. 2nd 905 (1983)

3) People v. Reilly, Court of Appeal of the State of California, First District, Division Two, filed 25 March 1985.

4) Grunbaum, Benjamin W. "Physiological Stain Evidence: Guidelines to Assure Quality Analysis," California Defender, Vol. 1, Issue 1, Spring, 1985. Pp. 20-26.

service to the criminal justice system. This service is limited to the production of accurate data and the reporting of scientifically valid interpretations of that data. The bench analyst does not and should not experiment with evidence materials or offer untested hypotheses to explain ambiguous or inconclusive results.

The criminalists' findings are reported and put to use within the criminal justice system without peer review or review by a scientific community and without validation by a process of replication. The expected criticism which characterizes a scientific community is absent. Challenges in court by attorneys who do not understand the scientific principles or techniques from which the findings were derived is ineffectual in uncovering fraud, bias, error, or incompetence.

Apart from their membership in the large professional community of criminalists, analysts of physiological stain evidence should properly belong to a specialized community of criminalist-biologists. Such a community would be characterized by its commonly-held scientific knowledge and technical skills and its shared professional values and attitudes.

The hallmark of a professional community is self-regulation. If they are to be regarded as professionals, the analysts must be able, as a community, to articulate and observe appropriate standards of expertise and performance. This community must be able to monitor itself and to provide the criminal justice system with proofs of proficiency and reliability.

Unfortunately, a tradition of autonomy and a philosophy of generalism in United States' crime laboratories have acted as deterrents to the development of such a professional community.

Most government laboratories enjoy an autonomy that they do not care to relinquish. They range from municipal police department laboratories to county sheriff's department laboratories, to state and federal laboratories, most of which have developed without coordination. They differ from each other in physical facilities, staff size, analyst educational requirements, and caseload. These laboratories do not embrace common standards of practice or common requirements for the education and internship of analysts. Most of them are prosecution oriented.

In many so-called "full-service" laboratories, criminalists are expected to be generalists with expertise in several diverse areas such as drug chemistry, arson and explosives, ballistics, toxicology, and trace evidence analysis. Few laboratories start with trained immunologists or biochemists on their staffs. Consequently, criminalists who lack basic knowledge of analytical biochemistry are sometimes enlisted to learn and apply techniques of genetic marker typing. Most crime laboratory analysts learn these techniques on the job or in workshops that are oriented toward the transfer of technical skills. There is no uniform or core curriculum that leads to the practice of this sort of analysis. There are no minimum educational requirements and there is no agreement as to what the educational requirements should be. The analysts work in comparative isolation from each other, often supervised by laboratory directors who may have little or no background in hemogenetics.

Reviews of crime laboratory bench notes show professional responsibility and a high level of expertise on the part of some analysts, but they also show carelessness, incompetence, and basic disregard for the rules of scientific procedure on the part of others. At present, no

PROFESSIONAL RESPONSIBILITY: DEVELOPMENT AND ENFORCEMENT OF STANDARDS WITHIN UNITED STATES CRIME LABORATORIES FOR THE GENETIC MARKER TYPING OF PHYSIOLOGICAL STAIN EVIDENCE.

Benjamin W. Grunbaum (University of California, Berkeley CA 94720, USA)

There have been recent challenges in United States' courts to the admissibility of evidence derived from genetic marker typing of physiological stain evidence. In at least two instances, higher courts have ruled that the expert witness who has offered such evidence is unqualified to testify in regard to its reliability. These opinions raise some interesting questions regarding the status and role of the crime laboratory analyst within the criminal justice system and his relationship to the scientific community.

In California and most other states, the legal standard for admissibility of scientific evidence is a 1923 decision by the Court of Appeals for the District of Columbia in the case of Frye v. United States (1). The decision holds that ". . .while the courts will go a long way in admitting expert testimony deduced from a well-recognized scientific principle or discovery, the thing from which the deduction is made must be sufficiently established to have gained general acceptance in the particular field in which it belongs."

In 1983, while retaining jurisdiction in the case of People v. Young (2), the Michigan Supreme Court remanded the case to the trial court to determine whether the results of "serological electrophoresis" has achieved general scientific acceptance for reliability among impartial and disinterested experts of the relevant scientific community. The Supreme Court held that the testimony at trial of the people's expert witness, an analyst from the Michigan State Police Crime Laboratory, had been insufficient to establish reliability.

In 1985, a California Court of Appeals reversed a seven-year-old murder conviction (3), holding that the Frye requirements for admissibility of the bloodstain evidence had not been met. An analyst from a city police department crime laboratory had presented the bloodstain evidence at trial. The Court of Appeals held that a sole prosecution witness was insufficient to attest to the views of the scientific community regarding the reliability of the methods used. Secondly, the court held that this witness could not be regarded as a detached and neutral observer since she was not employed in an academic or other research setting that reinforces objectivity. Finally, the court held that the witness appeared to be "a technician and law enforcement officer, not a scientist."

The status of an analyst as a technician or a scientist is not likely to be settled to everyone's satisfaction. However, the compelling issue is whether or not the analysts of physiological stain evidence in the United States comprise a professional community.

A forensic biologist who is by profession a research scientist belongs to a community that holds as its central value the production of new scientific knowledge. He is accountable only to his fellow scientists. His findings belong to the community of science and are published. Findings and data are subject to organized scepticism until they can be validated through a process of replication.

In the United States, the analyst of physiological stain evidence is a criminalist employed in a government laboratory or by a private laboratory which sells its services to the criminal justice system. The primary value of the professional community of criminalists must be

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The answer to the question whether there is any particular advantage in using A (= exclusion chance) or W_A (= probability of paternity using the exclusion chance as information) in cases of disputed parentage is simple: there is no apparent advantage, neither for normal nor special cases.

SUMMARY:

The only correct way to use the exclusion chance A as a serostatistical parameter is to express it as a probability:

$$W_A = \frac{1}{2-A} .$$

This W_A however
- is deficient in information in comparison with the full informative Essen-Möller W_{EM};
- is neither easier to understand nor easier to calculate than W_{EM};
- requires - like W_{EM} - a prior probability;
- cannot interpret W_{EM};
- is unsuitable for setting a decision limit because the W_{EM}-value of an individual case can be lower than the limit.

$100-W_A$% cannot provide a reliable expectation of error in an individual case because the phenotype of the putative father is not taken into full account.

There is no reason that W_A and W_{EM} converge in higher degrees.

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2. Hummel, K.: Zum biostatistischen Vaterschaftsbeweis aufgrund von Blutgruppenbefunden. Eine allgemeine Betrachtung, zugleich eine Stellungnahme zur Methode von Sachs und Hoppe, sowie von Fiedler, Hoppe und Pettenkofer. Bundesgesundh.-bl. 1969, p. 379-383.

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vidual W_{EM}-values associated with a specific W_A-value approaching this value, until they all meet at infinity; and, the lower a W_A-value the greater the scatter of the associated W_{EM}-values. Is this true? The prime cause of the scatter of the W_{EM}-values for fathers and non-fathers - for given child-mother combinations - is the respective homozygosity or heterozygosity of the putative fathers: the W_{EM}-values of the homozygous men are higher than those of the heterozygous. The scatter is independent of the exclusion chance. Hence one cannot expect that W_A and the individual W_{EM}-values will converge at infinity.

W_{EM} contains all the information held in A. The converse, however, is not true; for, W_{EM} always contains more information than A. For, using W_A instead of W_{EM} is tantamount not only to destroying information but also to accepting unsystematic distortions of the reality, i.e. stronger or weaker indications of paternity than are actually the case.

Occasionally a Court questions the evidential value of W_{EM} when a cohabitor has been named but cannot be found. Such doubts are unwarranted. W_{EM} applies to the putative father, $1-W_{EM}$ to the cohabitor, regardless of whether the latter has been named or is only assumed, whether there is only one cohabitor or several, whether the child's mother admits to cohabitation with men other than the putative father or not, or whether proof of cohabitation is provided or not.

Even though W_{EM} provides full biostatistical information in cases involving a known though missing cohabitor, some experts and judges resort, in addition, to the exclusion chance and argue, e.g., as follows: "If the cohabitor were included in the opinion, the probability of his being excluded from paternity would be e.g. 99%. Hence, one could expect with great certainty that his non-paternity would be established". Conclusions of this nature presume that the cohabitor is in fact not the father - for, as father he could not be excluded. The correct argument in such a situation is the following: "If one regards the defendant with e.g. W = 99.73% as the real father of the child, then it can be expected with a probability of e.g. A = 99% that the unknown cohabitor will be excluded". This knowledge, however, is useless for the Court's decision.

The information A can be used - to distinguish between the hypotheses X and Y - only in the formulas $W_A = \frac{1}{2-A}$ (for X) and $1-W_A$ (for Y). However, by "adapting" A in this way to the Bayes' principle one destroys the "advantage" of greater "clarity" of A. With other words: Once A (the exclusion chance) is transformed to take account of a probability of paternity it becomes "unclear"; for this raises not only the problem of applying statistical behaviour to an individual case but also that of a prior probability. And precisely these are the "difficulties" to understand and to accept Essen-Möller's W-value.

W_{EM} is nothing else but a W_A-value "corrected" for the individual serotype of the putative father. The father's chance to have contributed to the serotype of the child is largely dependent on the serological similarity between mother and child. For this reason the differences between the values of the two terms are not systematic. In one case W_A is smaller than W_{EM} and in another larger - and by varying degrees. The differences can be considerable. E.g. in 14 cases of biostatistically evaluated HLA-A,B findings the value for A was consistently well above 50%, and that for W_{EM} very much lower (3):

A%	W_{EM}%	A%	W_{EM}%
68	40	89	4.5
80	8.3	91	30
81	9.2	91	41
85	24	95	15
85	31	95	41
88	4.4	95	44
88	42	96	42

In deficiency cases A can be 0; W_A is then 50% - this in all isolated grandmother and grandfather cases; W_{EM}, however, can have high values and sometimes also low ones. Apparently, there is no "natural" relation between W_A and W_{EM}. However, from a statistical point of view, there is some correlation: as the A-values increase so do the mean W_{EM}-values. But this is irrelevant in the individual case.

Some supporters of the exclusion chance maintain that at the upper end of the scale A approaches the W-value obtained. In other words, the higher the range the closer the mass of indi-

ADVANTAGES OF THE EXCLUSION PROBABILITY IN THE BIOSTATISTICS OF BLOOD GROUP OPINIONS ?

Konrad Hummel (Institute of Blood Group Serology, University of D-7800 Freiburg/FRG, POB 820)

By exclusion one does not recognize all non-fathers. Hence, it is necessary to establish a plausibility for or against paternity. If one wishes to use the fact of non-excludability as information, one has to work with the term 1-A = non-exclusion chance for non-fathers.

It represents the frequency for the counter-hypothesis Y (= non-paternity)

$$f(Y) = 1-A.$$

The frequency of the null-hypothesis X (= paternity) is

$$f(X) = 1$$ (which means: 100% non-exclusion of the true fathers in real triplets).

The likelihood ratio using information A is then

$$f\left(\frac{Y}{X}\right) = \frac{1-A}{1} = 1-A.$$

By substituting for this in the Essen-Möller formula (1) one obtains a "probability of paternity W_A" (2)

$$W_A = \frac{1}{2-A}.$$

The only information that W_A contains - besides a neutral prior probability - is the exclusion chance A.

W_A% states how many men among 100 non-excludable men for a given mother-child combination are the real fathers; 100-W_A% gives the percentage of non-excludable non-fathers.

This assumes that the material on file contains either as many fathers as non-fathers or as many cases of kinship as of non-kinship.

Because A can never be negative W_A is never less than 50%. Hence, the information A can never produce any W_A-values which would speak against paternity - this in contrast to Essen-Möller's W-value (W_{EM}).

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Fig.2 Mean ΔEM values and their scatter in four groups displaying relative black frequencies of <80%, 80...95%, 95...99%, and >99%, respectively

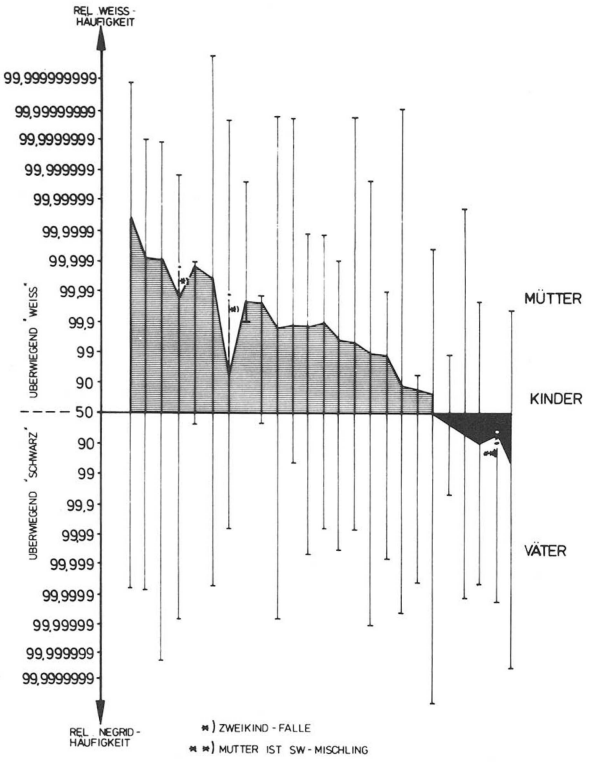

Figure 1

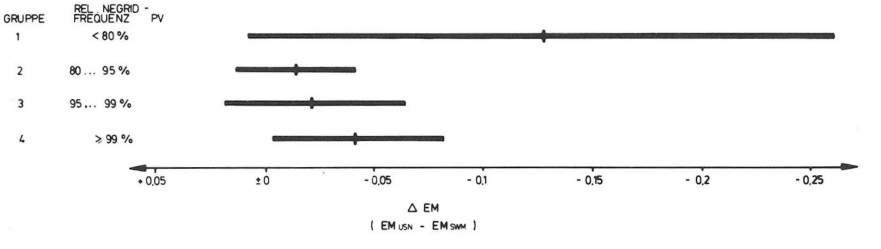

Figure 2

well as for S > 95%, and especially for S > 99%, ΔEM is too large. The scatter is considerable, as might be expected with limited data. Correspondingly, the difference between the mean values is generally insignificant. Using STUDENT's test the assumption of a real $\overline{\Delta EM}$ difference between groups 1 and 2 is associated with an error of 30%, between groups 2 and 3 of > 50% and between groups 3 and 4 of 20%. Thus, the $\overline{\Delta EM}$ differences for the groups 1/2, 2/3 and 3/4 are not significant. But the mean values of the groups 2 and 4 ($\overline{\Delta EM}_2$ = -0.01138; $\overline{\Delta EM}_4$ = -0.0410), with an expectation of error of only 0.05%, do differ significantly from one another.

Summary

From these comparative studies we conclude that in cases involving US Negroes it would be useful to seroanalytically determine the relative "blackness" and to use an appropriate frequency mix in the biostatistical evaluation, in particular when black frequencies are < 80%. One should act analogously in respect of members of other mixed populations. This applies, for obvious reasons, particularly to the Americans.

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1. Hummel K.: Biostatistical evaluation of blood group findings in cases in which the usual tables based on (Central) European frequencies cannot be applied because one or other of the persons involved is an alien. In: Inclusion probabilities in parentage testing. Am.Ass.Blood Banks. Arlington/Virg. (U.S.A.), 1983, p. 234-237.

2. Hummel, K.: Blutgruppeneigenschaften zur Definition menschlicher Grundrassen und zur Analyse von Mischbevölkerungen. Wienklin.Wschr. 92, 768-774 (1980).

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Legend to the figures:

Fig.1 25 black-white married couples and their 27 children; relative serotype frequencies among Whites and West Africans on a logarithmic scale (as "probabilities")

(3) about half of the probands had relative black frequencies of over 99% (and possibly 100%). Only 4 (= 10%) had a relative Negroid frequency of below 50%. The mean frequency was 87.2%, i.e. a mean white relative frequency of 12.8%. This agrees well with the "mean value" of 15% based on the literature (2).

Even if one uses frequencies for US Negroes to evaluate cases where the putative father is a US Negro, these will be adequate only in a certain percentage of the cases. Most US Negroes have a far smaller "white portion" than the mean, a minority a larger.

A simple means of taking account of the distribution in individual cases would be to use US Negro frequencies for black frequencies between 80% and 99% (= approx. 35% of all US Negroes), and West African frequencies for black frequencies above 99% (= approx. 50% of all US Negroes). Hence, only in cases where the PV had a black frequency of less than 80% (= approx. 15% of all US Negroes) would it be necessary to construct an appropriate frequency mix to take account of the particular blood composition.

Evaluating the 44 cases with

a) US Negro frequencies, and

b) with adequate frequencies

provided an EM difference of <0.1 in 38 cases and >0.1 (up to 0.888) in 6 cases. In all the latter 6 cases the black frequency was $<80\%$. A change in the EM value resulted in a change in the verbal predicate in only 2 cases, of 1 grade in one case and of 2 grades in the other.

Fig.2 correlates for individual cases Δ EM and the black frequency of the PV.

If we consider the position of the mean Δ EM values and their scatter we notice that all mean values lie in the "negative" range ($\Delta EM = EM_{USN} - EM_{SWM}$). In addition, the scatter of all the individual values also lies in the negative range. This means that in virtually every single case an evaluation based on US Negro frequencies will produce unduly low W values. The greater the difference between the analytically established black frequency (S) and the "mean" the larger Δ EM. The deviation is least for S = 80 - 95%; the black portion more or less corresponds to that previously obtained from mean frequencies. For $S<80\%$ as

There are two possible explanations for the unsatisfactory correlation for the above-mentioned 13:
either there is little correlation between appearance and serotype or the seroanalysis has for some reason produced unrealistic results in a number of the cases;
perhaps the explanation may lie in a combination of both causes.

To study this problem we analyzed 25 black-white married couples and their 27 children (fig.1). In this fig. the 25 families are listed in order of decreasing frequency of the children's serotypes among whites. There does not appear to be any correlation between the children's frequencies and the respective Negroid or European frequencies of the parents. But it is significant that the frequencies of 22 of the 27 children are more typical for Europeans than for Negroes, i.e. appear in serotype to be more European than their actual racial mix warrants. Whatever the explanation, the blood of the white mother seems "dominant" over the blood of the black father. Maybe this would explain the unsatisfactory correlations in cases 1-7*).

The serotype of the 27 children all lie "between" those of their parents. This would indicate that the method of establishing racial composition by comparing serotype frequencies does not provide "arbitrary" values but values not too far removed from reality. The comparisons between the physical appearance of and the seroanalytical findings for 9 individuals of mixed blood and 65 of pure blood support this conclusion. This should justify the practical application of the method within the serostatistics of cases of disputed parentage.

One possible field of application is cases involving US Negroes as putative fathers. Although gene frequencies for US Negroes are available for most of the common polymorphous blood systems, they are only mean values which, in individual cases, may not apply to the defendant without qualification.

My calculations (2) using frequencies in the literature give the mean portion of white blood in US Negroes as 15 - 20% (the remainder is West African blood). In a seroanalysis of 44 US Negroes

*) We lack sufficient data to say whether this applies to cases of a white father and black mother; initial data does suggest so.

nationality or origine	frequency among dutch	poly-micro-nesians	black-africans	appearance
8. Polynesia	pract.zero	pract.zero	0.9999992	negroid
9. Indonesia	0.6667	0.3333	pract.zero	european-polynesian

We were able to compare physical features of each of the 9 persons - especially skin and hair colour, hair type and facial build - with the results of the seroanalysis. The autopsies bore out the sero-analytical results.

In other cases, usually involving Negroes, there were some discrepancies:

place of origine	black-white ratio	appearance
Gambia	0.7 : 0.3	purely negroid
USA	0.18 : 0.82	" "
USA	0.045 : 0.955	" "
USA	0.005 : 0.995	predominantly negroid
former Fr.Afr.	0.0033 : 0.9967	" "
West Africa	0.0013 : 0.9987	" "
USA	0.0000002 : 0.9999	plainly negroid

Our sample of people of German origin included 5 individuals with a Negroid frequency >0.01, though the appearance of each was devoid of any negroid features. The black-white ratios were as follows:

0.0187 : 0.9813
0.0197 : 0.9803
0.045 : 0.955
0.19 : 0.81
0.836 : 0.164

A further person from Germany had an American-European ratio of 0.027 : 0.973, though no noticeable American features.

In contrast to these 13 cases in which seroanalytical data and physical appearance did not correlate well, there were 65 cases with a good correlation:

- 32 Germans with a black-white ratio of 1:99 and less, and
- 33 US and African Negroes with a black-white ratio of 99:1 and more.

None of the 65 exhibited Negroid or European features respectively.

SEROTYPE FREQUENCIES IN DIFFERENT HUMAN POPULATIONS; RACIAL COMPOSITION OF INDIVIDUALS; STATISTICAL EVALUATION WITH A FREQUENCY MIX

K. Hummel (Institute for Blood Group Serology, University of D-7800 Freiburg/Br., FRG., POB 820)

An individual's serotype has a specific and different frequency in each and every population. A high frequency indicates that the person is "typical" for the population with which he is compared, a low frequency that he is "atypical". These frequencies can be expressed as probabilities; the sum of probabilities for any individual is always 1 (1).

Frequency comparison requires widely differing reference populations, e.g. Caucasian, Negroid, Ameridian, East Asian or Oceanian. The prior weighting is the same for all the frequencies compared. The probabilities obtained from the frequency comparisons tell (in Bayes' terms) how often one would be right if one were to assign the individual concerned to one or other race:

$$W_X = \frac{1}{1+\frac{f(Y)}{f(X)}} \; ; \; W_Y = 1-W_X.$$

W_X is the relative frequency of a person's serotype in population X, and hence the probability that he/she is a member of this population; W_Y is the relative frequency of that serotype in population Y, and hence the probability that the person belongs to population Y; f(X) is the frequency of the serotype in population X, f(Y) that in population Y. - The following table lists the results of seroanalyses and the physical appearance of 9 non-caucasions:

nationality or origine	frequency among spaniards	amerindians	black-africans	appearance
1. Bolivia	0.9997	0.0002	0.0001	european
2. Puerto-Rico	0.9920	0.0080	pract.zero	european
3. Angola	0.9774	pract.zero	0.0226	fairly negroid
4. Mexico	0.9679	0.0001	0.0320	"
5. Paraguay	0.5690	0.4260	0.0050	european-indian
6. Mexico	0.1600	0.1600	0.6800	"
7. Panama	0.0020	0.9980	pract.zero	indian

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ship with another man, F_2, but she denied a consummated sexual intercourse. F_2 denied having had even genital contact with the mother, because she resisted him. He had not taken off his underwear, and he did not ejaculate. The combined blood group and anthropological index of F_2 was 13.4, which indicated his paternity with a weight of 93 % against 7 % in favour of an unknown paternity possibility. The City Court stated in its judgment that there was not sufficient evidence to the effect that the mother had had sexual intercourse with F_2, and acquitted F_1 as well as F_2. During the proceedings before the High Court the HLA-system was applied, and an increased total combined index for F_2 of 400 to 1 or 99.7 % to 0.3 % appeared. The High Court now stated in its judgment: According to the opinion of the medico-legal institute the burden of proof for sexual intercourse between F_2 and the mother is fulfilled; and F_2 was convicted.

I should like to ask: Can such a use of the biological evidence be justified in relation to the scientific methods and in relation to the public conception of law?

cal and anthropological index was 0.007 and spoke against the paternity of the man compared to an unknown paternity possibility in the Danish population with the weight of 140 to 1 or 99.3 % to 0.7 %. The Danish High Court convicted the man to paternity. The Supreme Court acquitted the man with 4 votes against 3.

In cases concerning paternity of children born out of wedlock where there are two or more paternity possibilities, who have not been excluded, the court can convict one of them to paternity, if - as laid down in the law - there is a considerably higher degree of probability for the paternity of one of the men in comparison with the degree of probability of each of the other men. In these cases a degree of about 95 percent for one man against 5 percent for the others is required in order to reach a conviction to paternity.

In cases of children born in wedlock the limits of the law concerning acquittance of the paternity of the husband are considerably restricted, caused by the fact that the acquittance of the husband deprives the child of a certain status.

If the wife has denied having had sexual intercourse with other men than the husband, an acquittance of the husband requires that it is assumed certain that the husband is not the father of the child. According to the motives of the law it is required that the paternity is excluded according to several blood group systems, including one of the most reliable, but even in that case the judge may sustain the paternity of the husband, if the mother's evidence concerning conjugal fidelity carries complete conviction.

If it has been proved that the mother has had sexual intercourse with another man, the husband will be acquitted if it must be assumed that the child has been concepted by this other man. On the basis of the motives of the law and of the legal practice I venture to set the limit of the total weight of probability for the paternity of the third party against the paternity of the husband to at least 99 % against 1 %, unless the mother should have married the third party in the meantime.

In addition to this, it shall be mentioned that in cases where there are doubts as to whether the said limits are reached by means of blood group determination, the courts will nowadays require so-called anthropological determination, especially the HLA-system and the chromosome examination. In most cases this seems to remove any remaining doubt.

Finally, it shall be pointed out that in Denmark we have some court decisions in which the weight of the bio-hereditary evidence has been so predominant that it has influenced the piece of evidence as to whether the mother has had intercourse with a particular man. In a case from 1977 the mother gave evidence to the effect that she had had sexual intercourse with one man, F_1. He was excluded. Subsequently the mother stated that she had had one single relation-

First of all there will be cases in which biological evidence is of no importance, for instance because new evidence reveals the impossibility of a sexual intercourse between the mother and the man in question. In other cases, however, the assessment of biological evidence will be of great importance. This may occur when it is very likely that the mother has only had sexual intercourse with two men, but very often throughout the whole conseption period with both of them, and where there is no particular evidence concerning these men, for instance about reduced fertility. In this situation the choice between the two possibilities must be justified on the basis of the biological evidence solely.

In most cases it will be necessary to combine the weight of the biological evidence with the weight of all the other kinds of evidence present in the case. It is generally accepted that the probabilities of different pieces of circumstantial evidence must be multiplied, when each piece - independent of other pieces - causes a probability of the theme of the evidence with a certain strength. I will give you an example: In a case with no more than two paternity possibilities one of the possibilities is known, the other, which belongs to the same population, is not identified. The blood group statistical index of the tested man is 20, which indicates his paternity with a weight of 95.2 % against 4.8 % in favour of an unknown paternity possibility. All other evidence in the case indicates the paternity of the tested man with a weight of 80 % against 20 % in favour of the unidentified man, which can be expressed by a combined index of 80 for all circumstantial evidence in the case, or 98.8 % against 1.2 %.

Yet, this can only be a principle. It is not possible for the judge to perform a numerical classification of the non-biological evidential factors of the case. Instead, the judge will try to estimate the evidential strength of the most important and relevant factors and to compare this estimation with the numerical information in the opinion of the biological statement.

Which degree of probability for or against the paternity must be obtained in order to convict or to acquit a man?

In Denmark such limits are described in the law in common linquistic expressions and clarified partly by the motives of the law and partly by the legal practice.

In a case concerning paternity to a child born out of wedlock where the mother has only had sexual intercourse with one man, this man must be convicted unless the circumstantial evidence makes his paternity very improbable. An exclusion based on one of the blood group systems used in Denmark as a routine - which gives a reliability of at least 99.9 % - will be sufficient according to legal practice. In 1970 a limiting case appeared before the courts: There was only one man involved. The only sexual intercourse had taken place very close to the end of the conception period, 195 days. A combined blood group statisti-

LEGAL CONSIDERATIONS

Finn TAKSØE-JENSEN, Professor at the University of Copenhagen, Retsvidenskabeligt Institut, DK-1455 Copenhagen

Paper to be read by Dr. Taksøe-Jensen:

In paternity cases concerning children born out of wedlock it is the mission of the court to try to determine who is the biological father of the child.

After the conception period has been stipulated the mother is questioned before the court about her sexual relations during the conception period. If she gives evidence to the effect that she has only had sexual intercourse with one man, and if this man in his evidence confirms sexual intercourse with the mother, and if he is also willing to accept the paternity, the judge can - according to Danish law - close the case without use of bio-hereditary evidence, provided that no additional circumstances challenge the paternity of the man. At this stage of the case a decision to obtain bio-hereditary evidence might however be justified: The man can refuse to accept the paternity and claim his firm belief that the mother has had other sexual relationships without he being able to identify the latter. If a blood group determination shows that this man cannot be excluded, and if a statistical valuation concerning blood groups gives an index of 10 for this man compared to an unknown paternity possibility in the population in question, the judge will estimate the weight of the biological index against the probability of the mother having had sexual intercourse with other men. An entirely different situation arises when the man is excluded. In this case the point will be to find other possibilities. When the judge has explained to the mother the meaning of the principle of exclusion she will often point out the next man or men. Here the issue of the first bio-hereditary evidence effects the possibilities for the judge of obtaining perhaps decisive evidence in the case.

In cases with more than one paternity possibility the judge will always decide on biological evidence, although the mother and a certain man both should wish this man to accept the paternity. The picture of the circumstances of the case may change many times during the proceedings.

In cases concerning children born in wedlock the proceedings will be performed in similar ways, but be influenced by the strong presumption of the paternity of the husband as laid down in the law.

The judge will decide to close the proceedings either when the limits laid down in the law concerning conviction or acquittance of the men involved are reached and new evidence cannot be expected to have any effect, or when new evidence simply cannot be obtained.

How is the weight of bio-hereditary evidence now combined with the weight of all other kinds of evidence?

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by definition to all conceivable problems, should be the basis of the judges decision.
As conclusion I would like to cite J. Morris' remark - "If it's not broken let's not fix it" - but from previous experience I have little hope that this will happen.

	Kind of case	P_E	P_E vs. W (X/Y,PI)
I	One man not excluded	high	$P_E \sim W$
II	One man not excluded	low	$P_E < W$
III	Several men not excluded	not informative	$P_E < W$
IV	"Family" cases	P_E "complex"	$P_E < W$
V	"Family" cases	$P_E = 0$	$P_E < W$

" $\sim$ " almost equally informative

" $<$ " less informative

Table 1 Comparison of P_E and W (X/Y,PI) in different situations

in contrast to the use of the paternity index X/Y, which may or may not be converted to a posterior probability W by way of the bayesian approach using either a standardized or an estimated prior probability.
Whereas the argument that the decision rule "all non-excluded men are fathers" has a small error rate of

$$(1-P_O)\ (1-P_E) < 1-P_E$$

relative to all cases, given a powerful battery of tests, it should become obvious from table 1, that the likelihood ratio X/Y as well as W have the same or better properties (because they use the total information) and are applicable for all conceivable situations.

Admittedly, in case of a high exclusion chance (I), there is little difference in the information provided by W as well as P_E. Contrary, though, with a low exclusion chance (II) for the given mother-child combination the parameter W, which is based on the total genetic evidence, is far more powerful to differentiate and consequently the amount of error comitted is smaller, if based on W. In case of several non-excluded men (III) P_E is of no information at all, because it is equal for all of them, and only W correctly quantifies the difference due to differing phenotypes. In case of complex family situations (putative father not available for testing) the correct calculation of P_E may be extremely complex (IV) in contrast to X/Y and W, and in many situations $P_E = 0$ due to the structure of the given data (V) as for the example of information only from one of the putative father's parents. In both cases (IV and V) the likelihood ratio X/Y and W correctly quantify the often powerful information at hand.

As a consequence of this comparison there is no doubt, that the statistic W (or equivalently X/Y), which are applicable

$$P_t = \frac{P_o}{P_o + (1-P_o)\,(1-P_E)}$$

is equal to

$$P_t = \frac{1}{1 + \frac{1-P_o}{P_o}\;\frac{1-P_E}{1}}$$

which is immediately identified as the bayesian approach by Essen-Möller on the basis of less information than available. Basically all that is proposed, is to lump the most powerful knowledge of the possible phenotypes together into the two subclasses of exclusion phenotypes and non-exclusion phenotypes and use this reduced information in the well established Essen-Möller approach.

As to the discussion of the methods presented in this panel it has been already pointed out that the tail probabilities used in the Neyman-Pearson approach are conditional and can only be converted to an overall error rate by introduction of the same prior probabilities used in the bayesian approach. The use of a tail probability conditional on the varying subset of non-excluded non-fathers is strongly objected and it seems that the standardized paternity index of Martin uses exactly this approach. Both methods seem to be based on the believe, that the statistical argument begins after there has been no exclusion, which by no means is justified, because all likelihood arguments are based on all possible phenotypes.

The final argument concerns the use of the exclusion chance P_E only, because it is not based on a prior probability,

nifying the condition, that he is the father, whereas in the denominator he has been picked at random. Simple cancelation of P(A) and P(M) in this well defined likelihood ratio reduces the computational effort to evaluate

$$\frac{P(C/M\ A=F)}{P(C/M)}$$

but in no way changes anything with regards to its original property as likelihood ratio. Their next objection is to the fact that $X/Y > 1$ in a given example for all non-exclusion phenotypes. This is true, but they do not state that $X/Y=0$ for the exclusions. Their third objection that a true probability of paternity should monotonically increase with increasing number of tested systems and no exclusion only holds by expectation over all phenotype classes and definitely is no necessity for each single non-exclusion phenotype.

After this sequence of (non acceptable) objections they proceed to state, that on the basis of N previous court cases with a theoretical exclusion chance P_E and an observed number of exclusions N_E the prior probability P_O can be estimated by

$$\hat{P}_O = 1 - N_E/NP_E$$

There statement that this parameter "may be easily estimated.... and we are surprised that no such investigation has been made until recently" again shows ignorance of numerous such investigations carried out during the last 20 years and in addition ignores the more powerful estimation of P_O by way of expectation maximization applied to the posterior probability as a function of P_O.

Their final proclamation of a new "method" to calculate a posterior probability on the basis of exclusion - non exclusion by way of

a rare silent allele s and a case of

Child:	1	Mother:	1
P 1:	1	P 2:	2

the likelihood ratio strongly supports the hypothesis of paternity for putative father P1 on the basis of the given genotype-phenotype relations, although in some cases P2 could have genotype 2-s and be the father of a child 1-s. This objection is not acceptable, because the likelihood ratio correctly quantifies this likelihood in relation to a 1-1 homozygous child with a 1-1 homozygous father.

More serious in the way of confusion is the paper of Li and Chakravarti (2), whose mayor objection is based on their statement that the ratio X/Y is not a likelihood ratio, but merely a segregation probability over a weighted average of segregation probabilities written as

$$\frac{P(C/MF)}{P(C/M)}$$

The original definition of the likelihood ratio conditional on the two alternative hypotheses (which they state themselves) is again

$$\frac{P\ (\text{obs. phenotypes/fatherhood})}{P\ (\text{obs. phenotypes/non-fatherhood})}$$

which is equal to

$$\frac{P(A) \times P(M) \times P(C/MA=F)}{P(A) \times \left[P(M) \times P(C/M)\right]}$$

with A being the phenotype of the alleged man and A=F sig-

of doubt with regards to a well established method, namely the use of the paternity index X/Y as the only statistic containing all the genetical information at hand.
Aickin (1) in his paper correctly defines X/Y as the likelihood ratio conditional on the two alternative hypotheses

$$\frac{X}{Y} = \frac{L\ (\text{obs. phenotypes/fatherhood})}{L\ (\text{obs. phenotypes/non-fatherhood})}$$

His first objection states that this likelihood ratio can not distinguish between different men with identical phenotype. He draws the conclusion that all statements consequently correspond not to a specific man but to all males with this given phenotype. This objection would have its merrit, if paternity testing was performed by way of screening all men of the given population giving equal prior probability to all of them ignoring the information given from the mother. His second objection concerns the assumptions for the calculation of the denominator

$$Y = L\ (\text{obs. phenotypes/non-fatherhood})$$

First of all this conditional likelihood depends on the ethnic background of the true father, which may not be well defined. The effect caused by possibly differing genefrequencies in differing ethnical or geographical populations is small, though, if a sizeable number of systems has been tested (as Aickin states himself). Furthermore he would rather redefine Y on the basis of a population of "plausible fathers", which is a correct point, if knowledge about such a group exists. But given this knowledge we are no longer dealing with a one man case and proper statistical handling will yield the correct conditional likelihood(s). Aickin's third objection is the one least understandable. On the basis of a constructed example he argues that in case of a system with two common codominant alleles 1 and 2 and

can be calculated, but it must be stressed that the total error of this procedure, which is

$$(1-P_0)\ \alpha\ +\ P_0\ \beta$$

also is a function of the prior probability P_0 for paternity. α and β are error rates conditional on the subsets V (cases with the true father) and $\overline{V}$ (cases with a man falsely alleged) (Baur and Rittner (3)). Chakraborty and Ryman use for comparison not the subset $\overline{V}$ but - with no justification - the subset $N\overline{V}$ (non-excluded non-fathers), thus reducing one subset trough increase of the number of genetic systems tested. The consequence is a redefined conditional error rate α' which increases to a large relative value, while the absolute error equals

$$(1-P_0)\ \ \alpha'\ \ (1-P_{E)}$$

with P_E equal to the exclusion chance of the tested systems. It is apparent that α' can have a large numerical value while the absolute error is minimal. Furthermore α' errors from different tests are not comparable unless exactly the same battery of systems has been used for testing.

With regards to the discussion of the two critical papers by Aickin (1) and Li and Chakravarti (2) it must be stated that as a consequence of the legal situation the use of statistical methods in paternity testing is relatively new in the United States in comparison to Europe. Due to lack of knowledge of the literature from the last thirty years (admittedley not all available in english) it seems that methods are reinvented as in the case of the tail probabilities of Chakraborty and Ryman, which were already discussed by Hummel as well as Koller and introduced by Schulte-Mönting and Walter. More serious, though, is the creation

Panel Discussion: Biostatistics

Statistical Considerations to the Methods Used in Paternity Testing

Max P. Baur

Institute of Medical Statistics, University of Bonn, West-Germany

As requested by the organiser, there were three topics, which hat to be dealt with in this contribution:

1) The method of Chakraborty and Ryman

2) Two recent "critical" publications by M. Aickin (1) and C.C. Li and A. Chakravarti (2)

3) The "Pros and Cons" of the methods presented in this panel

The method of Chakraborty and Ryman is based on the well known paternity index X/Y and was originally introduced into paternity testing by Schulte-Mönting and Walter. The method uses the Neyman-Pearson principle to compare the conditional distribution functions for a given test statistic.
In this case the test statistic is the paternity index X/Y and the conditional distribution functions for both hypotheses can be explicitly derived on the basis of the gene frequencies of the systems used for testing. Consequently the conditional tail probabilities α and β for the errors of the first and second kind given a decision threshold

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(6) Finally we should take into consideration that biostatisical data can not be given without the risk of error. May be the error is very very small but it exists.
In the individual case that is to be decided in court the error is zero or 100%.

THE INDIVIDUAL EXCLUSION CHANCE

by V. Sachs *

In my opinion the individual exclusion chance related to a given mother - child - pair is a highly suitable parameter for paternity estimating by blood groups.

(1) The individual exclusion chance (IEC) for non fathers is defined by the sum of phenotype frequencies of a blood group system leading to the exclusion of non fathers in a given mother-child-pair.
This procedure is continued successively with the respectively remaining frequencies of not excluding phenotypes and all blood proup systems being determined. The difference of the last remaining frequency to one is the IEC.
The IEC can be calculated independent of a given presumtive father.

(2) The following conclusion is plausible: The higher the IEC the greater the probability that a non excluded man is the father. Thus it is also a reasonable conclusion to assume that a non excluded man is the father if the IEC tends to one.

(3) With respect to the high number of 24-28 blood group systems used for paternity testing especially when HLA is included the parameters of all biostatistical methods converge, as it has been shown by MARTIN et al.(1984). Thus the parameters of the different methods in nearly all cases lead to the same result.

(4) In contrast to the parameters of other biostatisical methods for paternity estimating. The IEC is simple and understandable also for non mathematicians. The parameters of other methods are not easily intelligible to all.

(5) In case the IEC does not reach a value in the nearest neighbourhood of one it stands to reason that other parameters are calculated e.g. the paternity index (Pi), the standardized PI, the "W"-value of Essen-Möller or prohabilities of error according to Schulte-Mönting and Walter.

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* Prof.Dr.V. Sachs, Transfusionsmedizin d. Univ.
Klaus Groth Platz 2, D-2300 Kiel

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Table 2

Case result, 25 systems including HLA

PI = 2711 L = 0.000369

PI*s = 904

(W*s = 99.889%) W = 99.96%

Table 3

Verbal Predicates PI*s - A

I A >= 99,73 %
PI*s >= 400

Paternity practically proved

II 99,73 % > A > 90 %
400 > PI*s > 10

Indication of paternity

III A < 90 %
PI*s < 10

The statistical evaluation of the blood group findings did not deliver usable contributions to the ascertainment of paternity.

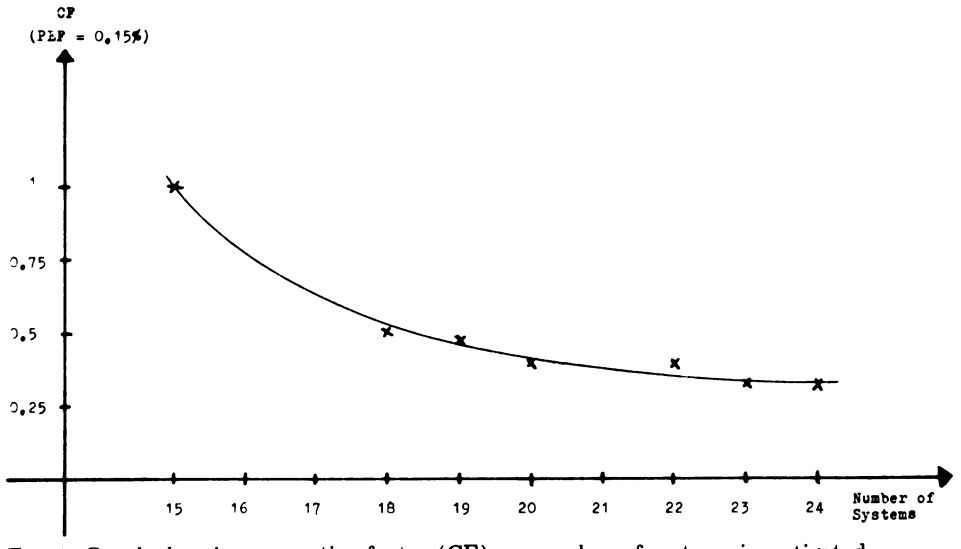

Fig. 1. Graph showing corrective factor (CF) vs. number of systems investigated.

TABLE 1

CF, PEF = 0.15%

Mean value according to the curve shown

No. of Systems	*CF*
15	1
16	0.78
17	0.63
18	0.53
19	0.46
20	0.41
21	0.38
22	0.35
23	0.34
24	0.33

3. The employment of the proposed verbal predicates delivers the explanation of the mathematical result.

Finally, it can be stated that the use of the likelihood ratio in most cases is no longer necessary. At a test volume of about 25 informative systems including HLA (as it is performed in our and in many other laboratories), the chance of exclusion for non-fathers reaches values exceeding 99.73% in more than 90% of the cases. In all these cases, the proof of paternity can be based on the fact of non-exclusion alone. The power of the test, then, is so high that the possible error (1-A. non-exclusion of a non-father by accident) is minimal and can be neglected. Because of the power of the test, this is also valid. if PI*s does not reach or exceed a value of 400.

Consequently, our statistical expertises are given as follows:

1. Calculation of A: if A >= 99.73%, report of A alone with the corresponding verbal predicate.
2. If A < 99.73%, calculation of PI*s, if PI*s >= 400, report with the corresponding verbal predicate.
3. If A < 99.73% and PI*s < 400, recommendation of further serological investigations, if possible.

as follows:

$$CF = \frac{PI\ 400\ \text{(with PEF 0.15\% with 15 systems)}}{PI\ X\ \text{(with PEF 0.15\% with Y systems)}}$$

The CF for the increasing volume of tests is summarized in Table 1 and graphically shown in Fig. 1 . It can be seen that the CF is just slightly changed at a volume of tests from 23 to 24 systems. The CF will, therefore, remain constant with the addition of further systems. An example for a result of a case is given in Table 2. .

The PI*s is the first parameter in the statistical evaluation of blood group findings in Paternity Testing giving complete information of the likelihood ratio and, moreover, regarding the test volume based on the probabilities of error according to Schulte Mönting and Walter. At the same time, the statement of high percentage values can be avoided, which might easily pretend a non existing safety to a layman.

With PI*s, a parameter is given which is only understandable for the layman by a corresponding explanation of the expert. The result influences the court decision only by the expert's professional explanation but not by possibly misleading relatively high values of percent. The best way to give this explanation is by verbal predicates with four ranges as listed in Table 3 . The limit of the standardized PI is given but neither a certain probability of error nor a W value.

The most important aspects are emphasized as follows:

1. By using the Paternity Index as parameter, the indication of plausibilities of paternity in percent can be renounced without loss of information.
2. By standardization of the PI in the proposed way, the test volume is also taken into consideration.

Hence. it follows that on an average W increases for all not excluded men, independent of their being fathers or not. Consequently, the requirements for the height of the W value have to increase with increasing number of systems tested, corresponding to the probabilities of error changing at equal PI values with the number of systems tested. A certain probability of error being attributed to a PI value at a fixed number of systems has to be allocated to a continually increasing PI value with increasing number of systems.

Consequently, it has to be demanded that the paternity index has to be standardized in dependency of the number of systems tested. Hereby, the use of the W value is no longer necessary and the complete information of the findings is considered including the volume of tests. The tables on the probabilities of error according to Schulte Mönting and Walter as to Umbach and Walter are the basis here. The table on a volume of tests for 15 systems of the German guidelines for paternity testing is the starting point. At a higher volume of tests, the likelihood ratio PI is multiplied by a corrective factor calculated from the distribution tables with the effect that the test volume is considered in the evaluation and the calculated values become comparable with those calculated in cases with a different number of systems tested.

A PI value of 400 (L = 0.0025, W = 99.75 %, PEF = 0.15 %) in the 15 systems of the German guidelines is the starting point for the corrective factor (CF) which is here 1. With an increasing volume of tests the value of PEF * (0.15 %) is allocated to a continually increasing PI value. As a result of this, the CF is calculated

* PEF = probability of error for the assumption of fatherhood

The method for the statistical evaluation of blood group findings developed by Schulte Mönting and Walter in 1972, delivered for the first time the possibility to record probabilities of error without using Bayes theorem. Hereby, the full information of the likelihood-ratio X/Y is involved in the calculation. In this method, the distributions of the likelihood ratio are seen in special defined collectives or partial collectives, respectively. The knowledge of these distributions and the consideration of the area or partial area under the distribution make it possible to determine limits, the exceeding of which allows the indication of probabilities of error for correctness or incorrectness of certain hypotheses. The consideration of the area must be regarded as an essential and pregnant completion to the punctual statement based on Bayes' theorem. This theorem starts out from, in a single case, an unprovable presumption that an alleged father has equal (some times also unequally shifted but always to 100 % complementing) chances for or against paternity. The decisive fact of the method developed by Schulte Mönting and Walter, based on the Neyman-Pearson principle, is that the number of systems tested is considered in this calculation for the first time. The number of systems tested stays disregarded in the interpretation of the W value, while at equal PI values the probabilities of error change with the number of systems tested.

The addition of further systems of genetic markers shifts the summation distributions of the log X/Y values more and more into the region of positive values. Moreover the distribution curves assimilate more and more so that the differentiation between true fathers and not excluded non-fathers becomes more and more difficult.

For the statistical evaluation of blood group findings in paternity testing, the likelihood ratio Y/X (L) or X/Y (PI) provides the full information on blood testing. The X/Y ratio indicates the relation of the frequency of begetters to that of any (unrelated) man. PI indicates how many times more frequently the phenotype of the alleged father occurs in true father trios than in non-father trios. It is possible, therefore, by means of this ratio to assign an alleged father in the different groups (begetters or any unrelated man). This assignment has risks of error: it may happen that any man can, by mistake, be taken as begetter or a begetter by mistake as any unrelated man.

The likelihood ratio alone or its transformation to W (according to Essen Möller - Hummel) do not allow a realistic statement on the probabilities of error for the alternatives paternity or non-paternity, respectively. The probabilities of error can only be stated according to Schulte Mönting and Walter (based on the Neyman-Pearson principle).

Recent results showed that the probabilities of paternity according to W lead - at a great volume of tests - to values which may not be regarded as realistic in a single case. This is probably one of the main reasons why X/Y (PI) is more and more used as parameter. In this way the suggestive effect of high values of percent can be avoided without loss of information. In general, PI corresponds better to the common understanding than L (Y/X), since the chance to have to deal with a true father rises with increasing values.

Summary

The standardization of the paternity index (PI, X/Y) is based on the probabilities of error according to Schulte Mönting and Walter. By using the suggested standardization, the test volume is taken into account including the full information of the blood group findings. The interpretation of the mathematical result is given by verbal predicates.Besides the essential fact that the test volume is taken into account, the most important advantage of this procedure is that the mathematical result is included in the court decision only by the PI and its verbal predicate and not by sometimes relatively high percentages, that may be misunderstood by laymen. At the present stage, the use of the chance of exclusion for non-fathers (A) alone is sufficient in most cases. At a test volume of 25 systems including HLA, more than 90 % of the mother/child pairs reach or exceed a value of A = 99,73 %, indicating proof of paternity by the fact of non-exclusion of a man alone.

The Standardized Paternity Index for the Statistical Evaluation of Blood Group Findings in Paternity Testing

W. Martin

Aus dem Bundesgesundheitsamt

Robert Koch-Institut

Berlin

Zusammenfassung

Die Standardisierung des Paternity Index basiert auf den Irrtumswahrscheinlichkeiten nach Schulte Mönting und Walter. Durch die vorgeschlagene Standardisierung wird neben der vollen Information der Blutgruppenbefunde auch der Untersuchungsumfang berücksichtigt. Die Interpretation des rechnerischen Ergebnisses erfolgt durch verbale Prädikate. Neben der wesentlichen Tatsache, daß der Untersuchungsumfang berücksichtigt wird, ist ein Hauptvorteil dieses Vorgehens, daß das rechnerische Ergebnis in die Gerichtsentscheidung nur durch den PI und sein verbales Prädikat eingeht und nicht durch mitunter relativ hohe Prozentwerte, die von Laien falsch verstanden werden können. Beim gegenwärtigen Stand reicht bei den meisten Fälle die alleinige Verwendung der Ausschließungschance für Nichtväter (A) aus. Bei einem Untersuchungsumfang von 25 Systemen einschließlich HLA wird bei über 90 % der Mutter-Kind-Paare ein Wert von A = 99,73 % erreicht oder überschritten, was den Beweis der Vaterschaft eines Mannes allein durch die Tatsache des Nicht-Ausschlusses bedeutet.

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may be important on the evaluation of disputed paternity, it is incorporated into the PI. Once the PI has been computed, it would be unnecessary and misleading to consider A again. The same is true of the issue of father versus non-excluded nonfather.

For those who come to the field of disputed paternity from an experimental, rather than mathematical, background I offer the following observations. The paternity index, being a quotient of population frequencies, can be obtained directly from experimental data, as well as by computation from gene and haptotype frequency tables. The posterior probability of paternity is a mathematical expectation. This expectation can be experimentally verified for actual case material by testing a number of cases with a limited test battery, noting the expectation of paternity, and confirming that expectation by testing in further genetic systems. Confirmation can also be obtained by computer simulation (Monte Carlo methods). Despite several decades of controversy regarding paternity probability, no practical advantage has ever been shown for any biostatistical summary of genetic evidence different from, or in addition to, the paternity index. We should heed the old adage "If it's not broken, don't fix it."

It is clear that the tailed probabalist and the exclusion probabalist cannot both be right. There is no point in trying to decide which argument is correct, as they have equal merit. This can be appreciated by considering the third important property of the PI; it can be factored:

$$PI = \frac{1}{1-A} \cdot \frac{P(\text{phenotypes of trio/paternity,nonexclusion})}{P(\text{phenotypes of trio/nonpaternity,nonexclusion})}$$

The first term contains only the exclusion probability (A). The second term, which looks very much like the PI, is the likelihood ratio which tests the hypothesis that a nonexcluded man is the father relative to the hypothesis that he is a nonfather. As it is computed only with regard to trios in which the alleged father is not excluded, the second term is independent of the exclusion probability (A). The exclusion probabalist is arguing that the first term is larger for Case #1 than for Case #2; the tailed probabalist is arguing that the second term is larger for Case #2 than for Case #1. These two terms are like the sides of a rectangle.

Suppose two rectangles are presented. The first measures 6 units by 4 units; the second measures 8 units by 3 units. The question is posed: Which rectangle is larger? The straightforward approach is to note that the two rectangles are equal in area, so the question is moot. One can imagine that the following argument could be made by a "length probabalist": "On the basis of area there is no difference. However, length is important in determining the size of a rectangle, so that rectangle #2 must be larger." The reader no doubt can produce the argument that would be made by a "width probabalist."

Of course, these arguments are silly. Once the area has been computed, the length and width need not be considered again. In the same way, while the exclusion probability (A)

statements regarding the hypotheses, the court must consider the prior probability of paternity. Thus, there is no justification for withholding the paternity index from the court, as the posterior resulting probability of paternity directly addresses the issue before the court.

The question then arises: Should additional statistics be submitted to the court? Can the court improve on its decision making process if it has, in addition to the PI, the exclusion probability (A) and/or information regarding the issue of father vs non-excluded nonfather (e.g. tailed probabilities)? These questions can be answered by considering the following example. Suppose we are given two cases:

Case #1	Case #2
PI = 99	PI = 99
A = 0.999	A = 0.90

and the question is posed: In which case is the biostatistical evidence in favor of paternity stronger? The exclusion probabalist would argue as follows: In terms of PI, there is no difference. The alleged father in Case #1, however, was subjected to a more powerful exclusionary battery (a statement with which we all should agree). The exclusion probabalist would conclude that the evidence in Case #1 is stronger than in Case #2.

The tailed probabalist would argue as follows: In terms of PI, there is no difference. The alleged father in Case #1, however, looks more like a non-excluded nonfather because the tail of the distribution of the PI for non-excluded nonfathers is larger for Case #1 than for Case #2 (a statement with which we all should agree). The tailed probabalist would conclude that the evidence in Case #2 is stronger than that in Case #1.

We can create a simple counterhypothesis H_1: "the coin has two heads." Now a likelihood ratio can be computed as follows:

$$L = \frac{P(\text{10 heads in 10 flips}/H_1)}{P(\text{10 heads in 10 flips}/H_0)} = 2^{-10} \approx 10^{-3}$$

A meaningful statement regarding the truth of the null hypothesis is still not possible. If, for example, Professor Nijenhuis were to hand me a coin, inform me that it was either true or two-headed, and I were to obtain 10 heads in 10 flips, I would conclude that the coin probably is two-headed because knowing Professor Nijenhuis, under such circumstances I would judge the prior probability of a two-headed coin to be substantial. On the other hand, were the coin to come from my pocket and I were to obtain the same result, I would conclude that the coin is probably not two-headed because over the years I have pulled many thousands of coins from my pocket and have yet to encounter one with two heads.

As further example, consider a null hypothesis tested by χ^2 (a typical tailed probability method). If the tailed probability is less than 0.05 without further assumptions one can "reject" the null hypothesis, as this is not a conclusion about the truth of the null hypothesis, but only a statement about the tail value. However, one cannot logically conclude that the null hypothesis is unlikely to be true without assuming that the prior probability of the null hypothesis is significantly less than unity.

These examples demonstrate that meaningful statements regarding the truth of hypotheses cannot be made without explicit or implicit statements regarding prior probabilities.

As the court has the responsibility in a disputed paternity matter to make meaningful

weekend is 30%, the switchboard does not light up at the television station with complaints by statisticians that the concept of probability is inapplicable to this unique event, and nobody calls the station to complain that they do not understand the weather report. Dislike of priors explains in part the attractiveness of tailed probabilities, which are prior free. However, one can ignore priors only at the cost of ignoring the issue at hand as the following examples will demonstrate.

Suppose a coin is presented. The question to be addressed is whether or not the coin is biased; the evidence to be evaluated consists of a trial of flipping the coin. The null hypothesis (H_0) is that the coin is true (i.e. when flipped, the probability of heads and probability of tails is each ½). If the experimental result is that the coin produced ten heads in ten flips, the null hypothesis can be tested by classical Neyman-Pearson methods as follows:

$$P(\geq 10 \text{ heads in } 10 \text{ flips/true coin}) = 2^{-10} \approx 10^{-3}$$

This result can be labeled "significant" or whatever, and the null hypothesis can be "rejected" or not, but a meaningful statement regarding the truth of the null hypothesis is not possible, in part because the counterhypothesis has not been tested. In this case, the counterhypothesis H_1(the coin is biased) cannot be tested because it is not a simple hypothesis. To say that the coin is biased is not to specify in what manner it is biased. For example, if "biased" means "biased in favor of tails" then the experimental result (10 heads in 10 flips) actually favors the null hypothesis that the coins is true, despite the "significance" of the result.

expert's error of assuming that the problem of disputed paternity is subject to similar limitations, when, in fact, the ideal solution can be obtained in a straightforward manner. Thus, they bring their biostatistical cannons to attack the impregnable wall of the fortress, when in truth they possess the biostatistical key which unlocks the front gate.

2. Other statistics which can be computed in disputed paternity cases are intuitively attractive. For example, the observation that an alleged father is not excluded by a powerful combination of genetic tests is powerful intuitive evidence that he is, in fact, the biological father. It is thus natural to conclude that the exclusion probability (A) is an important statistic in disputed paternity. On the other hand, if the man is nonexcluded, he is either the father or a non-excluded nonfather. This issue also needs to be addressed. One way to do so is to examine the distribution of the PI for fathers, or for nonfathers, or for non-excluded nonfathers. This leads naturally to computation of inverse ("tailed") probabilities and to inferences of the Neyman-Pearson type.

3. Many statisticians don't like prior probabilities. They correctly view such a probability as a probability concerning an event. As in an absolute sense every event is unique, one can assert that the concept of probability does not apply to events. This seems to me to be an excessively academic point of view. In our daily lives we are accustomed to making inferences regarding events; events which may be regarded as unique. We are also accustomed to expressing and understanding our expectations of unique events in terms of probabilities. Thus, when the television meteorologist announces that the chance of rain this

H_0: The man is a nonfather of the child

H_1: The man is the father of the child.

The paternity index is defined as

$$PI = \frac{P(\text{phenotypes of trio}/H_1)}{P(\text{phenotypes of trio}/H_0)}$$

The PI is a classical likelihood ratio which has a large number of interesting properties, of which only three will be discussed here. The first two are:

1. In the typical case, both hypothesis are counterhypothesis are simple, in the sense that each completely specifies the frequency distribution of the phenotypes of the trio. Given the phenotypes of the trio the PI is, therefore, a number free of any arbitrary parameters.
2. Given the prior probability of paternity and the PI application of the rules of conditional probability yields W, a conditional probability of the form

   W = P(paternity/prior, PI)

   Comparison of this expression with the ideal solution to the problem of disputed paternity indicates that W is, in fact, that solution. Moreover, as the prior probability is independent of the phenotypes of the trio, the evidence relating to the question before the court has been factored: the PI contains all of the relevant genetic information relating to the probability of paternity.

All of the above has been known for many years. It is natural to ask: why is there still controversy? Other than confusion caused by failure to comprehend the fundamental aspects of the problem, there appears to me to be three contributing factors:

1. Most statisticians are accustomed to inferential problems about which only incomplete and indirect solutions can be obtained. Some of them make the

Paternity Index (PI)

Jeffrey Morris, M.D., PhD.

Department of Pathology

Memorial Medical Center

2801 Atlantic Ave.

Long Beach, CA 90806

As we are discussing the solution to a problem of biostatistical inference it is best to begin by defining the problem. The problem of disputed paternity can be reduced to the following question:

Is this man the father of this child, or not?

Many other questions can be and are addressed during the course of a disputed paternity case, but when the time comes to make a decision this is the only question which remains.

In absolute terms we can never be certain of paternity (or of nonpaternity). It follows that the answer to the above question can only be given in probabalistic terms. The ideal solution to the question posed above is a conditional probability of the form

P(paternity/evidence), the posterior probability of paternity.

During the course of the evaluation of disputed paternity a great many statistics can be defined, and a great many probabilities related to these statistics can be computed, but only the posterior probability of paternity (and its complement) are of direct relevance to the question before the court. In general, it is uncommon for a problem in biostatistical inference to admit to a solution in the form of a posterior probability. When such a solution is not possible, a variety of indirect biostatistical methods can be applied, none of which directly address the question. Whatever the biostatistical method utilized it is customary to define a null hypothesis (H_0) and counterhypothesis (H_1). For the problem of disputed paternity, these are:

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For reasons of neutrality of the "utility" principle - i.e. equality before the law - Essen-Möller recommended neutral and standardised priors. Realistic prior plausibilities are frequently higher or lower than these. Thus W-values in reality represent orientation data and not absolute data.

It is possible to obtain a realistic plausibility from a given W-value by including a realistic prior value. However the latter should only be used in exceptional cases. Usually it suffices - especially in cases of promiscuity or questionable bipaternity - to aim for the highest W-value possible so as to eliminate doubts arising from a low realistic prior.

Most German experts as well as those in other German-speaking countries have regarded Essen-Möller's W-value as the most valuable of all possible serostatistical parameters for at least the past twenty years. In 1966 a survey was conducted among 111 German experts (7); 75% replied, and of these 80% used the Essen-Möller plausibility. Only one used the exclusion probability. In another survey conducted this year - 19 years later (8) - 98,5% of the respondents use the Essen-Möller plausibility. Only one regards the exclusion chance as sufficient.

It is unlikely that the practice of twenty years and more - which has moulded the practice of justice in this field right up to the German Supreme Court - will change in Germany in the future. The serostatistical probability of kinship will continue to be determined in accordance with the principle of Essen-Möller, and in this form will be reflected in the Court's judgements.

high or low: a W-value always corresponds to the plausibility of the X-hypothesis in one hundred equivalent cases, provided

a) that the files contain prior to the blood group opinion as many cases with a correct null-hypothesis as cases with an incorrect one;
b) that adequate genetic frequencies were used in obtaining W.

The third merit: In a given case each hypothesis is not only related to a certain family-tree frequency but also to a prior plausibility. The latter can easily be included in the computation of probabilities of the hypotheses.

In a two-hypotheses case the following formulas are valid:

$$W_X = \frac{f(X) \cdot aprW_X}{\left[f(X) \cdot aprW_X\right] + \left[f(Y) \cdot aprW_Y\right]} ;$$

$$W_Y = \frac{f(Y)\ aprW_Y}{\left[f(X) \cdot aprW_X\right] + \left[f(Y) \cdot aprW_Y\right]} .$$

To avoid prejudice Essen-Möller recommended using only neutral prior plausibilities irrespective of the nature of the case in question. Thus one assumes that in all imaginable cases the realistic prior plausibilities are approximately equal. However such an assumption may be unjustified for at least the following three groups:

a) in cases where the mother was a prostitute at the time of conception,
b) in cases where the mother is non-commercially promiscous,
c) in cases of questionable bipaternity.

The observations of Dr. Weber/Cologne (5) indicate that a neutral prior plausibility is justified in prostitute cases.

In cases of non-commercial promiscuity this cannot be assumed as easily. As no adequate statistics are available for these cases, the Court should be recommended to accept biological paternity only at $W \geq 99{,}73\%$. By analogy this applies to cases of questionable bipaternity (6); superfecundation is so rare that its plausibility must be at least $W = 99{,}73\%$ to be accepted as proved.

If $f(X)+f(Y_1)+f(Y_2)+f(Y_3) = 100\%$, then

$W_X = 0{,}13\%$
$W_{Y_1} = 99{,}86\%$ } Sisters 1 and 2 have the same father;
$W_{Y_2} = 0{,}005\%$
$W_{Y_3} = 0{,}005\%$ } sister 3 has a different father.

These examples emphasize that the serostatistical information is derived from pedigree frequencies; these are related to the hypotheses that have been set up in the respective case. Furthermore it can be seen that 2-hypotheses cases can be solved quite easily by means of a formula where the sum $f(X)+f(Y) = 1$. Multiple hypotheses cases, however, may require summing of hypotheses which may result in more than two plausibilities of kinship - e.g. in a "two-men" case there are three plausibilities of paternity: one for the defendant, one for the witness and one for an unknown man (3).

By defining the X and Y frequencies Essen-Möller paved the way to solving two-hypotheses as well as multiple-hypotheses cases.

The second merit: A likelihood ratio $\frac{Y}{X}$ (Essen-Möller) or $\frac{X}{Y}$ (Gürtler, 4) is suitable for simple 2-hypotheses but not necessarily for multiple-hypotheses cases. If, however, probabilities are used (as recommended by Essen-Möller) every imaginable hypothesis can easily be allotted an individual probability.

Unlike likelihood ratios, probabilities are familiar notions in anybody's daily life. Thus lawyers and participants in a lawsuit have a clearer idea of the value of the evidence if W-values are used instead of the rather esoteric likelihood ratios.

Regardless of whether a W-value concerns a normal case, a deficiency case, an incest case, a sibling case or any other, of whether this W-value has been obtained from the findings for a few or numerous genetic systems and of whether the probability of exclusion for non-fathers is

of the cumulated frequencies for X and Y calculated by using our "kinship program" (2) shows that

W_X = 97,6%: indicating that it is "very likely" that the plaintiff is the father of Th, and

W_Y = 2,4%: indicating that the paternity of an unknown man is "very unlikely".

After rejecting hypothesis Y we compared the 4 X-frequencies one with the other and came to the conclusion that - with a probability of 90% - either the children Ma and Na are full siblings or that each of the 4 children has a different father.

In another case (from Professor Henn, Innsbruck) the question was whether or not three sisters have the same father. Here, there is one X-hypothesis,

"All three sisters have the same father",

and are four Y-hypotheses:

"Sisters 1 and 2 are full sisters"
"Sisters 1 and 3 are full sisters"
"Sisters 2 and 3 are full sisters"
"All three sisters are half-sisters".

On the basis of other evidences the fourth Y-hypothesis may be excluded.

The following X and Y frequencies are calculated:

$$f(X) = 8.343\ 10^{-31}$$

$$f(Y_1) = 6.185.000\ 10^{-31}$$

$$f(Y_2) = 0.035\ 10^{-31}$$

$$f(Y_3) = 0.035\ 10^{-31}$$

$$\Sigma f(X,Y_1,Y_2,Y_3) = 6.193.410\ 10^{-31}$$

The ESSEN-MÖLLER Method

Konrad Hummel (Institute for Blood Group Serology, University of D-7800 Freiburg, POB 820; FRG)

If a method is known by a person's name, as is the case with the Essen-Möller method (1), one can wonder where exactly lie the author's merits that justify such an allocation. In my opinion Essen-Möller has three merits:

1. introducing the frequencies X and Y as biostatistical parameters;
2. recommending a W-value indicating the plausibility of paternity - and not using a likelihood ratio;
3. weighting each hypothesis by a neutral prior plausibility.

I shall discuss the importance of these three merits in turn:

The first merit: For each possible hypothesis in a case of disputed parentage, a corresponding family tree can be set up. In the simplest case the X pedigree represents the null-hypothesis: "The man is the child's father"; the Y pedigree represents the counter-hypothesis: "Another man is the child's father".

The W_X-probability of the null-hypothesis and/or the W_Y-probability of the counter-hypothesis can be obtained by comparing the respective frequencies of the two family trees:

$$W_X = \frac{f(X)}{f(X)+f(Y)}\ ,\ W_Y = \frac{f(Y)}{f(X)+f(Y)}.$$

In a case from Dr. Hirtz, Oldenburg, the child Th has three siblings; these, however, have not been fathered by the plaintiff, but by an unknown man or even different unknown men.
This results in four family trees for the X-hypothesis "The plaintiff is Th's father" and as many as ten for the Y-hypothesis "An unknown man is Th's father". Comparison

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that is generated when conflicting statements from various publications are presented. Recently some papers in the American Journal of Human Genetics [4,5] have raised several questions about the validity of inclusion estimates. It is unfortunate that the rationale on which these conclusions were based is faulty. It is my hope that the discussions in this symposium will address some of the issues raised in these articles. I hope that through the diversity of opinions expressed here we can reexamine several critical questions without undermining the credibility of the methods used by others.

In conclusion, the following statement, made over thirty years ago, is still applicable.

> "[I]n the field of contested paternity... the truth is so often obscured because social pressures create a conspiracy of silence or, worse, induce deliberate falsity.
>
> The value of blood tests as a wholesome aid in the quest for truth in the administration of justice in these matters cannot be gainsaid in this day. Their reliability as an indicator of the truth has been fully established."[6]

mind who will use the information and how it will be perceived. In my country (U.S.A) parentage is often decided by a jury after an adversarial proceeding. In each case the individuals asked to determine the meaning of the biostatistical evidence will have little or no experience on which to base their decision. They will also have listened to a series of convincing arguments by each side which will attempt to extoll and discredit the tests and their meaning. In my opinion it is important that each advocate of particular approach to the calculation be mindful of the potential this process has for confusing the users of information which, though obtained differently, has essentially a similar meaning.(3)

A recent case of mine illustrates what can happen when a jury is presented with different experts' opinion on the biostatistical estimate of paternity. My findings indicated that the likelihood of paternity (using a 50% prior probability) was greater than 99%. The defendant then produced a biostatistician as a witness who claimed that in his opinion (method of calculation not known) there was only a 90% chance the man was the father. After hearing all the testimony, the jury decided the alleged father was not the father. When questioned after the trial it was apparent that the two estimates confused the jury, which was reluctant to declare the man the father because he had only been casually involved with the mother.

The confusion that can occur when two experts seem to have different opinions is minimal compared to the misunderstanding

Biostatistical Evaluation of Paternity

Herbert F. Polesky, M.D.
Director Memorial Blood Center of Minneapolis
2304 Park Ave. , USA-Minneapolis MN 55404

Introduction

"In my experience of these cases, apart from one other class - namely fish poaching cases - there is no class of case in which there is a greater degree of perjury in the courts. In those cases which are fought at all there is always flat denial on the one side or the other of the facts at issue."

- Lord Merthyr (1)

"There is nothing more shocking than that injustice should be done on the basis of a legal presumption when justice can be done on the basis of fact."(2)

The purpose of this symposium is to present various approaches for evaluating the results of genetic marker testing in cases of disputed parentage when the tested man has not been excluded. Although each of the speakers advocates a slightly different way of calculating his results, all of us are in agreement that the goal of testing is to exclude all non-fathers and to provide information that will help to establish the identity of the true father. The genetic tests now available make it theoretically possible to achieve this goal in almost all cases; however, as scientists we recognize that the information provided by testing is not absolute.

As one looks at the various approaches to biostatistical evaluation of genetic marker tests it is important to keep in

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which would, at the same time, reduce costs.

The legal validity and safekeeping in the preservation of the samples, which implies the fractioning of the different components of the blood, must be solved with accuracy. Each sample must be kept in a proper manner so as to guarantee the viability of the same and taking into account future field of investigation (as, for example, polymorphism of DNA).

The usefulness of identification by means of the study of the proteins in the hair and dental casts shall have to be decided upon.

In the cases for which it is possible to recover the remains of the murdered parents, it is of great interest to establish the possibility and the degree of usefulness in investigating the genetic markers of these remains. In order to set up a National Bank of Genetic Data, the support of the international scientific community is fundamental. It is necessary to establish channels of scientifical advice and exchange that would allow reaching a solution with respect to the multiple problems that will, without doubt, arise in carrying out this monumental task. Furthermore, due to the scarce economic resources of the Durand Hospital, it is fundamental to count with support in order to provide the necessary reagents in order to carry out the studies of the National Bank of Genetic Data.

Due to the volume and complexity of the genetic data to be dealt with and, taking into account the experience accumulated in this field, it is necessary to count with proper computers and computer programmes in order to carry out this task.

Due to the fact that about 20% of the relatives of the Missing Children reside abroad, it shall be necessary for these persons to be analysed at Centers of an acknowdleged scientific level that shall have to send the results and a blood sample to the National Bank of Genetic Data.

Even today, we are still having many difficulties in carrying out our task. Some of them are linked with the powerful interests which we have to face in the task of returning their true identity to these children. On the other hand, the method of identification by means of genetic markers is totally unknown to Argentine judges who, many times, are reluctant to give these studies the importance which they deserve. In this way, the court orders are postponed, both in the case of the children and the grandparents. Many grandparents have died without the corresponding judge having upheld the petition to order an expert witness's exam to be carried out. These studies are also unknown to the experts that form part of the Forensic Medical Staff who, in one case, confused in their report the concepts of "inclusion" and "exclusion" and thus reduced the percentage of the index of grandparentship.

The provisions establishing that the relatives of Missing Children have to pay for the studies of the genetic markers are still in force. The Military Government was responsable for the disappearance of children, Grandmothers of Plaza de Mayo are able to get the reagents due to the scarce economic resources and, in the end, the relatives of missing children have to pay for the studies. This situation requires an immediate change.

The identification of the Missing Children and their restitution to their legitimate families is an ehical duty which, keeping in mind the restoration of principles and order, society has the unavoidable obligation of promoting supporting.

This situation shall benefit not only the Missing Children but also the children on the whole, who shall never again have to undergo the violation of their most essential human rights when being deprived of their identity, their family and their freedom.

In order to achieve the monumental task of the National Bank of Genetical Data, the taking part and support of the Argentine government, the rigorous cooperation of the affected relatives and the help of the international scientific community are essential. Taking into account the absence of parents and, taking into consideration that the relatives will not be at reach to repeat the studies, it is necessary to work out a reasonable record

essential. For said purpose, it is necessary to keep all the genetic information of these children's relatives. To achieve this, Grandmothers of Plaza de Mayo, by means of petitions presented before the Executive, Legislative and Judicial Powers, have worked ardously in order to create a National Bank of Genetic Data of Relatives of Missing Children. Likewise, the National Bank of Genetic Data shall also have to be applied to the relatives of stolen children, thus constituting a useful measure to prevent the commercial traffic of minors.

This genetic information shall have to be safely and properly kept for a period of at least sixty years.

The studies of the genetic markers must be carried out on every child suspected of being a Missing Child. It must be possible to crossmatch the results, by means of proper computer programmes, with the data filed in the National Bank of Genetic Data. It must be possible to crossmatch the information of children suspected of being missing but whose relatives have not been found through the National Bank of Genetic Data, with the data of transplant banks or similar institutions.

The Affiliation Team of Grandmothers of Plaza de Mayo has prepared and/or received up to now, the family-trees of 107 family groups (71 % of the non-found children). A scheme of the family tree including three generations, together with instructions of whow there are to be filled with the name and surname, adress, country and health conditions, was sent to all the family groups.

Only 101 families shall count with the "Index of Grandparentship" to prove, when the time comes, that a certain child belongs to its biological group.

A little over 500 persons are to be studied immediately. According to the frequency in the reception of reports and to the calculation of the total number of Missing Children made by Grandmothers of Plaza de Mayo, we estimate that the total number of persons to be analysed adds to 2000.

Up to now, 79 persons, members of the family groups of 15 Missing Children, have been studied for the files of the National Bank of Genetic Data.

in the above mentioned cases.

In our country, due to the petition put forward by the Grandmothers of Plaza de Mayo, these studies are carried out in the Service of Immunology of the Durand Hospital located in Buenos Aires. This is an official institution that counts with skilled professionals and an adecuate functional structure. Eight systems of blood groups and the systems HLA-A, B and DR which add up to an exclusion average of 98 %, are been studied. In a chort while it will have a staff skilled in the study of plasmatic proteins and erythrocytic isoenzymes. Frecuency charts of Argentine populations are used to assess the results of the systems HLA and the great mayority of the blood groups. Caucasian charts are used for those groups for which information is lacking due to being a mayority in the origin of the Argentine population.

Up to now, it has been possible to confirm the identity of three missing children and of one abandoned child. In one of the cases, the biological link with the petitioning group was completely set aside. Another case is still being studied. Except in the case of the abandoned child, where we could study the mother, due to the absence of parents, the rest of the investigations were carried out, according to relatives available, with grandparents, uncles, and brothers of the supposed missing child.

In none of the cases, the families who had these children, in spite of alleging being the biological parent, allowed their genetic markers to be studied. There exist court orders to carry out the studies on six children, but these have not yet been done due to the objection of the imposed parents. In two of the cases the court order was upheld by the Court of Appeals. One of the decisions authorized the compulsory taking of blood samples to the minor notwithstanding of the reluctancy of the imposed parents.

It is impossible to say when the last Missing Child will be found. Some will be identified soon, while others will wait years. In some of the cases, it shall be the children themselves, upon adulthood, who shall find out about their real origin. To ascertain the proper conditions to enable the identification of these children beyond illness, absence or death of relative, is

Nine children have been returned. In one of the cases, the return was ordered by the Court of Appeals. The way in which the restitution is carried out, has been adapted considering the circumstances of each case in particular, counting with the support of a group of persons belonging to different professions formed by doctors, psychologists, lawyers, etc. None of the children suffered psychological descompensation whether at the time of restitution, nor afterwards. In all the cases, the restitution has been a positive and therapeutical event for the child, and all the children enjoy both physical and mental health.

The need of counting with an efficient method to prove the identity of a child, of whom, many times, not even its sex was known, was necessary from the start of the search. A system capable of verifying with validity the biological link of a child with its corresponding family group and, at the same time, capable of excluding false parents, was necessary.

Different possibilities were investigates. Finger prints and foot prints are useful only for the cases of kidnapped children and when the same have been properly taken. The anthropobiometrical methods, under investigation in U.S.A., have no legal validity. The machine of Age, that would permit to rebuild the face of the child in time, is useless for children born in prison.

Due to the phenomenon of the disappearance of children in Argentina and the fact that Grandmothers of Plaza de Mayo set forth the problem, different scientifical groups, that joined in the cause, have been working on the finding of methods and concepts that would enable them return their stolen identity to these children.

Due to its great variety and stability in time, the study of genetic markers has shown to be an adecuate method to prove the identity of these children. On the other hand, already since 1971, there exist precedents in world-wide literature in the field of affiliation, showing how to establish the biological link between a child and his family for those cases of absence of one or both parents.

These efforts have allowed to reach to the concept of "Index of Grandparentship", which has been applied successfully in

GENETICAL IDENTIFICATION OF "MISSING" CHILDREN IN ARGENTINA.-

Jorge Berra, Diana Grinspon, Norberto Liwski, Morris Tidball Binz (Equipo de Filiación Abuelas de Plaza de Mayo, Montevideo 459, Capital Federal, República Argentina).

Between the years 1975 and 1983 in Argentina, hundreds of children were kidnapped with their parents or were born during the imprisonment of their mothers in secret detention camps and were then given to persons or families generally connected with the repression body. This monstrousity was the product of a deliberate, organic and systematic plan put into execution by the Military Dictatorship in order to produce terror among the people and achieve their objectives. Important pediatric specialists have compared the situation to slavery. In general, the parents of these children are still missing.

In 1977 the Grandmothers of Plaza de Mayo is created, an organism for human Rights, recognized world-wide because of the efforts in trying to return these children to their legitimate families.

Up to now, the Grandmothers of Plaza de Mayo have received reports related to 189 missing children, of whom 58 were kidnapped and 131 were born in captivity.

Thirty-five children have already been found as a result of a continous and anxious search, counting with the support of the people. Four children had died, 12 are still living with their "foster" family and 9 have been returned to their legitimate families. The cases of 10 children presumably missing and under the charge of persons connected with the repression body, are pending before Courts of Justice with the purpose of verifying their identity and then return these children to their legitimate families.

Four of the children found had died, three of them murdered by the Armed Forces and the other was left to die, abandoned in a hospital.

Prior agreement between the families, 12 children are still living with their "foster" family. These children have their real names, know their origin and keep in touch with their real family.

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FIGURE 1

TEST	DISEASE	
	HLA PREDICTS	
	NOT EXCL (-)	EXCL (+)
PI < X (+)	False positive	True positive
PI $\geq$ X (-)	True negative	False negative

$$PV+ = P(\text{Disease} \mid \text{Pos test})$$
$$= P(\text{HLA excludes} \mid PI < X)$$
$$= TP/(TP + FP)$$

$$PV- = P(\text{No disease} \mid \text{neg test})$$
$$= P(\text{HLA does not exclude} \mid PI \geq X)$$
$$= TN/(TN + FN)$$

TABLE 1.

PI Before HLA Test	No. Tested	HLA Excludes #	HLA Excludes %
> 10	194	20	10.3
10 - 25	53	7	13.2
25 - 50	47	4	8.5
50 - 100	27	1	3.7
< 100	92	2	2.2
	413	34	8.2

TABLE 2.

	PREDICTIVE VALUE (%)	
PI CUTOFF	HLA EXCL (+)	NO HLA EXCL (-)
10	8.3	93.3
25	9.8	95.9
50	9.5	97.5

TABLE 3.

PI CUTOFF	CHANCE OF EXCLUSION WITH ADDED HLA TESTING (%) (1-PV neg)
10	6.7
25	4.1
50	2.5

The results of our data (see Table 2,3) indicate that the prior PI is useful in predicting whether additional tests (HLA) might exclude the already tested man. This same approach should prove useful for other marker systems.

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CHANCE OF EXCLUDING PATERNITY BY HLA IN MEN NOT EXCLUDED BY OTHER SYSTEMS. H. F. Polesky, Jane M. Souhrada, Dale D. Dykes, and Margaret Helgeson. Memorial Blood Center of Minneapolis, Minneapolis, Minnesota, 55404, U.S.A.

In our laboratory we routinely test all cases of disputed parentage with a battery of 14 to 16 genetic systems (red cell antigens, serum proteins and red cell enzymes) that in accordance with the AABB Standards for Parentage Testing should exclude 95% of falsely accused men.(1) In selected cases the test battery used is expanded to include several additional systems. The reasons for doing more testing include cases with a PI $\leq$ 10 after routine tests are completed, cases with a single indirect exclusion, cases where there is a missing or deceased parent or when two men are tested and neither is excluded. In some cases, despite what appears to be a conclusive result, the court will order more testing. On other occasions we have been asked to justify why more testing is not considered necessary.

In order to evaluate the possibility of predicting the chance of obtaining an exclusion by doing additional testing we have compared the number of men only excluded by HLA -A,B with those not excluded by HLA or any other system (2). For each case the paternity index (PI) was calculated for all systems except HLA. A matrix using various ranges of PI for cases not excluded or excluded by HLA was used. (Figure 1) From these data the predictive value of a negative test, HLA will not exclude given a PI value for all other tests, was determined.

The study group consisted of 413 Caucasian trios (see Table 1). Testing prior to HLA included multiple systems (14-20). Only cases with a CPE $\geq$ .95 were included. Thirty-four men in this group were excluded by HLA only. In twenty (59%) of these cases the PI was less than 10 based on all other tests. In two (6%) cases the initial PI was greater than 100. In one of these cases the brother of the man excluded by HLA was not excluded. In the other case, two of several accused unrelated men were not excluded. Both had PIs > 100. One was and one was not excluded by HLA.

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Results

Approximately 18 A and 25 B locus specificities were recognized using multiple antisera for definition. The total number of artificial trios examined, the number of observed exclusions, and the power of exclusion, expressed as a percentage, are displayed in the table below:

Observed Exclusion Frequency in Artificial Trios

	No.		%
Race	Artificial Trios	Observed Exclusions	Power of Exclusion
White	461	431	93.5 ± 1.1
Black	128	118	92.2 ± 2.4
	589	549	93.2 ± 1.0

Discussion

Examination of artificial trios is ideal in that it uses actual reported phenotypes rather than theoretical values and therefore considers serological and technical failures of antigen definition. It assumes that the artificial trios are indeed false and that the alleged fathers are always non-fathers, a rather safe assumption. The method is not reliable if different HLA reagents were used to define the antigens of the alleged father than were used for the mother and child. This problem is circumvented by the use of contiguous sequential cases from the same laboratory which utilized identical lots of reagents for all persons tested.[6]

The PE value of 93.5% for whites for the HLA-A,B system observed in this study does not differ significiantly from the 95.7% value reported by Mayr.[4] This difference could be due to chance random variation, gene frequency differences, failure to recognize some of the antigens present as a result of technical problems in processing, antisera failures, the rather strict criteria employed for the definition of HLA antigens, or a combination of two or more of these possibilities. In spite of the increased frequency of blanks in blacks, their PE value was comparable to that of the whites, probably due to the greater phenotypic heterogeneity in blacks at the A locus. This study does underscore the powerful utility of the HLA system in laboratory tests utilized for the resolution of parentage disputes.

PROBABILITY OF EXCLUSION (PE) OF THE HLA-A,B SYSTEM IN NORTH AMERICAN WHITES AND BLACKS

R.H. Walker, M.A. Meyers, L.M. Phillips

William Beaumont Hospital, Royal Oak, Michigan USA

Introduction

Although it is well known that the HLA system is the single most powerful genetic system commonly used in parentage testing, its efficiency with currently available antisera in excluding a falsely accused man has not been well documented. Various estimates have yielded values which range from 86-96%.[1-4] This value will, of course, vary with the number of known alleles, their gene frequencies, the extent of testing, and the criteria used to define the antigens.

Methods

Two commercial HLA typing trays were used for each individual typed. Criteria utilized for antigen definition conformed to the Standards for Parentage Testing Laboratories* of the American Association of Blood Banks.[5]

An observed estimate was obtained by moving the alleged father forward one case in the chronological sequence of paternity test cases in our archives from the last five years. The assumption was made that all of the alleged fathers were then false fathers and the HLA system was utilized alone to determine if these false fathers could be excluded. The artificially created trios were examined individually by each of the co-authors for evidence of an exclusion.

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*Each HLA antigen must be tested on two different trays and be defined by at least two different operationally monospecific sera, or by one monospecific serum plus two multispecific sera or by three multispecific sera.

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CONCLUSION

The regulation of paternity and maternity is very important in the internal legal intercourse of the SFRY (the interrepublic and the interprovincial) as well as in its international legal intercourse. But with the passing of both discussed federal laws respectively their collision regulations on the admitting, ascertaining and contesting of paternity and maternity the conditions are given for a successful solving of the contestable cases which appear in Yugoslav international legal intercourse because of the difference of the legal organizations of foreign countries.

ZUSAMMENFASSUNG

Die Regelung von Vater- und Mutterschaftsangelegenheiten ist sehr wichtig sowohl im inneren juridischen Verkehr der SFRJ (im interrepublikanischen und interprovinziellen) als auch in ihrem internationalen juridischen Verkehr. Mit der Annahme beider diskutierten Bundesgesetze, beziehungsweise ihrer Kollisionsregeln über die Erklärung, Feststellung und Widerlegung von Vater- und Mutterschaft sind die Bedingungen gegeben für eine erfolgreiche Lösung von Streitfällen welche wegen der verschiedenen Einrichtungen innerhalb Jugoslawiens auftreten und Fällen welche wegen der verschiedenen Rechtsordnungen fremder Länder im jugoslawischen internationalen juridischen Verkehr auftreten.

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III. REGULATION OF PATERNITY AND MATERNITY AFFAIRS WITH AN INTERNATIONAL ELEMENT

1. General

Until the passing of the Law on the settlement of the collision of laws with the regulations of other countries in certain relations in 1982 Yugoslav law had no collision regulations for regulating legal relations between parents and children with an international element. Because of the lack of collision regulations Yugoslav law theory proposed various solutions. And from 1956 on Yugoslavia signed bilateral agreements on international legal help with Bolgaria, Czechoslovakia, France, Greece, Hungary, the GDR, Poland, Rumania and the USSR. In the continuation we shall discuss the collision regulations from the new law and the regulation of paternity and maternity in the mentioned bilateral agreements.

2. Regulation of paternity and maternity with an international element according to collision law

In chapter 2 of the Collision Law entitled " Law which must be used " we find in articles 40 - 43 the collision norms for regulating the relations between parents and children and for the liability of maintenance between blood relations and in - laws. But for the admission, ascertaining and contesting of paternity and maternity the following collision norm is known: the law of that country is used of which that person, whose paternity resp. maternity is admitted, ascertained or contested, is a citizen at the time of the child's birth (art. 41).
Thus the lex nationalis of the presumable father resp. mother at the time of the child's birth is decisive for these relations. This point of connection is quite unusual since Yugoslavia determined with the bilateral agreements signed with the mentioned countries that the child's lex nationalis was decisive for these relations. Since cases may come up in which according to the native law of the presumable father it may not be possible to ascertain his paternity, Yugoslav law theory proposes in such cases to turn to the institution of public order. According to this institution the legislation of a foreign country is not used if its effect would be in opposition to the basis of the social order determined by the Constitution of the SFRY (art. 4 of the Collision Law). [8]

In the bilateral agreements on international legal help which Yugoslavia signed with Bolgaria, Czechoslovakia, France, Greece, Hungary, the GDR, Poland, Rumania and the USSR we have the collision regulations for the admitting, ascertaining and contesting of paternity resp. maternity. Decisive is the child's lex nationalis, which is by some agreements bound to the time of the child's birth. But in the bilateral agreement signed with Czechoslovakia and the GDR we find also points of connection regarding the form of the admission of paternity and maternity. The prescribed form of that country is regarded as satisfactory within which the admission took place.

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8. See: M. Ilešič, A. Polajner - Pavčnik and D. Wedam - Lukić, Mednarodno zasebno pravo (International private law), Zakon s komentarjem (Law with comments), p.64.

2. Collision regulations for the establishing and contesting of paternity and maternity

The collision norm from article 28 of the interrepublic collision law distinguishes the case when all parties are Yugoslav citizens from the case when the presumed father or the presumed mother is a Yugoslav citizen, but according to the regulations of international law the Yugoslav law must nevertheless be used. It must be stressed that this collision norm uses the expression presumed father or presumed mother for cases of establishing and for cases of contesting paternity or maternity.

In the first case, in which all parties are Yugoslav citizens, the law of that republic respectively autonomous province is used within which the presumed father respectively mother has his or her fixed residence. [5] If a person does not have the permanent residence within Yugoslavia then subsidiarily the law of that republic is used of which he is citizen. [6]

In the second case, in which the presumed father resp. mother is not a citizen of Yugoslavia, but according to regulations of international law the Yugoslav law must be used [7], the law of that republic resp. autonomous province is used within which the presumed father resp. mother has his or her permanent residence.

The primary points of connection are thus equal in both cases i.e. the permanent residence of the person who is presumably the father resp. the mother. The subsidiary point of connection is in the first case the republican citizenship of the person who is presumably the father resp. the mother and in the second case the permanent residence of the child and the republican citizenship of the child as the next subsidiary circumstance.

The interrepublic collision law has no special regulations on the recognition and execution of individual deeds issued in various republics and autonomous provinces. Here the constitutional stipulation from art. 250 of the Constitution of the SFRY is in force, from which ensues that any decrees, documents or other individual deeds issued by state organs and authorized organizations in one republic resp. autonomous province are equally valid also in other republics resp. autonomous provinces.

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5. A fixed residence is a place in which someone actually settles (corpus) with the purpose of making it his permanent domicile (animus manendi). A temporary residence is a place in which someone stays for a shorter or longer time, but without the intention of staying there permanently.
6. In SFR Yugoslavia we know Yugoslav and republican citizenship. The republican citizenship is regulated by laws on citizenship of individual republics. Only a citizen of Yugoslavia can have a republican citizenship. The Yugoslav citizenship is regulated by the Law on the citizenship of SFRY, publ. Ur.list SFRJ, No. 58 / 1976.
7. Under " regulations of international law " we understand: collision regulations from the Yugoslav collision law (esp. art. 41), stipulations from bilateral agreements and collision regulations from foreign legislations.

INTERREPUBLIC AND INTERNATIONAL REGULATION OF PATERNITY AND MATERNITY IN YUGOSLAV SOCIALIST LAW

Geč - Korošec Miroslava, Glaser Edvard, Univerza v Mariboru, Višja pravna šola, Oddelek za transfuziologijo in imunohematologijo, Splošna bolnišnica Maribor, Jugoslavija

I. INTRODUCTION

The necessity for the introduction of the interrepublic collision law arose with the year 1971 when the federal constitutional amendments from XX to XLII[1] were issued, which established new relations between the federation, the republics and the autonomous provinces. The legal competence of the republics and provinces was extended so that each republic and autonomous province regulates its field of family law and others independently and originally, except those belonging under the exclusive competence of the federation. That was a reflexion of the achieved degree of development of a socialist, selfgoverning and social system and an expression of the democratization process in legislation and other fields. Today we have eight different family law regulations in the SFR of Yugoslavia.[2] The interrepublic collision of laws and competences is therefore a new phenomenon in Yugoslav socialist law. In 1979 a federal Law on the settlement of collisions of laws and competences in status, family and inheritance relations was issued.[3]

For the first time in its law history the SFRY passed a law in 1982 which regulates international collisions and carries the name Law on the settlement of the collision of laws with the regulations of other countries in certain relations.[4] Yugoslavia settled the establishing of paternity and maternity also in some bilateral agreements signed with foreign countries.

II. THE REGULATION OF PATERNITY AND MATERNITY IN INTERREPUBLIC COLLISION LAW

1. General

The Law on the settlement of collisions of laws and competences in status, family and inheritance relations from 1979 includes in its chapter III entitled " Law used in marriage relations and the relations between parents and children, adoptions and guardianship " in articles 22. - 28. the collision norms which are used in the relations between parents and children. The interrepublic collision law brings in article 28 a collision regulation which is used in establishing or contesting paternity and maternity.

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1. Publ. in Ur. list SFRJ, No.29/1971 of 6.7.1971
2. The SFRY is composed of: the SR Bosnia and Herzegovina, the SR Montenegro, the SR Croatia, the SR Macedonia, the SR Slovenia and the SR Serbia, as well as the two autonomous provinces Kosovo and Vojvodina belonging in the composition of SR Serbia. Each republic and auton. province has its own laws in the field of family law.
3. Publ.in Ur. list SFRJ, No.9/1979 of 2.3.1979,further stated as: interrepublic collision law.
4. Publ. in Ur. list SFRJ, No. 43/1982 of 23.7.1982, further stated: collision law.

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The choice of the more likely frequency is a difficult one . In areas with mixed populations one has to consider the effect on matings of ethnic differences, of social stratification, and must evaluate the real opportunities of meeting for the involved persons.

A solution to the problem of frequency of the abnormal gene is that of taking the average frequency observed in the larger area in which the female can have the opportunity of finding a partner: this solution will avoid the risk of giving an excessive weight to the abnormal character in positivo or in negativo.

On excluding as a father a man with normal haematological characteristics, carrying the mother a beta-thalassaemia trait and showing the child beta-thalassaemia disease, one has to take into account the exceptional occurrence of a beta-thalassaemia silent gene.

Absolutely unreliable is the exclusion of a "normal" man, when the child has evidence of an alpha-thalassaemic disorder.

On the contrary Hb globin variants offer a perentory criteria for paternity exclusion.

The contribution of a gene to the value of Probability of Paternity is inversely proportional to the frequency of this gene: the abnormal haematological genes have a wide geographical variation of frequency (Tab. V).

Tab. V a. FATHER WITH A LOW FREQUENCY GENE

	Fr GENE	Pr P	I.P.	Fr Ex
Thalassaemia	0.08	86.21	6.25	84.64
	*(0.92)	35.21	0.54	0.64
	0.04	92.59	12.5	92.16
	*(0.96)	34.25	0.52	0.16
	0.013	97.47	38.5	97.42
	*(0.987)	33.62	0.51	0.02
Hb Hasharon	0.004	99.21	125	99.20
	*(0.996)	33.42	0.5	0.00
Hb S	0.00025	99.95	2000	99.95
	*(0.99975)	33.34	0.5	0.00
HbC, Hb Lepore, H.S.	0.0001	99.98	5000	99.98
	*(0.9999)	33.34	0.5	0.00

*low frequency gene absent in the Child.

In consideration of the dispersion of values of Probability and Frequency of Exclusion observed with limited genetical typing, the contribution of an abnormal gene is calculated only for trios and duos typed for 19-20 polymorphic systems (Tab. III).

Tab. III

17 FAMILIES - 26 CHILDREN

TESTED FOR 19-20 POLYMORPHIC SYSTEMS

L Father with an abnormal gene

observed in 20 Children - not observed in 6 Children

	abnormal gene of L Father			
	not included		included	
	Pr P%	Fr Ex%	Pr P%	Fr Ex%
20 Children with a.g.	96.4	93.9	99.9	99.8
6 Children without a.g.	98.9	97.7	97.9	97.7

For comparison the values observed in the 56 trios and duos with or without paternal transmission of an abnormal gene are reported in Tab. IV.

(Tab. IV)

Mean values for 56 (C-M-LF) and (C-M)

Tested for 19-20 Polymorphic Sistems

Pr P	Fr Ex
97.1%	94.4%

No paternity exclusion due to an abnormal gene was observed in our series. In 3 families, not included in the present series, the exclusion of 1 child came out from common polymorphic systems testing.

An alleged father can be excluded from paternity of a child with a beta-thalassaemia trait, if this man and the mother do not carry the trait.

Tab. I **87 FAMILIES-142 CHILDREN TESTED FOR 8-20 POLYMORPHIC SYSTEM**

45 Families-45 Children **29 Families-58 Children** **13 Families-39 Children**

18 Families - 28 Children tested for 8 - 9 polymorphisms
20 Families - 38 Children tested for 11 -14 polymorphisms
15 Families - 20 Children tested for 15 -18 polymorphisms
34 Families - 56 Children tested for 19 -20 polymorphisms

26 Families - 40 Children

L Father with an abnormal gene
observed in 26 Children
not observed in 14 Children

5 Families - 7 Children

L Father & mother with an abnormal gene
6 Children with the 2 interacting
abnormal genes

24 Families - 44 Children

Mother with an abnormal gene or
Mother & L Father with the same abnormal gene
Children etherozygous for the abnormal gene
or without the abnormal gene

32 Families - 51 Children

Mother and L Father
without abnormal genes

In 55 out of 87 families the diagnostic suspect was confirmed:the haematological abnormalities encountered are reported in Tab. II

Tab. II 55 FAMILIES WITH AN ABNORMAL GENE

Abnormal gene	Families	Children	Children with a.g.	Children without a.g.
β -thal	35	52	38	14
α-thal	2	5	4	1
Hb Lepore	1	3	1	2
Hb S	8	17	10	7
Hb C	2	2	2	-
Hb Hasharon	2	4	2	2
Other variants	3	5	2	3
Sickle-cell thal	1	2	1	1
H. spherocytosis	1	1	1	-
TOTAL	55	91	61	30

ABNORMAL HAEMATOLOGICAL CHARACTERISTICS IN DISPUTED PATERNITY.

Bestetti A.*, Assali G.*, Ripamonti M.*

Domenici R.**, Giari A.**, Bargagna M.**

* Servizio di Immunoematologia - Ospedale di Monza (MI)

** Istituto di Medicina Legale - Pisa (PI) ITALY

The thalassaemias, disorders of Hb globin synthesis, and the Hb globin structural variants are hereditary characters with a simple modality of trasmission. Therefore they can be usefully employed in paternity testing.

A preliminary remark of these genetic markers comes out from very simple laboratory tests:

- a blood count showing normal or elevated number of red cells along with a low M.C.V. and M.C.H. points out to a thalassaemic disorder
- the electrophoretic separation of red cells isoenzymes brings out at the same time the separation of Hb components of red cell lysate: therefore the presence of a haemoglobin variant can by easily noticed.

Further investigations are necessary only in the few cases positive at this preliminary screening.

In order to evaluate in concrete terms the use of these markers in paternity testing, along with the haematological study, we tested for 8-20 polymorphic systems the 316 members of 87 families investigated for a possible hereditary haematological disorder (Tab. I).

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FIGURE TWO

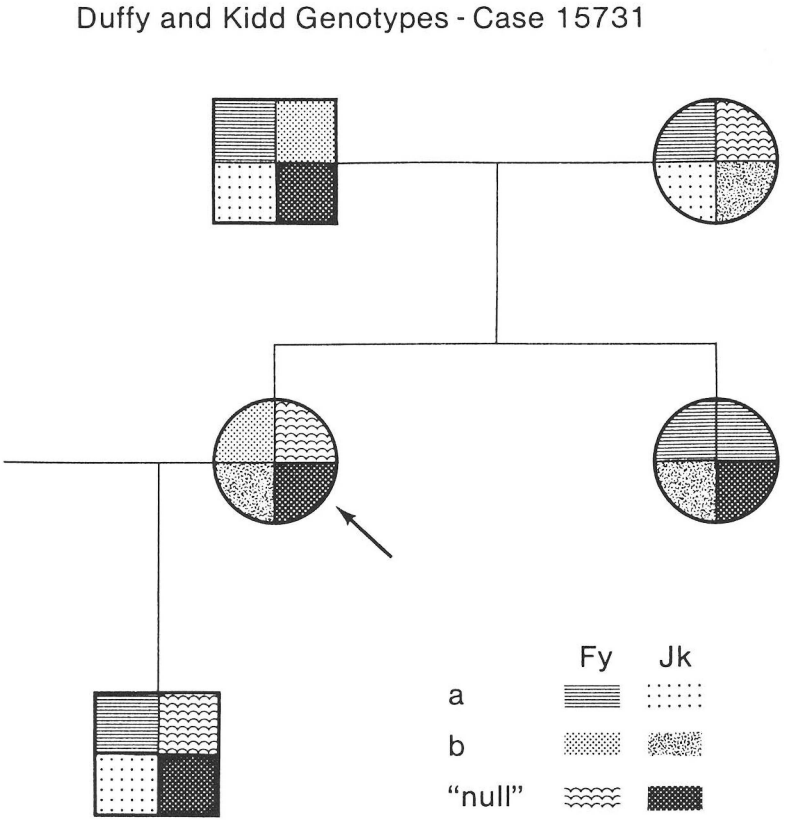


Table 1

Duffy and Kidd Typing on
Original Trio (Case 15731)

	Fy			Jk		
	a	b	Titer	a	b	Titer
AF II-3	+	−		+	−	
M II-4	−	+	NI*	−	+	Hetero
C III-7	+	−	NI	+	−	Hetero

Adsorption and elution studies

II-4 negative anti-Fy^a, - Jk^a
III-7 negative anti-Fy^b, - Jk^b

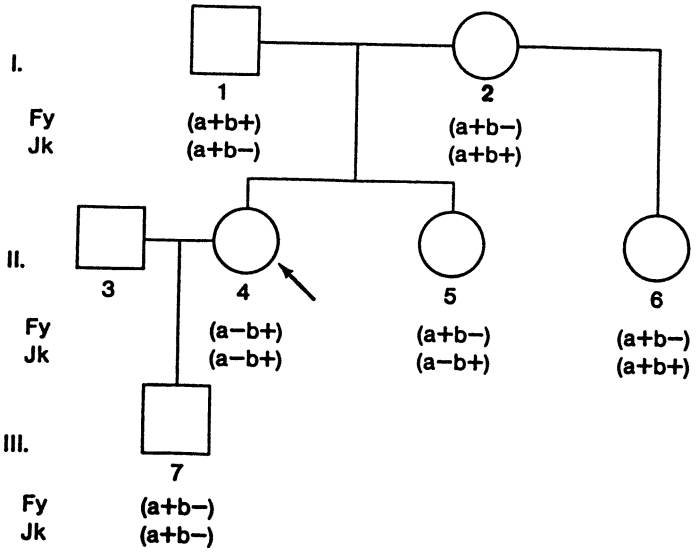
* Not informative

FIGURE ONE

FAMILY STUDY

Case 15731

(Fy & Jk typing)



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APPARENT EXCLUSION OF MATERNITY BY BOTH DUFFY AND KIDD.

Jane M. Souhrada and H. F. Polesky. Memorial Blood Center of Minneapolis, Minneapolis, Minnesota, 55404, U.S.A.

An apparent exclusion of maternity was found in two independent systems (Duffy and Kidd) in a Caucasian trio tested because of disputed paternity (see Table 1). No other contradictions to the rules of inheritance were observed in extensive testing (ABO, Rh, MNSs, Kell, GC, BF, HP, TF, PLG, Gm, Km, ESD, ACP, PGM1, and GLO).

New samples were obtained from the child and mother as well as from members of her family. The results of Duffy and Kidd testing are shown in Figure one. Based on this testing it appears that the propositus' father (I-1) is excluded from her paternity in the Kidd system. A similar apparent exclusion is also present in her sister (II-5). The propositus' mother (I-2) appears to be excluded in the Duffy system. No other inconsistencies in the expected inheritance patterns were observed in tests on the family members.

Our interpretation of the testing in this family is that the propositus has inherited null alleles in two systems and passed both to her child. In the Duffy system the *Fy* appears to be present in the propositus' mother and in the Kidd system the *Jk* is present in her father. (Figure 2).

The chance (P) of one individual having "null" alleles in more than one genetic system is the product of the frequency of the allele in each system. In this case using *Fy* = .005 and *Jk* =.001, $P = 5 \times 10^{-6}$. (1) The chance of finding a child in a paternity case with both "nulls" is 1.25×10^{-6} since the chance of both parents passing one or one parent passing both alleles is 0.25.

We have not been able to find reports of cases with two "null" alleles in one individual except where the systems are on the same chromosome. (2) These unusual cases do not invalidate the use of multiple indirect exclusions in determining non-paternity. (3) Findings in families like the one reported here support using frequencies for "nulls" in calculating estimates of paternity.

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TABLE 1

Optimized sequences of some genetic markers used routinely in our Institute, according to total and 1st rule exclusions

	Exclusion powers (%)		Ordination according to	
System	Total	1st rule	Total	1st rule
ACP1	22.69	15.45	1	1
GPT	20.03	8.03	2	5
GLO	18.54	6.45	3	9
HP	18.28	6.69	4	8
ME2	18.19	6.76	5	7
PGM3	14.64	8.29	6	4
C3	12.80	9.11	7	3
ESD	12.33	9.14	8	2
PGP	8.66	7.90	9	6
ADA	3.35	3.11	10	10

TABLE 2

Examples of outcomes from classical and first rule exclusion strategies in the choice of genetic systems for paternity testings.

Nr.of systems with maximum total excl. power	Nr.of systems with maximum excl.power by 1st rule	Total exclusion probability Pex (%)	1st rule excl.prob. P1 (%)	P1/Pex
20	0	98	72	.73
15	5	98	75	.77
10	10	97	78	.80
5	15	96	80	.83
0	20	95	82	.86

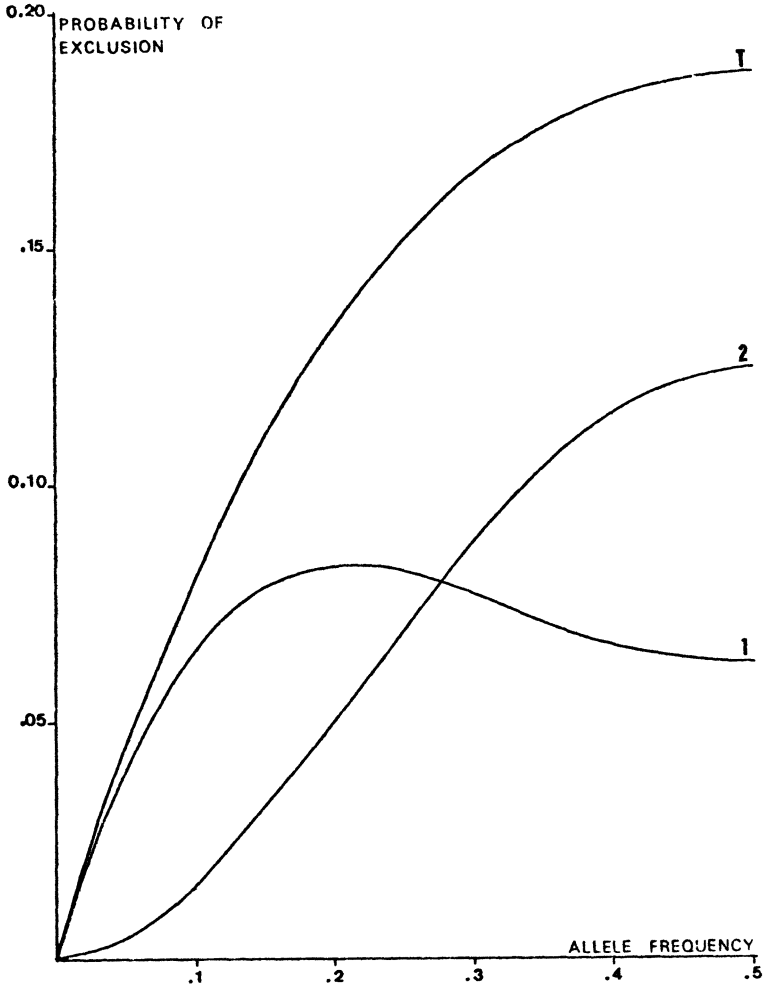

Fig. 1. Probabilities of exclusion in codominant 2-allele systems. (T: total; 1: first rule and 2: 2nd rule probabilities of exclusion)

$$\sum_{i=1}^{k} \left(pi^2 \times \sum_{\substack{j=1 \\ j \neq i}}^{k} pj^2 \right)$$

For 2-allele systems, the results are easily visualized in the graphic form (Fig. 1).

The main fact to underline from this distribution is that while total power of exclusion is maximum for equally frequent alleles, first rule exclusion reach a maximum when the alleles have very assymetric frequencies, decreasing afterwords.

DISCUSSION AND CONCLUSIONS

From these results it turns out obvious that the choice of the genetic systems to be included in a battery of genetic tests is a much more delicate operation than just ordering them by their total power of exclusion. Indeed, it is contradictorious to construct a battery of tests optimized on the basis of the total power of exclusion, if a single exclusion by the second rule (or, according to some authors, two) is not considered sufficient. Therefore it seems justified to use the formula derived above, to materialize another criterion for optimization of a sequence of markers: the exclusion efficiency by the first rule.

In order to demonstrate the contradiction between the two criteria, in Table 1 we compare the ordination of some polymorphic systems currently used in paternity testings, according to each of them. It is symptomatic that only 2 of the systems do not change their position in the sequences, those with both maximum and minimum total and 1st rule exclusion powers. However, the practical implications of the distribution properties of total and 1st rule exclusion chances are only realized when the "optimized" sequence excludes some of the technically available markers.

In order to simplify the calculations we assumed a very ideal situation in which 20 polymorphic markers can be freely chosen (i.e without technical or financial limitations) from systems with maximum power either total or according to the 1st rule.The results of the application of the referred oposite strategic choices are shown in Table 2. Again it is clear that optimization based in the total exclusion efficiency has a serious drawback: the low reliability of many of the obtained exclusions. On the other hand, when the oposite criterion is used, an apparent decrease in the total exclusion power, is compensated by the high reliability of the obtained exclusions.

Thus it seems to us that the only unambiguous criterion for optimization of a sequence of genetic markers for paternity testings is the exclusion power by the first rule.

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TYPES OF EXCLUSION AND EFFICIENCY CRITERIA FOR PATERNITY TESTINGS.

António Amorim, Jorge Rocha and Nuno Almeida
(Instituto de Antropologia, Faculdade de Ciências, 4000 Porto, Portugal.

More than a hundred different genetic systems suitable for paternity testings have been described in man. This fact, being a remarkable achievement in itself by the very high exclusion efficiency that can be attained in the expertises, is at the same time a source of problems. Indeed, no laboratory is able to perform (at least routinely) all of them, and - on the other hand - besides technical problems, the cost of such an investigation would be prohibitive.

Therefore, it is necessary to select from the available list of polymorphisms some to be included in a practical routine battery of genetic tests. Two kinds of criteria can be used for this choice: a) technical / economical costs or b) potential information content.

The analysis of the first type of criteria being outside the purpose of this work, we shall discuss only the ways to mesure the usefulness of genetic systems in paternity expertises under the assumption of identical costs. In fact, we do not believe that a standardized cost for any phenotyping can be calculated, even inside the same country, due to the enormous differences in personal, equipment and management between the different laboratories.

THE MEASUREMENT OF THE USEFULNESS OF A GENETIC SYSTEM

The usefulness of a genetic system in the field of paternity expertising has been measured by the exclusion power (exclusion efficiency or "a priori" probability of exclusion. The concept can be extended to a battery of tests, and is then defined as the probability of obtaining at least one exclusion, given a random mother-child pair and a random non-father. This parameter has been used under the assumption of equal costs and technical difficulties as a basis of decision for the elaboration of the list of genetic markes to be included in an optimized battery of tests (SALMON et al., 1980).However, this parameter overlooks the different reliability of the two types of exclusion according to LANDSTEINER's rules. In short words, it would make no sense to organize a battery of genetic tests in which most of exclusions are expected to be unique and by the second rule; in most cases, then, a reasonable doubt on the possible presence of a silent gene would prevent a veredict of true exclusion.

Thus, we made an atempt to study the properties of the distribution probabilities of the two types of exclusion together with the total power of exclusion in codominant systems.

1st AND 2nd RULE EXCLUSION: DISTRIBUTION PROPERTIES

The calculation of the total power of exclusion of a genetic system with k codominant alleles with frequencies p_1, p_2 ... p_k has been derived, for instance, by SELVIN (1980). Following the approach of this author, the expression for the probability of exclusion by the second rule is easily derived, since these can only occur in one mother/child type: AX / AA where X represents any of the k alleles, including A. If the frequency of this mother/child pair is given by p_1^2 and the corresponding excludable man by: $p_2^2 + p_3^2 + \ldots + p_k^2$. Therefore the general formula for second order exclusion probability in codominant system can be written as

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child 1 / child 2	AA	AB	BB
AA	$1 + \frac{q}{p} + \frac{q^2}{4p^2}$ or $\frac{1}{4} + \frac{1 + 2p}{4p^2}$ always >1		.25
AB	$\frac{1}{2} + \frac{q}{4p}$ or $\frac{1}{4} + \frac{1}{4p}$ always >.5	$\frac{1 + pq}{4\ pq}$ or $\frac{(p-q)^2 + pq}{4\ pq}$ always >1	$\frac{1}{2} + \frac{p}{4q}$ or $\frac{1}{4} + \frac{1}{4p}$ always >.5
BB	.25		$1 + \frac{p}{q} + \frac{p^2}{4q^2}$ or $\frac{1}{4} + \frac{1 + 2q}{4q^2}$ always >1

This shows that when the two subjects share two antigens the FI is always greater than 1; if they share one antigen it is always greater than .5 and if they do not share any antigen the FI is .25 and independent from the frequency of the genes.

CONCLUSION

A logic similar to that used to calculate a paternity index can be applied to the determination of a paternity index to quantitate the possibility that two individuals may be biological siblings. In this particular instance a calculation similar to the power of exclusion cannot be used since the power of exclusion will always be 0.

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Similar calculations for all genetic systems tested give the following results.

Blood Group	X/Y
ABO	9.953010752
Rh	.6684571344
MNS	.0927939075
Kell	.5224660397
Duffy	.5235727762
Kidd	1.16241347
HLA	.29831395
Total Product	.0585733327

Thus the final X/Y or FI (fraternity index) is .0587 or $\frac{1}{FI}$ = 17.1

It is 17.1 times more likely that they are not biological brother and sister. A probability of fraternity (PF) could be calculated as $\frac{FI}{1 + FI}$ = .055 or 5.5%.

Of interest is the MNS blood group system. II_1 is homozygous for $N\bar{s}$ and II_2 is homozygous for Ms. The two half-brothers are $MS\bar{s}$. If the third one was homozygous for either MS or NS that would completely exclude II_1 as a brother of II_2, since it would exclude the possibility that I_2 possesses $N\bar{s}$. The HLA system could also bring out an exclusion if the third untested half-brother would turn out to be entirely different from II_1, II_3, and II_4. Thus testing this person might bring out a certitude instead of a probability in this case.

GENERAL CALCULATIONS FOR A BIALLELIC CODOMINANT SYSTEM

Let the system have two genes A, B with frequencies of p, q. The X value is the probability of the same parents siring both children. The Y value is the product of the phenotypic frequencies of the two children. Example: Child #1 is AA, child #2 is AB.

Possible phenotypes of parents of both children	Frequency of Mating	Frequency of Child #1	Frequency of Child #2	Frequency of Combination
AB X AA	$4p^3q$	.5	.5	p^3q
AB X AB	$4p^2q^2$	.25	.5	$.5p^2q^2$

X = probability of same parents siring both children = $p^2q\ (p+.5q)$

Y = product of phenotypic frequencies = $2p^3q$

$$\frac{X}{Y} = \frac{2p+q}{4p} = \frac{1}{2} + \frac{q}{4p} = \frac{1}{4} + \frac{1}{4p}$$

Thus when the two children share one antigen $\frac{X}{Y}$ for that system is >1, when the frequency of the shared antigen is <.33. By applying the same logic to all possible phenotypic combinations the following formulas are obtained.

10) Calculate the relative frequency of such combination.
11) Calculate the probability that such combination could result in a second child of II_1 genotype: This is the X value (probability that II_1 and II_2 are sibs).
12) Calculate the frequency of II_2 phenotype in the random population: This is the Y value (probability that II_1 is a random unrelated child).
13) X/Y (fraternity index) is calculated for each blood group and the final result is the total product of each X/Y.

RESULTS OF CALCULATIONS

Below is an example of the calculation method for the ABO blood group using the genetic frequencies for U.S. whites published by the American Association of Blood Banks in 1977(5).

To have sired an A_1B, a B, and an O child I_2 has only two possible genotypes, A_1O and BO. If she is A_1O then I_3 must be BO; if she is BO then I_3 may be OO, AO, or AB (here A is $A_1 + A_2$). First we calculated the probability that I_2 is A_1O or BO.

I_2	I_3	Frequency of Mating	Frequency of O Child	Frequency of B Child	Frequency of Combination	Relative Frequency of Combination
A_1O	X BO	.0261663248	.25	.25	.0016353953	.0980099502
BO	X OO	.0438983267	.5	.5	.0109745817	.6577114428
BO	X AO	.0349327078	.25	.25	.0021832942	.1308457711
BO	X BO	.0100946228	.25	.75	.0018927418	.1134328358
				Total	.016686013	1.0000000000

Probability that I_2 is A_1O: .0980099502

I_2 is BO: .9019900492

Then we identify the possible genotypes of I_1:
if I_2 is A_1O then I_1 may be BB, BO, or AB,

if I_2 is BO then I_1 may be A_1A_1, A_1A_2, A_1O or A_1B

I_2	I_3	Frequency of Mating	Frequency A_1 B Child	Frequency of Combination	Relative Frequency of Combination
A_1O	X BB	.0005661055	.5	.0002830527	.0030577025
A_1O	X BO	.0137653015	.25	.0034413254	.037175225
A_1O	X AB		.25		
BO	X A_1A_1	.0350053318	.5	.0175026659	.1890741133
BO	X A_1A_2		.25		
BO	X A_1O	.2853734159	.25	.071343354	.7706929592
BO	X A_1B		.25		
			Total	.092570398	

The probability that the combination have another A_1B child is
X = .2980329539
Y = .029944
Thus X/Y for the ABO is 9.953010752

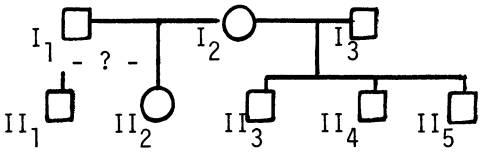
PARENTAGE TESTING FOR THE DETERMINATION OF SIBSHIP. Chantal R. Harrison and Sherrie L. Warner, Dept. of Pathology, Univ. of TX Health Science Center, San Antonio, TX 78284, USA.

INTRODUCTION

Genetic markers in blood have been used for paternity investigation and methods to quantify the genetic data into a paternity index or a probability of paternity have been described (1,2,3,4,5). We report here a case where the genetic investigation was attempted to determine whether two adults were biological siblings. A method to quantitate the genetic data and calculate a fraternity index using logic similar to that used to calculate a paternity index was devised for this particular situation. We also report a general method to calculate a fraternity index for a biallelic codominant genetic system.

CASE REPORT

Two adults (II_1 and II_2), North American Caucasians, a male and a female, had been adopted in the same family. Their adoptive parents are now deceased and they wish to know whether they are biological siblings. They now have children and there is a possibility that their children want to intermarry. Records of who the biological parents (I_1 and I_2) of the woman are available. Unfortunately they are also deceased. However, her mother had three additional children from a different man (I_3). Two are available for testing (II_3, II_4). These relationships are better described in the following pedigree.



The ABO, Rh, MNS, Kell, Duffy, Kidd, and HLA type of II_1, II_2, and II_3, and II_4 were determined with the following results.

II_1: A_1B, cDEe, Ns, K-, Fy^aFy^b, Jk^aJk^b, A1A2B3B27

II_2: A_1B, cde, Ms, K+, Fy^b, Jk^aJk^b, A2A29B12

II_3: B, cde, MSs, K-, Fy^aFy^b, Jk^a, A1A29B8B12

II_4: O, cde, MSs, K-, Fy^b, Jk^aJk^b, A1A29B8B12

II_5: Was not available for testing.

LOGIC OF CALCULATION

The following logic was used to determine a fraternity index in this particular situation:

1) Identify what possible genotypes I_2 must have had to sire her 3 known children (II_2, II_3, and II_4).
2) Identify which possible corresponding genotypes I_3 must have had to sire II_3 and II_4.
3) Calculate the frequency of such matings in the general population.
4) Calculate the frequency of the combination of each particular mating and 2 children of II_3 and II_4 genotypes.
5) Calculate the relative frequency of such combination.
6) Deduce the probability of each genotype for I_2.
7) Identify which possible genotypes I_1 must have had to sire II_2.
8) Calculate the frequency of the possible I_1 and I_2 matings.
9) Calculate the frequency of the combination of each particular mating and one child of II_2 genotype.

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With the 22 blood groups and for the mean cases, the percentage of excluded men went from 99.0 % to 99.990 %, it means one non excluded man out of 100 and one non-excluded man out of 10,000; the probability of paternity went from 99.0 % to 99.9990 %, it means one case of non-paternity out of 100 and one case of non-paternity out of 100,000.

When adding chromosome variants analysis, for the mean cases, the percentage of excluded men became higher than 99.90 % and the probability of paternity went from 99.990 % to 99.99999 %, it means one case of non-paternity out of 1 million and sometimes even more.

CONCLUSION

Chromosome variants bring disputed paternity questions a sure efficacy but with the blood group systems.

Used alone, chromosome polymorphism would not have excluded :
- 1 false father out of 5 when the child is a girl,
- 1 false father out of 20 when the child is a boy.

The detection of these chromosome variants requires a great experience of cytogenetics and has to be performed in specialized laboratories (cell cultures, banding stain for example).

On the one hand, the advantage of this polymorphism is that the variant is directly visible and is not the "translation" between several stages of an "invisible" gene. There is no silent allele .So are the second order exclusions as secure as the first order exclusions.

On the other hand, this polymorphism allows a new type of exclusion which is simple and accurate : exclusion by Y chromosome. The real father and the son must have a strictly identical Y chromosome.

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The total theoretical percentage of exclusion amounts to 88 % about paternity of girls and to 95 % for boys. Added to the probability obtained with blood groups (99.7 % - (7), it respectively amounts to 99.96 % and to 99.98 %.

STUDY OF 80 CASES

According to Table III, the 80 cases were distributed in 2 groups :
- excluded men (57 men)
- non-excluded men (30 men)

		BLOOD GROUP SYSTEMS		
		EXCLUDED MEN	NON-EXCLUDED MEN	
CHROMOSOME VARIANTS	EXCLUDED MEN	45	0	45
	NON-EXCLUDED MEN	12	30	42
		57	30	

57 excluded men

The mean number of blood group systems involved in exclusion was 4. It became 5 when adding chromosome variants.

30 non-excluded men

About non-exclusion cases, two indications of paternity help to decide if the man can be considered as the father or not :
- the proportion of excluded men in the couple "mother-child"
- the probability of the *a posteriori* paternity for alleged father

We calculated these two indications for each case (Table IV).

Values	proportion of excluded men Number of couple "mother child"		probability of paternity Number of trio "man-mother-child"	
	BLOOD GROUP SYSTEMS	+ CHROMOSOME VARIANTS	BLOOD GROUP SYSTEMS	+ CHROMOSOME VARIANTS
from 98 to 99 %	1	1	0	0
from 99.0 to 99.9 %	14	2	5	2
from 99.90 to 99.99 %	11	10	15	3
from 99.990 to 99.999 %	2	7	6	9
from 99.9990 to 99.9999 %	2	3	3	7
> 99.99990 %	0	7	1	9

MATERIAL AND METHODS

Our study was based on a sample of eighty paternity cases between 1980 and 1984 (73 trios "man-mother-child", 5 cases with 2 alleged fathers and 2 cases with 2 children).

The blood groups analysis concerned 7 erythrocyte systems, 14 protein systems and HLA-A, B.

The metaphase chromosomes were obtained by a 72 hours lymphocyte cell culture (5). We systematically used 3 types of banding :
G-banding (GAG) Q-banding (QFQ) C-banding (CBG)

THEORETICAL PROBABILITY OF EXCLUSION

Based on the Belgian frequencies, the theoretical rate, under which paternity exclusion may be obtained, has been calculated for each chromosome according to OHNO et al. (6) (Table II).

CHROMOSOME	REGION	NUMBER OF VARIANTS	THEORITICAL PROBABILITY OF EXCLUSION
1	qh	6	22.5 %
3	c	4	19.3 %
4	c	2	0.6 %
7	c	4	12 %
9	qh	6	28.5 %
13	cent-sat.	10	19.8 %
14	cent-sat.	6	11.1 %
15	cent-sat.	6	8.8 %
16	qh	7	16.5 %
19	c	4	19.9 %
21	sat.	4	18 %
22	sat.	4	15.6 %
y	qh	5	57.3 %
	TOTAL	GIRLS :	88.2 %
		BOYS :	95.0 %

EFFICIENCY IN DISPUTED PATERNITY CASES OF A NEW CATEGORY OF MARKERS : CHROMOSOME VARIANTS.

Ludmila E. KAMENEV, Armand C. ANDRE and Jacques L. FREDERIC *

SUMMARY

Using suitable cytogenetic techniques, chromosome polymorphism has been studied in eighty disputed paternity cases.

Based on the Belgian frequencies, the theoretical rate of exclusion is 88.2 % when a girl and 95 % when a boy. Thus chromosome variants added to 22 blood group systems increase the exclusion rate from 99.7 % to 99.96 and 99.98 %.

In case of exclusion, the mean number of involved systems increased from 4 to 5.

In cases without exclusion, the probability of paternity was often high and, in some cases, the paternity was almost certain.

INTRODUCTION

For controverted paternity cases, erythrocyte, enzymatic and plasmatic blood groups are used. Some laboratories are using the HLA system which gives very good results.

A new polymorphism - completely differing from those known until now - was lately described : chromosome variants (1, 2). These variants are observed by using cytogenetic techniques which reveal variable constitutive heterochromatin regions of human metaphase chromosomes.

These heterochromatin regions are supposed to represent highly repetitive DNA, thought to be genetically inert (3). Clinical abnormalities have not yet been correlated with such variations (4). These variants mainly concern the chromosomes described in Table I.

CHROMOSOME	REGION	VARIANT
1, 9, 16 7, 19	SUBCENTROMERIC HETEROCHROMATIN CENTROMERIC HETEROCHROMATIN	LENGTH VARIATION AND PERICENTRIC INVERSION
3, 4	CENTROMERE	INTENSITY VARIATION
D : 13, 14, 15 G : 21, 22	CENTROMERE SHORT ARMS SATELLITES	INTENSITY VARIATION LENGTH VARIATION INTENSITY VARIATION
Y	DISTAL HETEROCHROMATIN	LENGTH VARIATION

* Lab. des Groupes Sanguins Transfusion rue Dos-Fanchon 41 B-4020 Liege,

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VI. Paternity, Biostatistics and Quality Assurance

CONCLUSIONS

- Bf phenotyping in blood stains by IEF is possible
- The alleles are demonstrable in like manner on glass and on cotton cloth
- The quantity of detectable blood substance amounts to below 10 ul
- Bf marker can be detected within 2 weeks at room temperature

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RESULTS

Complete banding patterns of Bf from blood stains were well visualized. There are no differences between samples and normal serum of probands. No deviations were observed between the Bf patterns from blood drops on glass and blood soaked cotton clothes. On the other hand there were different results dependent on mode of application on gel surface:

- blood soaked cotton clothes directly: ++++,
- cotton clothes eluated with physiologic saline and application by filter paper pieces: ++++,
- dried blood drops directly: Ø,
- dried blood drops eluated with physiologic saline and applicated by filter paper pieces: ++++.

We achieved commonly the best results with only a little weted filter paper pieces.

The banding patterns staid steady within 2 weeks. Later the visualization of Bf types became problematically. We didn't observe any influence of storage temperature between $4^{o}C$ - $25^{o}C$.

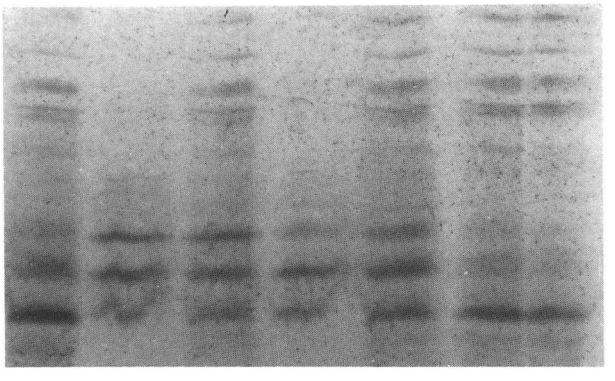

Fig. 1: Bf typing of blood staine

1 = Bf S; 1 day, $4^{o}C$
3,5 = Bf FS; 4 days, $4^{o}C$
6,7 = Bf S; 2 weeks, $25^{o}C$
2,4 = Bf FS; serum samples

- dried blood drop directly,
- eluated dried blood drop (20 ul physiologic saline, filter paper pieces Whatman No. 3).

The filter paper pieces were used in weted and dried condition.

Isoelectric focusing

IEF was carried out in an ultrathin layer polyacrylamide gel (200 x 100 x 0,3 mm; T=4,5%, C=3%) with 12,5 % glycerine. The pH gradient was established with carrier ampholytes by mixing 3 % Servalyte pH range 5 - 7, 3 % Servalyte pH range 6 - 8, and 1,5 % Servalyte pH range 3 - 10. Polymerization was accomplished chemically with 0,03 % ammoniumpersulphate in the presence of 0,25 % TEMED. For electrode solution 0,1 M glutamic acid in 0,5 M H_3PO_4 was used at the anode and 0,1 M NaOH at the cathode. An electrode distance of 9 cm was chosen. Refrigeratory temperature: $8^{o}C$.

Electric values and focusing times:

- 7,5 W; 7,5 mA; 1 500 V (max.),
- prefocusing 45 min. (dried sample) or 75 min. (wet sample),
- separation with samples 60 min. (dried) or 30 min. (wet),
- separation without samples 195 min. (dried and wet).

We worked with a self made equipment.

Immunofication

Immunofication was performed with monospecific Bf antiserum from Atlantic Antibodies (Scarborough, U.S.A.). According to GESERICK et al. after IEF we applied directly antiserum 1 : 5 in destilled water on the gel surface. After immunoprecipitation the nonprecipitated components were washed out in physiologic saline overnight. After washing in destilled water for 2 h the gel was stained with Coomassie brilliant blue (Serva, Heidelberg).

Bf TYPING IN BLOOD STAINS BY ISOELECTRIC FOCUSING

R. Wegener and J. Rummel
Institute of Forensic Medicine, Wilhelm-Pieck-University
Rostock, 2500 Rostock, G.D.R.

INTRODUCTION

On account of the reliability of Bf testing and the stability of Bf proteins this marker system is very useful in paternity testing. Isoelectric focusing (IEF) methods for typing of Bf polymorphism increase the discriminative power of this system (GESERICK et al. 1983, WEIDINGER et al. 1984).
These properties of Bf system indicate a potential fitness for the analysis of blood stains. The common applicability of ultrathin layer IEF in the investigations of blood stains was demonstrated by some authors (BERGHAUS and STAAK 1982, SCHMITTER and KISSLING 1983).
We tested the usefulness of ultrathin layer IEF of Bf marker for characterization of blood stains.

MATERIALS AND METHODS

For the determination of Bf phenotypes by IEF we used the methods similar to those described by GESERICK et al. 1983.

Artificial blood stains

The samples were collected from 12 probands with known Bf phenotypes (Bf S, Bf FS, Bf F). We prepared, dried and stored stains on glass and cotton clothes one day - two months at $4^{o}C$ - $25^{o}C$.
The stains were applied on the gel in following way:
- soaked cotton cloth directly,
- eluated cotton cloth (50 ul physiologic saline, filter paper pieces Whatman No. 3),

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proteins were transferred to nitrocellulose membranes, and allowed to react with specific antiserum and, after washing, with peroxidase-labeled second antibody. The immune complexes formed on the membranes were detected with 4-chloro-1-naphthol and hydrogen peroxide. Serum GC, AHS, C6, C7, F13B, and PLG could be phenotyped clearly. Furthermore, 6-month-old bloodstains could be AHS-typed correctly by this technique.

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be detectable. In contrast, the immunoblotting method allows detection of very low-concentration proteins with small amounts of antiserum. In the present study, the first antibody was used at 1:200 to 1:400, meaning a 100-fold increase in sensitivity over immunofixation methods. The high sensitivity resulted from the use of the enzyme-labeled second antibody the optimal dilution of which was 1:2000. Lower dilutions of the first or second antibody tended to visualize excess bands of proteins with which the antibody crossreacted.

Table 1. AHS phenotypes and gene frequencies in a Japanese population

Phenotypes	No.observed	No.expected	Allele frequencies
AHS 1	160	157.3	AHS*1=0.7456
AHS 2-1	102	107.4	AHS*2=0.2544
AHS 2	21	18.3	
Total	283	283.0	

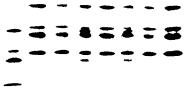

Fig.1. AHS typing of 6-month-old bloodstains. Anode at top. From left to right: AHS 2; 1; 1; 2-1; 1; 2-1; 1; 1.

Blotting by passive diffusion gives partial transfer of proteins, leaving part of the proteins behind. Thus F13B, a very low-concentration protein, could be detected only after complete transfer by electrophoresis. Since no posttransfer diffusion of proteins occurred on the membrane, the immunoblotting method gave sharper protein bands than print-immunofixation did.

Umetsu has found that treatment of serum with neuraminidase resulted in the loss of AHS polymorphism. This suggests that the allotypic determinants are located in the carbohydrate moiety, and probably explains, by analogy with the ABO blood-group antigens, why AHS is so stable in aged bloodstains as to be phenotyped clearly.

The present study demonstrates that immunoblotting is a useful tool in forensic science practice, and that AHS typing of bloodstains merits inclusion in crime laboratory casework.

Summary

Sera and bloodstain extracts were subjected to PAGIEF. The focused

(0.25u in 0.5M potassium phosphate buffer, pH 7.0, per 45µl serum) at room temperature overnight. For AHS typing of bloodstains, dried blood-stained filter paper applicators, 5X3mm, were soaked in 10µl of distilled water at 4°C for 2 h, and placed on the gel surface.

Focused GC, AHS, C6, C7, and PLG proteins were transferred to nitrocelluolose membranes nonelectrophoretically (by passive diffusion), whereas F13B was transferred electrophoretically according to the operating instructions of Bio-Rad. After transfer, focused proteins were visualized by incubation with a 1:200 or 1:400 dilution of specific antiserum and, after washing, with a 1:2000 dilution of peroxidase-labeled second antibody, followed by washing and the addition of a substrate mixture (30mg 4-chloro-1-naphthol, 10ml methanol, 50ml TRIS-buffered salione, and 25µl of 30% hydrogen peroxide). The washing solution and the diluent were 0.05% Tween 20 in TRIS buffered saline.

Results and Discussion

Serum GC, AHS, C6, C7, and PLG could be phenotyped with high sensitivity. Table 1 shows the distribution of AHS phenotypes in the population studied. The AHS*1 and AHS*2 allele frequencies were estimated to be 0.07456 and 0.2544, respectively. The observed numbers agreed with the numbers expected on the basis of the Hardy-Weinberg law (χ^2=0.72, df=1, 0.3<P<0.5). Describing the AHS polymorphism in a Japanese local population, Umetsu et al.[3,4,5] estimated the AHS*1 and AHS*2 frequencies at 0.7356 and 0.2639, respectively. No significant difference was noted between the two local populations (χ^2=0.2603, df=1, 0.5< P<0.7). All 6-month-old bloodstains could be AHS-typed correctly and clearly by the IEF-immunoblotting technique (Fig. 1).

Proteins fractionated by gel electrophoresis or IEF have been visualized conventionally by protein staining alone or in combination with immunofixation in gels or overlay cellulose acetate membranes. These immunofixation methods have one or more of the following disadvantages: (1) the processing of gels is time-consuming and entails handling accidents, (2) relatively large amounts of antiserum are consumed, and (3) the detection sensitivity is so low that proteins present in the serum normally in very low concentrations may not

APPLICATION OF IMMUNOBLOTTING TO SERUM PROTEIN PHENOTYPING WITH REFERENCE TO α2HS-GLYCOPROTEIN (AHS) TYPING OF BLOODSTAINS

Yoshihiro Tamaki (Department of Forensic Medicine, Medical College of Oita, Oita, JAPAN)

The use of the immunoblotting technique [1,2] has become increasingly popular in genetic typing of serum proteins by isoelectric focusing (IEF). This is because the technique is simple in operation, gives high detection sensitivity, and requires much less antiserum than immunofixation methods do.

Our laboratory has been making routine use of immunoblotting in group-specific component (GC), AHS, C6, C7, factor 13B (F13B), and plasminogen (PLG) phenotyping. The present paper reports the AHS typing of bloodstains along with the blotting methods used in our laboratory for the phenotyping of serum proteins.

Materials and Methods

Blood samples were collected from healthy Japanese residents of Oita Prefecture in the southwestern part of Japan. Bloodstains were made from bloods of 200 different subjects on Whatman No.3 filter paper, air-dried, and left at room temperature for up to six months. All antisera were commercially available. A Flat Bed Electrophoresis Apparatus FBE 3000 (Pharmacia Fine Chemicals) and a Power Supply 2103 (LKB) were used for IEF. A Trans-Blot Cell (Bio-Rad) and a Power Supply Model 250/2.5 (Bio-Rad) were used for electroblotting.

Sera and bloodstains were phenotyped by IEF in 14X10X0.05cm polyacrylamide gels (PAG) with pH ranges of 4.5-5.4 for GC, 4-5 for AHS, 5-7 for C6 and C7, 5-6 for F13B, and 6-9 for PLG. After prefocusing (except in GC subtyping) for 30 min, serum samples on pieces of filter paper, 5X3mm, were applied to the gel 1.5cm (for GC, AHS, and F13B) from the catholyte wick and 2cm (for C6, C7, and PLG) from the anolyte wick. IEF was performed at 5-10°C for 2 h at 2000 V max, 8 mAmax, and 8 Wmax.

For C7 typing, serum samples were pretreated with neuraminidase

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Fig. 3. Semen esterase activity on vaginal swabs taken at known times after intercourse.

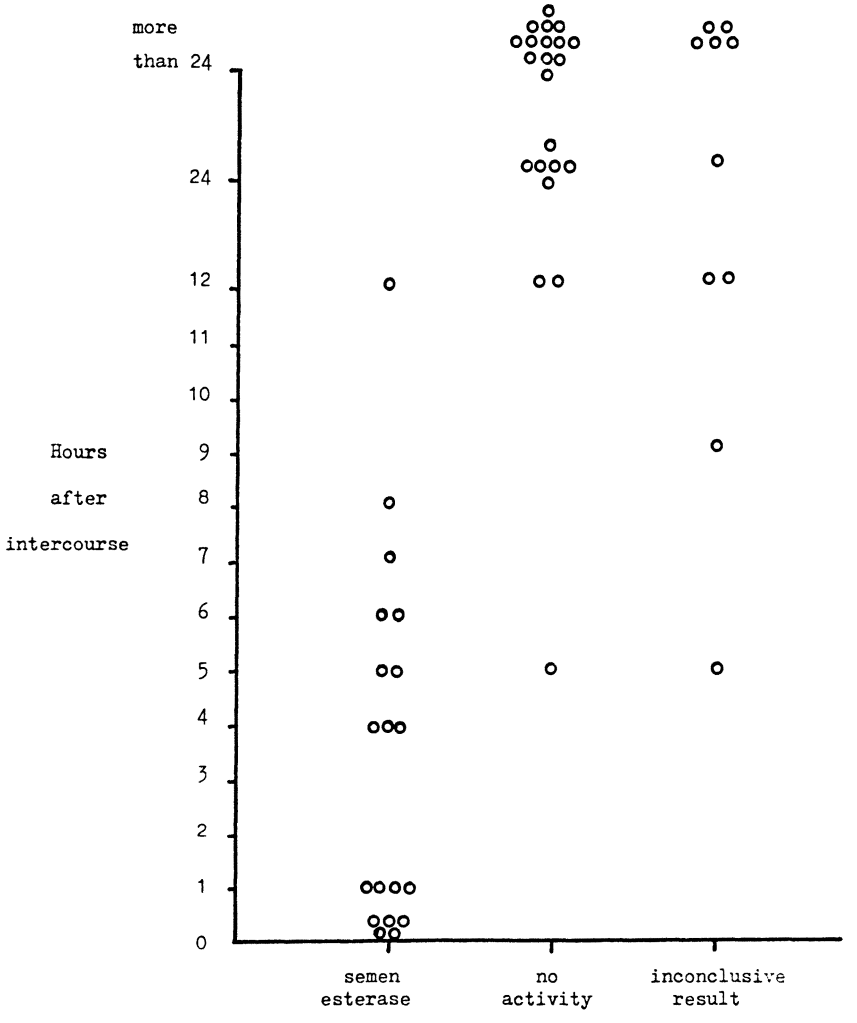

Fig. 1. Semen, vaginal secretion and blood esterases after polyacrylamide gel electrophoresis.

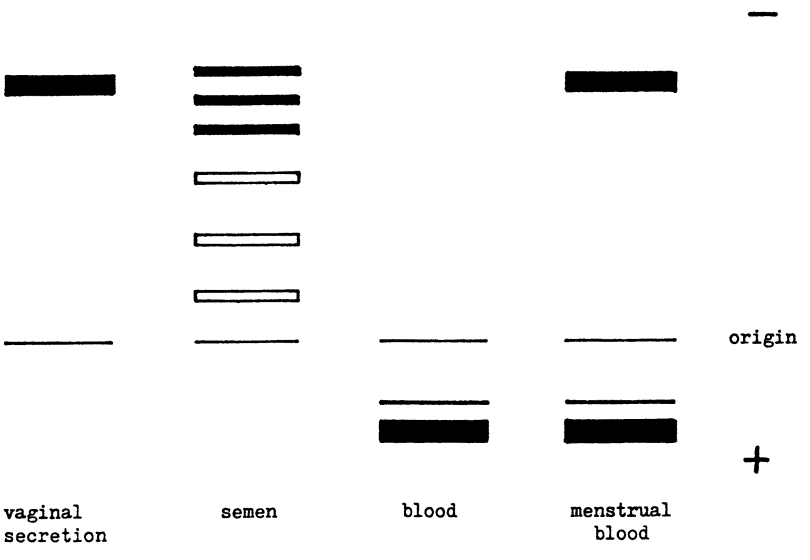

Fig.2. Reaction of semen and vaginal esterases with a number of substrates.

	Semen	Vaginal secretion
α-naphthyl acetate	++++	++++
propionate	+	+
butyrate	-	-
stearate	-	-
valerate	-	-
4-methylumbelliferyl-		
acetate	+	++
propionate	-	-
butyrate	-	-

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When electrophoresis was carried out in polyacrylamide gels of concentrations above 7%, the vaginal esterase was sometimes seen as two zones giving a possible, though unlikely, confusion with semen esterases. Hence, a concentration of 6.5% acrylamide was used.

A further attempt at distinguishing the esterases of semen and vaginal fluid was made by investigating their substrate specificities. A number of α-naphthol salts and 4-methylunbelliferyl salts were used to locate the esterase isozymes.

The results (fig. 2) showed that both semen and vaginal esterases show strong activity with acetate salts only, indicating that these are non-specific acetyl esterases.

Vaginal swabs

Vaginal swabs were donated by females aged between 20 years and 45 years, taken at known times after intercourse and examined for semen esterase and vaginal esterase activity.

Of the thirty-five swabs taken a minimum of seven days after intercourse, twenty-four showed vaginal esterase activity and the others either showed no esterase activity (four swabs) or were scored as inconclusive (seven swabs). No swab gave an isozyme pattern similar to that produced by semen.

Of the swabs taken within four days post intercourse, semen esterase was regularly detected up to about eight hours post intercourse. Swabs taken after eleven hours post intercourse normally showed no semen esterase activity (fig. 3)

Menstrual blood

Under these conditions, the presence of vaginal esterase and PCE from blood plasma is indicative, though not proof of menstrual blood (fig. 1)

CONCLUSIONS

The results so far in this investigation show that using the electrophoretic conditions described,

1) semen and vaginal fluid esterases can be distinguished.

2) On a vaginal swab, the presence of azoospermic semen can be strongly indicated up to eight hours after intercourse. (Although such esterase isozymes may be present in other tissues, these are unlikely to be found on vaginal swabs).

3) Vaginal fluid esterases cannot be reliably detected in the presence of semen.

4) The presence of vaginal esterases and PCE from blood plasma gives an indication, though not proof, of menstrual blood.

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FORENSIC APPLICATIONS OF HUMAN BODY FLUID ESTERASES

Brian H. Parkin (The Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE1 7LP, U.K.)

ABSTRACT

Esterases of human semen and vaginal secretion show different electrophoretic patterns when studied using polyacrylamide gel with a thin strip of starch gel at the origin. Vaginal esterase occurs as a single cathodal zone, while semen esterase is manifested as a number of cathodal bands and hence the method can be used to distinguish semen and vaginal secretion in sexual assault cases.

Under these conditions, pseudocholinesterase (PCE) from blood plasma is detected as anodal zones and hence a mixture of blood and vaginal secretion can be demonstrated, indicating, but not proving that a bloodstain is of menstrual origin.

Experiences with the technique in the examination of a range of vaginal swabs is presented.

INTRODUCTION

Multiple forms of esterases have been found in a variety of human tissues, most of which are non-specific, showing overlapping substrate preferences and varying inhibition characteristics. Some of these esterases have, however, been identified by the use of selected substrates and inhibitors, of which esterase D (EsD) and carbonic anhydrase (CA) are regularly exploited in forensic blood grouping. Pseudocholinesterase (PCE) also has a polymorphic variant which is demonstrated by electrophoresis, but its value as a forensic blood typing marker is low.

The isozymes of these non-specific esterases have been reported as varying from tissue to tissue (Coates et al, 1975) and since the physiological production of seminal plasma (Mann, 1964) and vaginal fluid (Raffi et al, 1977) is different, it appeared that a comparison of the esterase isozymes from semen and vaginal fluid may enable each to be distinguished.

METHOD

Semen and vaginal fluid esterases were examined by electrophoresis in a 6.5% polyacrylamide gel with a thin strip of 7% starch gel at the origin. The gel buffer was 0.015M succinic acid - TRIS, PH4.8 and the bridge buffer was 0.016M citric acid - sodium hydroxide PH4.8.

Electrophoresis was carried out at 5V/cm for 16 hours.

The esterase isozymes were located by the method of Stern and Lewis, 1962.

RESULTS AND DISCUSSION

After electrophoresis, the semen resolved into six zones of cathodal esterase activity (fig. 1), with zones 1, 2 and 3 the strongest. Vaginal esterase occurred as a single zone with a mobility between that of zones 1 and 2 of semen esterases. Under these conditions, blood plasma cholinesterase (PCE) occurred as strongly staining zones anodal to the origin.

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stains 1 to 4 can be assigned to the suspect and stain 5 to the victim. This is verified by IgD determination in 4 cases since the victim can not be regarded as a source of stains with a measurable IgD content. Sections of original plates of the case are represented in Figure 2.

Table 2: Results of serological bloodgroup typing and quantitative IgD determination in 5 bloodstains in a practical case

	1	2	3	4	5
ABO	A H				B H
Rh	ambiguous	ccD.Ee			cc(dd)ee
Gm	1-2-4+10+21-				
Km	1-				
PGM1	a2 - a1				a4 - a1
IgD(mg/l)	22	26	ambiguous	precipitate (beyond standard curve)	< 10

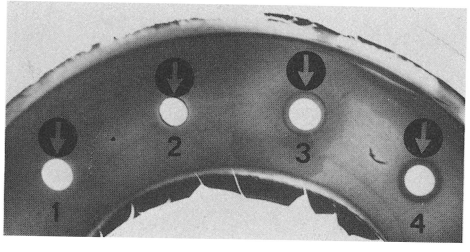

Figure 2:
SRID for IgD concentration determination. Sera and stains in a practical case.
1: victim serum <10 mg/l
2: stain 5 <10 mg/l
3: suspect serum 26 mg/l
4: stain 1 22 mg/l

More sensitive investigation techniques such as radioimmunoassay, could in some cases contribute to an improvement in differentiating stains on the basis of their IgD content. But our first results obtained by SRID also show that the IgD concentration can be a further marker in antibody profiling in bloodstains.

thus the stain IgD content could not be demonstrated because of the relative dilution of the eluates.

- serum IgD concentrations below the sensitivity of the investigation technique used (10 newborns, 2 children, 8 adults) so that IgD measurements in the stains were impossible.

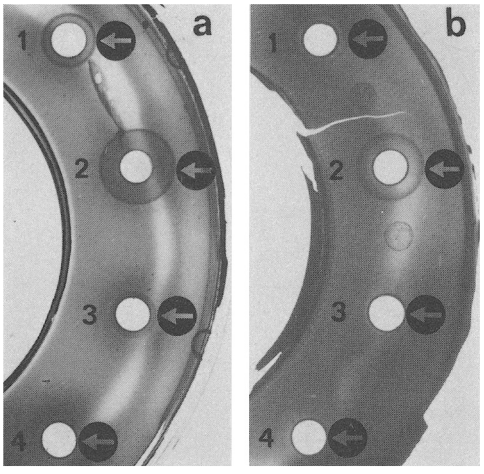

Figure 1:
SRID for IgD concentration determination. Adult samples (1-4).

a) sera (20 µl)
b) corresponding stain eluates (30 µl)

1/2: IgD level of (a) and (b) clearly measurable

3: IgD level of (a) little above sensitivity; IgD of (b) not measurable

4: IgD level of (a) below sensitivity; IgD of (b) not measurable

The best conditions for differentiating between stains of two individuals occur when one individual has a clearly measurable serum IgD concentration and the other has an IgD level below sensitivity. A person with no measurable serum IgD level can not be regarded as the source of stains with a measurable IgD content. Conclusions can generally be drawn in other cases, too, if the persons concerned come from different serum IgD concentration ranges.

Cases from forensic practice

The following example may serve as a case in point: A man shot his wife dead and afterwards inflicted a bullet wound on himself, but survived.

Blood properties of the suspect:

A_1 ccD.Ee Gm(1-2-4+10+21-) Km(1-) PGM_1 a2-a1 IgD: 26 mg/l

Blood properties of the victim:

B cc(dd)ee Gm(1-2-4+10+21-) Km(1-) PGM_1 a4-a1 IgD:<10 mg/l

The results of the investigation of 5 stains are shown in Table 2. Because of the bloodgroup-typing results, the

Determination of IgD concentrations was by SRID (partigen plates and IgD standard of Behring, FRG). Sample volumes were 20 µl (at the beginning 30 µl in the case of stain eluates). Diffusion period of 2 days. Staining with Amidoblack. Sensitivity at 10 mg IgD/l. Standard deviation 4.9. Along with the IgD determination, quantitative IgG tests were carried out using the same technique. Thus the dilution of the eluates in relation to the serum was determined by using a second marker with a high serum concentration so that the findings based on the IgD content of the eluates could be verified. The ratio between the dilution of the serum and that of the eluates was usually 1:3.

Results and Discussion

Artificial bloodstains

The results are summarized in Table 1.

Table 1: Quantitative determination of IgD in sera and corresponding bloodstain eluates

subjects (n)	age (years)	IgD-concentrations (mg/l)		storage time of bloodstains (days)
		serum	eluate	
newborns (10)	–	10 × < 10	10 × < 10	53-55
children (10)	1-13	2 × < 10 1 × 10 7 × 30-119	2 × < 10 1 × < 10 7 × 22-39	20-53
adults (20)	21-77	8 × < 10 7 × 14,5-27 5 × 31-174	8 × < 10 7 × < 10 5 × 24-87	9-14

The experimental subjects (Table 1) can be classified in three groups (cf. also Figure 1a and 1b):

- clearly measurable serum IgD concentrations (7 children, 5 adults) so that the stain IgD content could easily be measured and did not exhibit noticeable loss of activity even after storage periods of up to 53 days;
- serum IgD concentrations little above the sensitivity of the investigation technique used (1 child, 7 adults),

IgD CONCENTRATION: A MARKER IN BLOODSTAIN ANALYSIS

Marie-Louise Glowatzki[1] and Wolfgang Keil[2]

[1]Institute for Forensic Medicine (Director: Prof. P. Zink), Berne University, Switzerland

[2]Institute for Forensic Medicine (Director: Prof. O. Prokop), Humboldt University, Berlin, GDR

Introduction

The technique of antibody-profile investigation was introduced into bloodstain analysis by King (3) and was later extended (7). IgD antibodies have - as far as we know - not yet been investigated in bloodstains. However, the serum IgD concentration displays certain properties which could make its application in forensic practice possible:

- The serum IgD level of a particular adult is almost invariable with exceptional changes occuring only in cases of illness and towards the end of a pregnancy (6).
- The IgD values of different individuals may vary by more than a thousandfold. The IgD level of newborns can not be measured by single radial immunodiffusion (SRID) (4). The extremely varied adult values (ranging from 0.14 to 400 mg/l) are reached during childhood (4,5) and are obviously subject to a trimodal distribution (1,6). A genetic influence on the serum IgD level is considered very probable (1,2,4).

These properties of IgD encouraged us to experiment with quantitative IgD measurements in the differentiation of bloodstains.

Materials and Methods

First of all, artificial bloodstains (on cotton fabric, airdrying, storage at 22°C) and sera from 40 subjects were investigated (Table 1). This was followed by the investigation of practical forensic cases. Stain elution was with the necessary minimum of distilled water (12 h, 4°C).

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Titration of stain extract

Km1+3+	1	3
1/5	–	–
1/10	–	–
1/15	–	–
1/20	+	–
1/25	2	1
control	4	4

Fig. 3: comparison of relative intensities of Km(1) and Km(3) in stains

Storage Conditions

Room Temperature

37°C

Moist Chamber 37°C

56°C

Fig. 1: Experimental storage conditions for testing stability of Km(1) and Km(3)

Km(1,3) Microtitre

N=280

Km	N	%
1-3+	237	84,6
1+3+	39	13,9
1+3-	3	1,1
1-3-	1	0,4

Fig. 2: Distribution of Km(1) and Km(3) phenotypes in Münster

After incubation at 37° C in the humid chamber for 7 weeks, 3 samples gave unreadable results because the indicator cells did not form a button in the well.

In these experiments Km(1) and (3) showed approximately equivalent levels of activity (fig. 3).

Summary

Km(3) can reliably be used as a positive marker for a Km(1-)phenotype.

In casework it must be borne in mind that results can be affected and sometimes changed under extreme storage conditions but this also applies to most other systems.

The phenotype Km(1+3-) is rare but when possible it is wise to include this as a control as well as the normal antiserum controls.

The titration of the Km(3) was low and was therefore relatively expensive to use.

When stain extracts of Km(1+3+)phenotypes were titrated they appeared to show approximately equivalent strengths. In practice, so long as a titration series is made on stain extracts no mistake in typing should occur.

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of phenotypes was selected (a total of 12 stains) and stored under different conditions to test the relative stability and reliability of the two antigens (fig. 1) over a period of 7 weeks.

Results & Discussion

Although a slightly lower level of Km(1-) was observed, 85 % compared to 87 %, this survey was in reasonable agreement with other frequencies for a German population (fig. 2).
The occurence of the (1+3-)phenotype was extremely rare, only 1 % of the total. One sample was negative for both factors and this could possibly be due to a low level of light chains or immunoglobulins in the serum. No discrepancies were found between the sera and bloodstains, although the high frequency of Km (3+) could suggest nonspecific inhibition. A possible source of error could have been a lack of reaction between the antiserum and indicator cells, but this was eliminated by the use of controls throughout the experiments.

From the sample stored under different conditions the following results were observed:

Most stains retained their activity for the duration of this experiment.

Most stains showed no reduction of activity for either Km(1) or Km (3) although dilutions up to 1/10 of the stain extract were used.

One sample which was typed as (1+3+) gave varying reactions for Km(1) at 56° C and at 37° C in the humid chamber. This could be mistaken for a (1-3+)result.

THE USEFULNESS OF Km(3) TYPING IN BLOODSTAINS

Steven Rand[1], Petra Ritter, Bernd Brinkmann (Institut für Rechtsmedizin, Münster/Germany. [1]On loan from the Metropolitan Police Laboratory, London)

Known positive and negative controls should always be simultaneously tested when bloodstain grouping is carried out. In a multiallelic system such as Gm/Km it is also wise, if negative results are to be reported, to select a suitable antithetical marker as a control. One can then be sure that a negative result for a particular factor really is negative and not just that insufficient material is present.

When grouping bloodstains in the Gm system it is common to use Gm(4) and, or Gm(10) as a positive marker for a Gm(1-,2-) phenotype.
In the Km system approximately 87 % of caucasian and 50 % negro populations are negative for Km(1) and although guidelines to the reliability of a negative result, such as condition, age and colour of the stain can be employed, this is not an infallible judgement. Far better is the use of the antithetical Km(3) marker, so long as the antigens and, or antisera are of equal and compatible quality.
This investigation was carried out to assess these qualities in commercially available anti-Km(3) and anti-D (Km 3), with reference to blood stain grouping.

Method

The grouping was performed on microtitre plates.
The factors Km(1) and (3) were tested using dilutions of the stain extract and of serum samples.
A total of 280 samples were tested in parallel on stains and sera. Of these bloodstains a representative sample

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CONCLUSION

4.-

Enzyme-Immuno-Assay on nitrocellulose blots offers various advantages for bloodstain analysis :

HIGH SENSITIVITY, allowing to use serum diluted as far as 1/1280 or small amounts of bloodstains and to work on old bloodstains - with the urea extraction, results could be obtained on bloodstains ageing at least 17 months.

MULTIPLE REPLICATES, each blot can be stained for one or even more different systems, simultaneously or subsequently (1). Clear second copies were only obtained for recent bloodstains.

STABLE RESULTS, the blots can be stored (in the dark) as a permanent proof of analysis. They can even be stained later with a new detection method.

This technique is not more expensive than the immunofixation technique (3), because very diluted anti-sera can be used.
It is somewhat more time and work consuming : minimum 2 hours blotting and minimum 5 hours or 7 hours respectively for a 2 steps or 3 steps EIA method.

Acknowlegment

I gratefully thank Dr med. J. HALLENG for her help.

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ELECTROPHORETIC BLOTTING CONDITIONS

NITROCELLULOSE QUALITY : Nitrocellulose from MILLIPORE (HAHY 0.45 µm), SCHLEICHER & SCHUELL (BA 85, 0.45 µm) and BIORAD (A 598 0.45 µm) were used. The foils from SCHLEICHER & SCHUELL yielded the strongest results on the first replicate but a very weak second copy. The MILLIPORE and BIORAD foils gave good results for the first and second replicates.

TRANSFER PERIOD : Proteins were transfered during various periods from 1 to 10 h. Results were satisfying after minimum 2 h. but were better after longer transfer.

Gc TYPING OF BLOODSTAINS

Bloodstains from 57 persons of known Gc subtype were tested, some after different ageing periods. The results are summarised in the table.

AGEING PERIOD	NUMBER OF STAINS TESTED	RESULTS		
		CORRECT	FALSE	NO
< 6 months	34	30	4*	0
6 to < 9 months	19	16	2*•	1
9 to < 12 months	14	8	1*	5
12 to < 17 months	19	6	1•	12

* 6 stains Gc 2 read Gc 2-1S

• 2 stains Gc 2-1 read Gc 2

Two causes of Gc group mistyping were met :

1) In some Gc 2-1 (2-1S or 2-1F) samples - but not all of them - the Gc1activity disappears quicklier than the Gc 2 activity - So a weak Gc 2-1 sample can be read Gc 1.

2) In the five Gc 2 samples tested, an additional band usually appeared which, in the PAGIF conditions used, could not be distinghuished from the cathodal Gc 1S band. So a Gc 2 bloodstain can be misinterpreted as a Gc 2-1S. This band apparently is not in the same position as the Gc 2-actin complex described by SHINOMIYA (5).

RESULTS 2.-

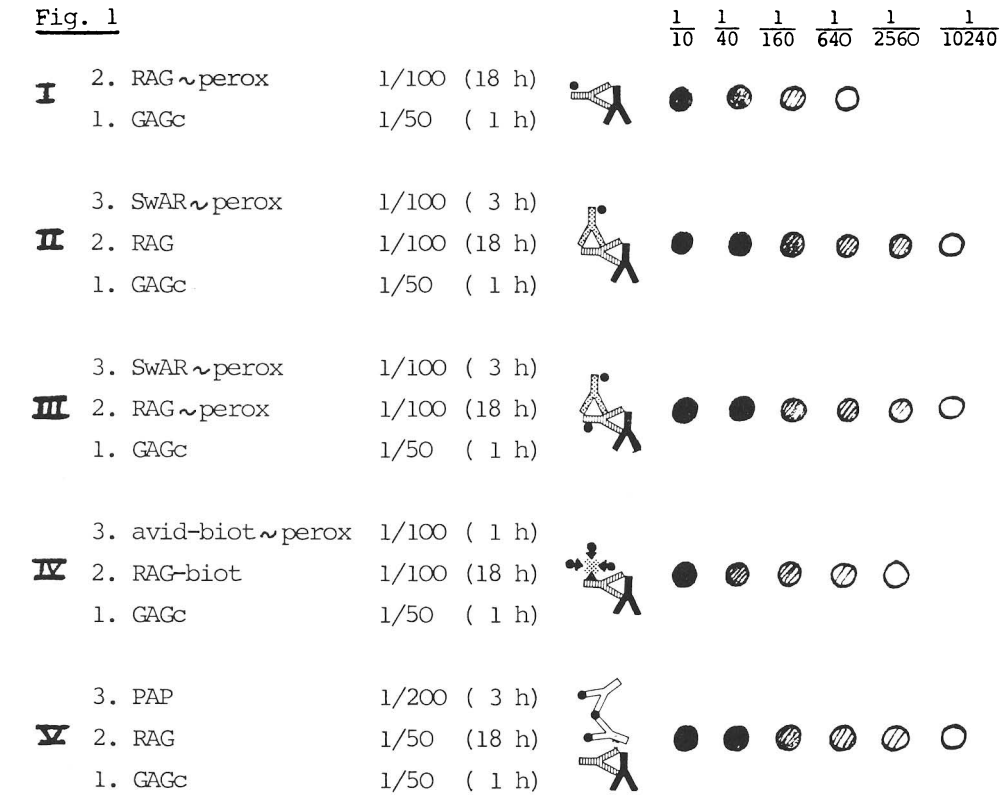
COMPARISON OF VARIOUS ENZYME-IMMUNO-ASSAYS BY DOT BLOTTING

The same two-fold dilutions of a Gc 1F-1S serum were tested as dots on nitrocellulose. For each immunoassay, a series of the reagent dilutions (from 1:20 to 1:1000) were tried in each step. Results are only shown for the optimal dilutions (Fig. 1). Another method (1. the same GAGc 2.protein A~peroxydase from GAMMA) was not tested further because our first results showed it very less sensitive.

From repeated assays it can be concluded that the sensitivity of method II, III and V was equivalent, with a detection limit around 10 ng. Method IV was slightly fainter and method I still a bit fainter.

For further work on Gc typing, method III was prefered because the third step can only be applied on very weak results, if necessary.

Fig. 1



GAGc : goat anti-Gc (ATAB)
RAG : rabbit anti-goat Ig (ATAB)
RAG~perox : rabbit anti-goat Ig, peroxydase conjugated (DAKO)
SwAR~perox : swine anti-rabbit Ig, peroxydase conjugated (DAKO)
RAG-biot : rabbit anti-goat Ig, biotine conjugated (VECTOR)
avid-biot~perox : complexes of avidine and peroxydase conjugated biotine (VECTOR)
PAP : peroxydase, anti-peroxydase from goat (DAKO)

ADVANTAGES OF ENZYME IMMUNO ASSAY AFTER BLOTTING IN BLOOD STAIN GROUPING. APPLICATION TO THE Gc SUBTYPES.

Bernadette M. HOSTE (Blood Group Laboratory and Transfusion Center, University of Liege, rue Dos-Fanchon, 41 B-4020 Liege, Belgium)

The available bloodstain amount is the principal limitation to analysises. Thus increasing the sensitivity of the methods and determining multiple groups in the same sample were the aims of this work.

METHODS

BLOODSTAIN EXTRACTION

Experimental stains of whole blood on cotton cloth were studied. 3 x 5 mm pieces were soaked in 6 M urea (6) during 1 hour for fresh stains and overnight for older stains. The cotton pieces were applied on the gel.

POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING (PAGIF) was performed with ampholines pH 4-6.5 (2)

DOT-BLOTTING

Nitrocellulose foils were soaked in transfer buffer and allowed to dry. Dots were applied, of 1 µl serum diluted in Tris buffer saline (TBS) 10 mM,pH 7.4 containing 0.6 % pure gelatine. The foils were then treated like after electrophoretic blotting.

ELECTROPHORETIC BLOTTING

According to TOWBIN (5) and the BIORAD protocoll, the nitrocellulose foils and the PAGIF gel were equilibrated 15 min. long in transfer buffer (Tris 20 mM, glycine 150 mM pH 8.3). The mylar sheet of the PAGIF gel was taken off and the proteins were transfered at 45 V during various periods in a BIORAD Trans-Blot apparatus.

ENZYME-IMMUNO-ASSAY (EIA) STAINING OF THE BLOTS

The free sites on the filter were blocked 1 hour at 37° C in TBS 10 mM pH 7.4 containing 1 % gelatine.
It was then incubated with the antibodies diluted in TBS + gelatine 0.6 % during 1 hour or 1 night. Each step was followed by 4 washings of 10 min. in TBS + gelatine 0.6 % + Tween 0.1 %. Finally the peroxydase activity was stained with 4-chloro-1-naphtol (according to BIORAD).

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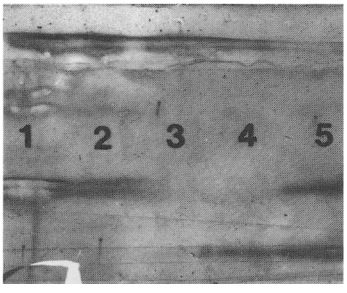
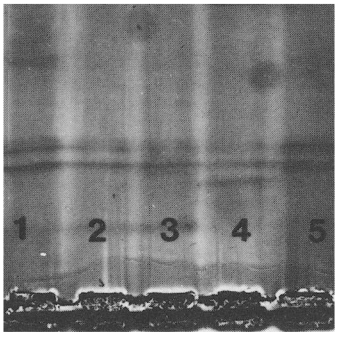
Fig. 4: Subtypes of Tf and Gc in blood stains by simultaneous IEF with immobilized pH gradient.



Upper part of the polyacrylamide gel: From left to right: Gc 1F1S reference, Gc 1F1S blood stain, no pattern blood stain, Gc 2 blood stain, Gc 2-1F reference.



Lower part of the same polyacrylamide gel. From left to right: Tf C1 reference, Tf C1 blood stain, Tf C3-1 blood stain, Tf C2-1 blood stain, Tf C3-1 reference.

Discussion

For the simultaneous demonstration of the genetically determined variability of the Tf and Gc system in blood stains, IEF with carrier ampholytes and IEF with an immobilized gradient were found to be suitable. IEF using carrier ampholytes shows a lesser degree of resolution than IEF with immobilized gradient.

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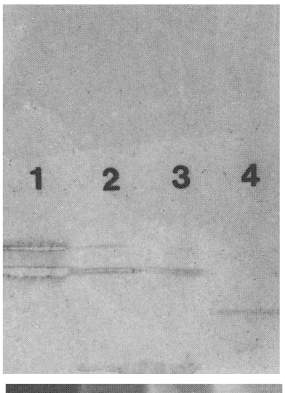
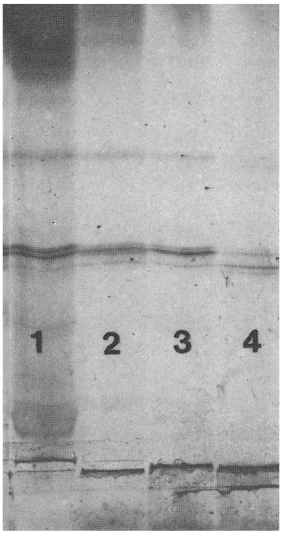
the Tf subtypes in the same polyacrylamide gel after TCA-fixation. Sample volume: 10 µl of a solution of 10 mg blood stain + 50 µl ferrous ammonium sulfate.

Fig. 4 is an illustration of the results of the IEF with immobilized pH gradient pH 4,8 to pH 5,8: In analogy to Fig. 3 in the upper part the Gc-phenotypes and in the lower part the Tf-phenotypes. Sample volume: 20 µl of a solution of 10 mg blood stain + 50 µl ferrous ammonium sulfate.

Fig. 3: Subtypes of Tf and Gc in blood stains, IEF with carrier ampholytes.



Upper part of the polyacrylamide gel: Gc-subtypes after immuno-fixation with monospecific anti-Gc antiserum. From left to right: Gc 1F1S reference, Gc 1F1F blood stain, Gc 1F1S blood stain, Gc 2 blood stain.



Lower part of the same poly-acrylamide gel: Tf-subtypes after TCA-fixation. From left to right: Tf C1 reference, Tf C3-1 blood stain, Tf C1 blood stain, Tf C2-1 blood stain.

Fig. 1: IEF-technique with carrier ampholytes for the simultaneous analysis of Gc and Tf subtypes.

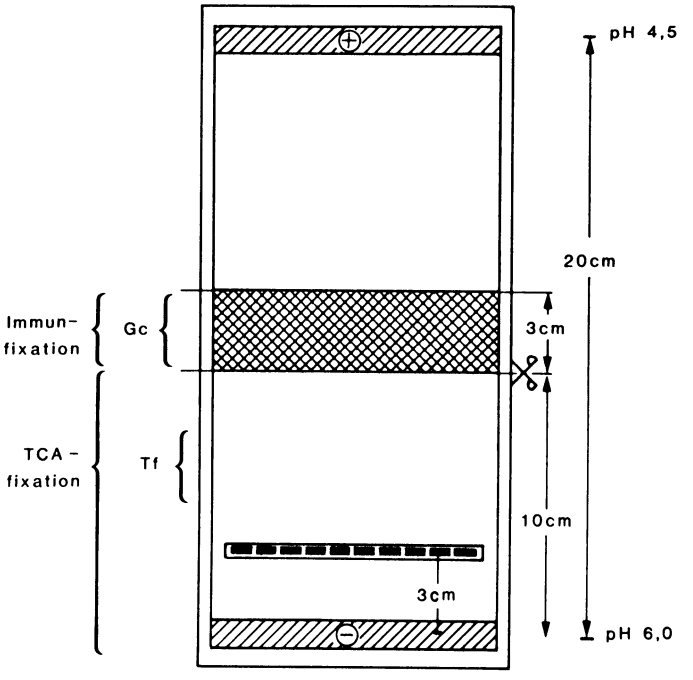
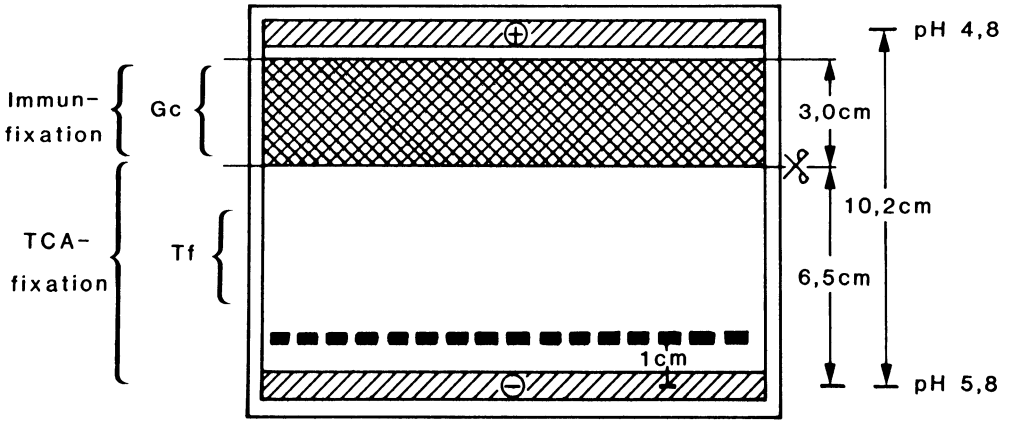


Fig. 2: IEF-technique with immobilized pH gradient for the simultaneous analysis of Gc and Tf subtypes.



PRACTICABILITY OF SIMULTANEOUS Gc AND Tf SUBTYPING IN BLOOD STAINS

Günter Berghaus and Michael Staak

(Institut für Rechtsmedizin der Universität zu Köln, Melatengürtel 60-62, D - 5000 Köln 30, BRD)

Gc and Tf subtyping techniques of blood stains by isoelectric focusing (IEF) has been developed in the past years (for example BERGHAUS and STAAK, 1982). Since there is considerable variation in gene frequencies IEF for simultaneous Gc and Tf subtyping of blood stains may be a useful tool that dramatically increases the discriminating capabilities of these polymorphisms. We therefore developed techniques (ampholyte gel, 20 cm separation distance; Immobiline^{R} gel, 10 cm separation distance) to analyse blood stains in one focusing.

Materials and methods

Serum specimens for subtyping had been pretreated by mixing 1 drop serum with 3 drops of 0,5 g% ferrous ammonium sulfate. Blood stains of known subtypes were made on glass plates allowed to dry and stored at room temperature. Prior to focusing blood stains were weighted and diluted in 0,5 g% ferrous ammonium sulfate. All samples had been stored overnight at 4° C.

The technique of IEF using carrier ampholyte on 0,1 mm polyacrylamide gels (RADOLA, 1980) has been modified as shown in Fig. 1. The technique of IEF with immobilized pH gradients (GÖRG et al., 1983) has been modified as shown in Fig. 2.

Results

The results of IEF with carrier ampholytes for the simultaneous analysis of Tf and Gc subtypes is shown in Fig. 3: In the upper part the Gc subtypes after immunofixation with monospecific anti-Gc antiserum and in the lower part

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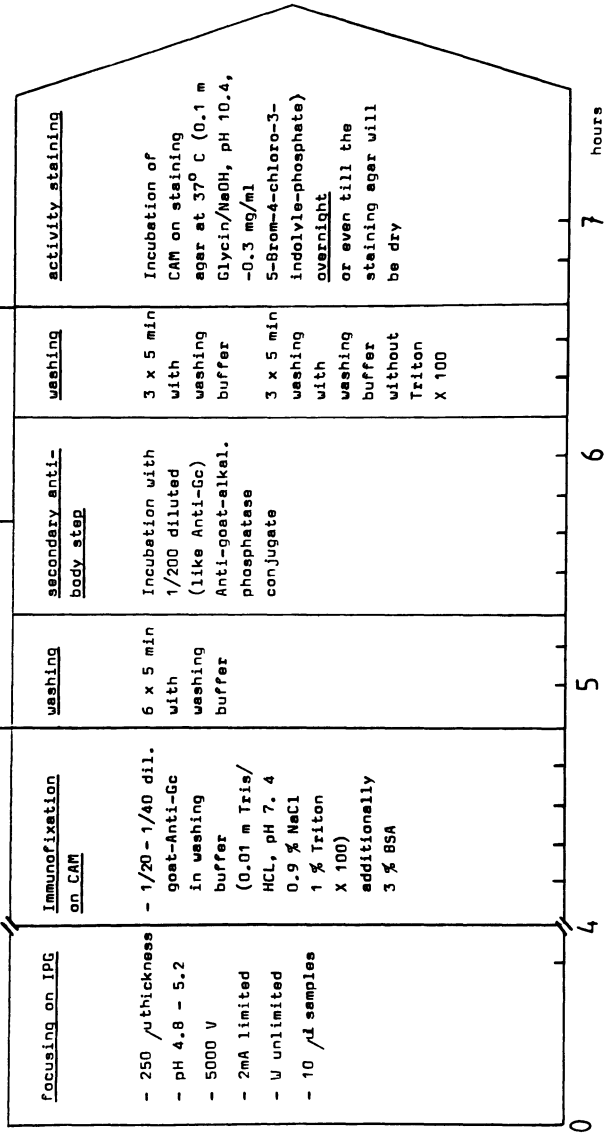
The last Table summarizes the results of our tests and shows all steps and the schedule of the Gc-subtyping procedure, which may be used as well for other systems like Tranferrin (Tf) or Protease Inhibitor (Pi).

Table 3: Single steps and schedule of Gc subtyping procedure



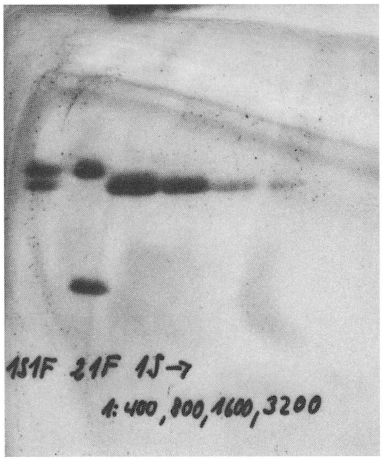


Fig. 3

Isoelectric focusing of Gc on immobilized pH-gradient gels and detection with the system described in Fig. 1: series of serum dilutions with a final titer of 1/3200.

In some cases we had problems with the gel preparation and the quality of the pH-gradient. Therefore we tried to reuse IPG's of good quality by washing it with water, detergents or NaOH after focusing.

The next Figure (Fig. 4) shows the results of a test series in which a gel was reused 5 times consecutively and washed only by means of bidistilled water between the different runs. In all cases there is no interference with samples of the earlier run. We are looking forward to present a final recycling procedure after additional tests with protein hydrolyzing substances like NaOH.

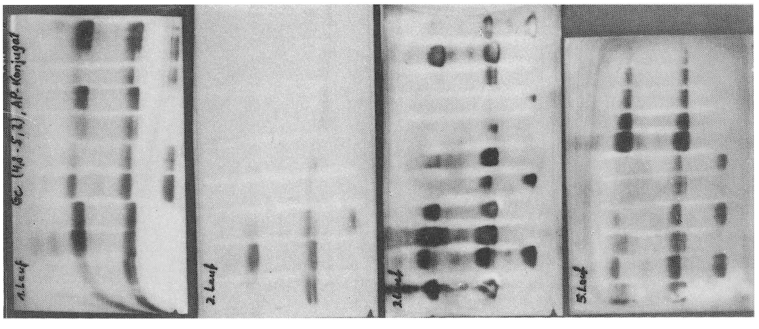


Fig. 4

Isoelectric focusing of Gc on immobilized pH-gradient gel with the detection system described above: reuse of the same gel 5 times after recycling with water between every run? The 1., 2., 3. and 5. run is shown.

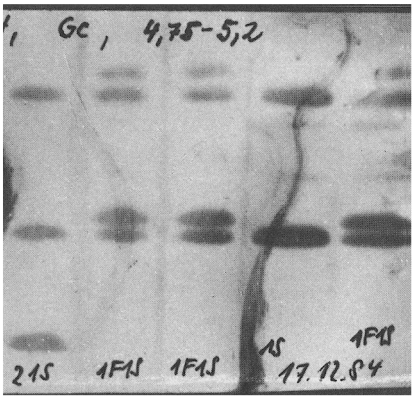


Fig. 1

Isoelectric focusing of Gc from diluted sera on immobilized pH-gradient gels and detection with an antibody conjugated alkaline phosphatase system after immunofixation on CAM.

The next Figure (Fig. 2) shows a comparison of immunofixation on CAM and capillary blotting on NC, where the sensitivity of the first is much better than that of the latter.

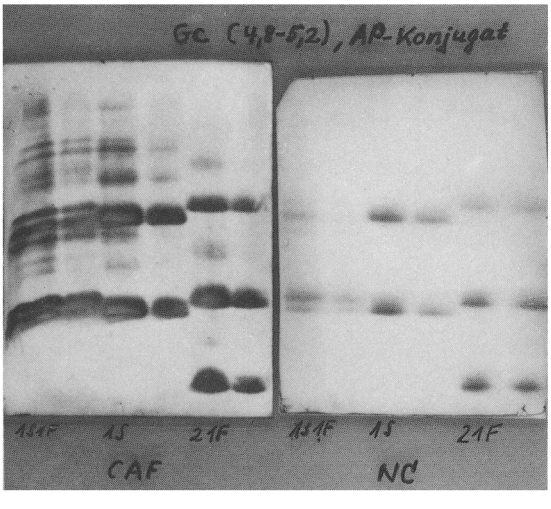


Fig. 2

Isoelectric focusing of Gc from diluted sera on immobilized pH-gradient gels: comparison of immunofixation on CAM and capillary blotting on NC using the antibody conjugated alkaline phosphatase system

The following Figure (Fig. 3) shows a series of dilutions of a two years old repeatedly frozen serum with a final dilution of 1/3200. In all dilution steps a typable activity can be seen. Recently we got activity even in 1/12 800 diluted serum samples and also in bloodstain extracts which were very diluted or for example in 5 years old badly soluble bloodstains kept at RT.

This demonstrates the efficiency of the method.

Table 2: Construction of the Gc-detection system with alkal. Phosphatase conjugated secondary antibody

a) Immunofixation on Cellulose acetate membrane (CAM) - 1/20 - 1/40 diluted goat-anti-Gc in washing buffer (0,01 m Tris/HCL, pH 7, 4) 0,9 % NaCl 1 % Triton X 100) additionally 3 % bovine serum albumin (BSA) b) capillary blotting on (NC) nitrocellulose with an additional blocking step (BSA)	6 x 5 min with washing buffer	Incubation with 1/200 diluted (same diluent as Anti-Gc) Anti-goat-alkal. Phosphatase conjugate	3 x 5 min with washing buffer 3 x 5 min washing with washing buffer without Triton X 100 activity staining overnight or even longer

The following three Figures show the results of our experiments. First (Fig. 1) a Gc pattern of several diluted 1 - 2 years old frozen sera. It is a great advantage of this method that no remarkable diffusion occurs if the staining period is prolonged for several hours or even days so that also very weak activities can be developed. The incomplete destaining of the background is due to the washing buffer system, which consists in this case only of saline.

Isoelectric focusing of Gc subtypes on reuseable immobilized pH-gradient gels followed by detection with antibody conjugated alkaline phosphatase

W. Pflug
Landeskriminalamt Baden-Württemberg
KTI
Taubenheimstraße 85
7000 Stuttgart 50
FRG

The best method now available for the subtyping of the Vitamin D-binding protein Gc is the isoelectric focusing on immobilized pH-gradient gels (IPG's) because very narrow and stable pH- gradients with high resolution and high loading capacity can be prepared (1). Cleve and coworkers showed, that by means of immobilized pH gradients correct subtyping of Gc from fresh sera is possible after protein staining with Coomassie Blue directly on the running gel (2). Their data for gel preparation and the running conditions with some modifications of our own are given in Table 1.

Table 1: Data for preparation and focusing of IPG's

Immobilines	pK 4.6 and 6.2
gel concentration	T 5%, C 3%
gel dimension	240 x 105 x 0.25 mm
focusing settings	5000 V 2mA limited W unlimited
cooling temperature	10^0 C
focusing time	4 h
Sample application	1 cm from cathode

When analyzing bloodstains, there are some problems in regard to the Gc subtyping. Firstly the Gc can form complexes with the actin of the cells which then migrate with a lower isoelectric point than free Gc (3). Therefore we dilute/extract all samples (frozen sera or bloodstains) with a mixture of 6 M urea (in order to split a possible Gc/actin-complex (3)) and 3% bovine serum albumin (in order to give a higher sensitivity by immunofixation - own results). Guanidine (4) instead of urea should only be used in combination with a dialysis prior to sample application (own results). Secondly, the sensitivity of direct protein staining methods is limited even after immunofixation and following silver staining since the highest possible dilution for detecting Gc in sera is limited to a titer of 1/64 - 1/128 (5). In forensic science casework there are often bloodstains with Gc amounts below this level. Therefore we developed a more sensitive secondary antibody conjugated enzyme system for the subtyping of very small amounts of Gc. The next table shows the construction of the test system (Table 2).

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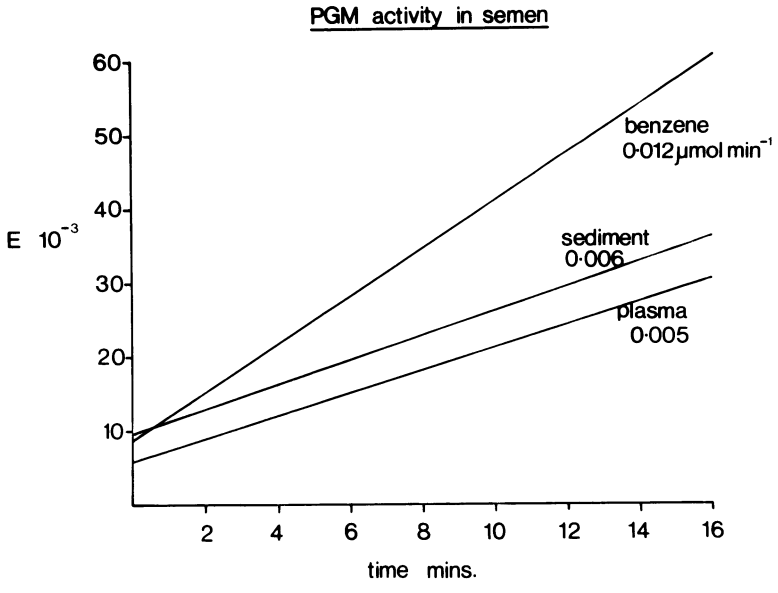

Fig. 1: Comparison of PGM activity in plasma, in spermatozoa after freezing/thawing and after benzene extraction

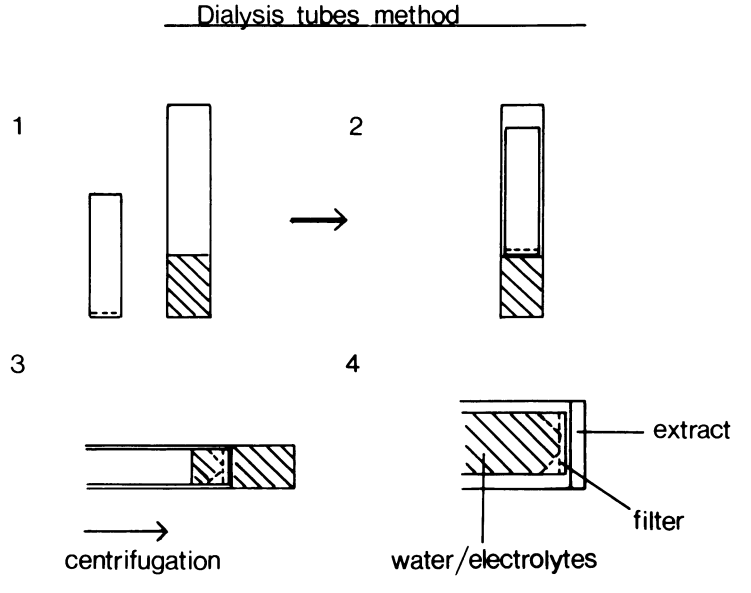

Fig. 2: Diagrammatic representation of procedure for dialysis of semen samples

treatment had strong activity but pronounced deformation of bands, making a clear identification impossible. It is hoped to improve the procedure sufficiently for use with the majority of samples.

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spermatozoa would therefore be small in comparison to the plasma. In the second stage, methods of improving the quality and quantity of activity found in bands after isoelectric focusing were investigated. It is not always the case, but strong activity is often observed with such distortion of the bands that it is not possible to identify the phenotype. In these cases systematic dilution of the extract in aqua dest or different buffer solutions resulted only in a weakening of complete absence of bands. One of the reasons for this distortion could be a high ion concentration in these samples causing an imbalance in the pH structure of an isoelectric focusing plate. With this in mind semen samples and stain extracts were dialysed using dialysis tubes, Centrisart^{R}1, produced by Sartorius, as follows:
200 µl semen or a 1 cm x 1 cm piece of dried seminal stain from the same sample was placed in 2 ml of aqua dest. After freezing and thawing, the liquid was placed in the outer tube (fig. 2). The inner tube with the attached membrane was then inserted over the sample and centrifuged at 2000 g, after allowing 5 minutes for the membrane to be uniformly wetted. The water is forced through the membrane leaving only a concentrated sediment between the inner and outer tubes. This sediment was removed and applied to the gel. After this treatment it was observed that the bands showed weaker activity than the untreated samples but were not distorted and a phenotype could be determined.

The results of the experiments were not completely satisfactory because of the decrease in activity and would not therefore be suitable for weak samples, but the procedure has been successfully used for samples which before

INVESTIGATIONS ON PGM ACTIVITY AND ELECTROPHORETIC PATTERNS FROM EJACULATES AND SEMINAL STAINS

Steven Rand[1], Gerda Winkelbauer, Bernd Brinkmann
(Institut für Rechtsmedizin, Münster/Germany,
[1]on loan from the Metropolitan Police Laboratory,
London)

PGM activity from semen samples was measured in vitro from the supernatant (i.e. seminal plasma) and from the sediment (i.e. spermatozoa) using equal volumes of both. The activity from the sediment was also tested after a 4 times freezing and thawing procedure and after treatment with various organic solvents in an attempt to perforate or dissolve the membrane. These solvents included benzene, toluene, xylene, chloroform, carbon tetrachloride, acetone and phenol. From the results of these experiments it was observed that in comparison to the purely mechanical method of membrane disruption, i.e. freezing and thawing, there was a further significant increase in the measured PGM activity after treatment with toluene and benzene, but only in the range of 50 to 100 %. Furthermore activity was found in both the plasma and the sediment, and the maximum activity for the sediment was achieved using benzene (fig. 1). The variation in activity between the sediment and plasma ranged from 1:0,5 to 1:4.
The case history of the individual semen samples was not made available to us so that the precise storage details and the possible presence of any abnormalities were not known. Some of these unknown factors would also be encountered in routine casework samples. The proportion of spermatozoa in a semen sample also varies enormously but is normally in the range of 1 % of the total volume, with an average 100 million spermatozoa per ml plasma (4). The percentage of the total PGM activity contributed by the

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Samples of the rabbit sera were applied to ULAGIF gels containing PGM from fresh human hemolysates and it was attempted to visualize PGM by conventional immunofixation in cellulose acetate membranes, washed with saline and water, and stained with Coomassie blue R250. No discrete bands were discernable.

Dale Dykes, Memorial Blood Center, Minneapolis, Minnesota, tested the rabbit sera by passive immunoblotting of ULAGIF gels with nitrocellulose membrane treated with enzyme-conjugated goat anti-rabbit IgG. At dilutions of the rabbit sera up to 1/250, faint bands were discernable on the nitrocellulose, but unfortunately, they did not correspond to the mobility of standard PGM locus 1 or locus 2 isozymes. In fact, there were a multiplicity of faint bands throughout the lanes of sample hemolysates.

There are a number of possible causes for the disappointing preliminary results of the experimentation thus far. However, numerous alternative courses of action remain to be pursued.

If recent history of the improvements made in protein detection enhancement by immunological methods is any indication, it should be obvious that the red cell enzyme markers traditionelly of forensic interest must soon be visualized by alternative detection methods in order to match the sensitivity now observed in serum protein analysis.

Any deterioration in the kinetic activity of PGM in stains results in the corresponding loss of detectability of the PGM isozymes, despite the probable migration of PGM isozymes to their usual respective locations in IEF and electrophoresis gels. Eventually, PGM kinetic activity will disappear completely and leave the isozyme bands which are present virtually undetectable.

An alternative detection method for PGM might be found by exploring other binding mechanisms such as potential immunologically reactive sites and enzyme-specific antibodies. That is to say, it is time to view PGM as an antigen as well as an enzyme.

The groundwork for the detection of numerous blood proteins by immunofixation and enzyme linked immune assays is already well-established. Antibody specific for human PGM ought to succeed in immobilizing PGM in a suitable matrix.

Unfortunately, there exists no human PGM antisera that we know of. Therefore, we are endeavoring to produce our own.

Purified human PGM is required as the immunogen. In order to obtain purified human PGM, Dr.Harvey Mohrenweiser, University of Michigan Medical School, Department of Human Genetics, proceeded to isolate approximately 10 mg of PGM from approximately 5 kg of human muscle.

Dr.Mohrenweiser determined by SDS gel electrophoresis that the purification yielded primarily a single band of protein corresponding in molecular weight to rabbit PGM (approximately 70.000 Daltons). He estimated that the end product was quite pure, showing in the gel only one additional faint band of unidentified protein.

The purified PGM was lyophilized and forwarded to two different laboratories for antisera production.
Dr.Robert Myers, Michigan Department of Public Health, immunized two rabbits. Dr. Henry Carwile, Bethyl Laboratories, immunized one sheep.

Both laboratories initiatd immunization employing Freund's Complete Adjuvant mixed with a solution of PGM consisting of 100 µg dissolved or suspended in 1.0 ml of saline. The animals were further stimulated according to their respective immunization protocols. Test bleedings from all three animals demonstrated antibody titers of no better than 1/4 by double diffusion against human hemolysate. The rabbits were hyperimmunized after six weeks raising the antibody titer to 1/8 and subsequently exsanguinated.

Extending the detectability of phosphoglucomutase in old bloodstains and seminal stains by immunofixation with anti-human PGM: a preliminary report.

Mark D.Stolorow, M.S., M.B.A.
Serology Coordinator
Illinois State Police Bureau of Forensic Sciences
515 E. Woodruff Rd.
Joliet, Illinois, 60432, USA.

Red cell polymorphic enzymes are vitally important in the individualization of dried bloodstains. Unlike those scientists who work exclusively with fresh liquid blood, the forensic serologist is unfortunately often prevented from interpreting phenotypes of particular red cell enzymes simply because the bloodstains are either

a. too small (or too diffuse), or

b. too deteriorated.

A natural question to explore thus emerges: how does the forensic serologist improve the detectability of red cell enzymes in stains whose size and degree of deterioration fall beyond his control?

For the analysis of small or diffuse stains, improved sampling techniques and sample application methods have expanded the capacity of the forensic serologist to determine phenotypes of the enzyme markers successfully. However, when the enzyme markers are too deteriorated (usually from age), there appears to be no solution at the present time.

It would seem the answer rests in locating a satisfactory alternative to the delicate detection procedures employing enzymatic staining currently used for red cell enzyme marker analysis virtually worldwide. In searching for an alternative detection method, it was observed that phosphoglucomutase would be an excellent candidate for such research because of its widespread application in the analysis of body secretion stains as well as bloodstains.

To date, PGM has been analyzed exclusively by electrophoresis or isoelectric focusing methods. The detection method employed to visualize the location of the PGM isozymes on the electrophoresis support media or blotting matrices is currently limited to some variation of a colored dye formation such as MTT, NBT, or meldola blue. The selective staining method unfortunately places a demand on perhaps the most labile property of an enzyme marker, namely its kinetic activity. In to be visualized, an enzyme such as PGM must maintain its conformation and activity at both its substrate bindingsite and its reactive site.

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2. The enhancing property is not unique to human blood. For example, red cell lystaes from goat, sheep and rabbit blood all have the ability to enhance the PGM activity of human saliva.
3. The factor appears to have a molecular weight of greater than approximately 1400. This was demonstrated by dialysing red cell lysates for 24 hours to remove any low molecular weight substances. The dialysed lysates were found to retain their PGM enhancing ability.
4. Red cell lysates retained their enhancing activity after being heated to 80^{o}c for 30 minutes.
5. Incubation of red cell lysates with trichloroacetic acid precipitates total protein and also removes the enhancing factor or factors.

Further attempts to identify and isolate this factor have been unsuccessful. Its nature and mechanism of action therefore remain speculative.

CONCLUSIONS

1. Red blood cells from a range of animal species contain a factor or factors which have the ability to enhance the PGM activity of human saliva.
2. The enhancing phenomenon could be a source of PGM mistyping when analysing blood/saliva stain mixtures.
3. The factor is non-dialysable and is relatively heat stable.
4. Further characterization and purification of the enhancing factor may provide a means of typing saliva stains in the PGM system.

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Sample no. 8 for example, consists of a PGM (1+) blood mixed with saliva from a PGM (2+) donor. Both the b+ and a+ isozymes can be clearly seen.

Sample no. 11 consists of PGM (1+) blood mixed with saliva from a PGM (2+1-) donor. A strong a+ isozyme can be seen with weaker bands in the b+ and a- positions.

Sample no. 17 consists of a PGM (2+1+) blood mixed with saliva from a PGM (2-1+) donor. The b+, a+ and b- isozymes are all visible.

One hundred and twenty-six blood/saliva mixtures were examined in this way and the extra PGM activity was observed in 90 (71%) of these samples. It was also clear from the results that the extra activity was derived from the saliva since in all instances it corresponded with PGM phenotype of the donor of the saliva. Since the saliva samples on their own contained little or no PGM activity it appeared that the blood contained a factor or factors which have the ability to activate or enhance the PGM activity of saliva.

These observations have two important implications when considering the analysis of body fluid stains. First, the phenomenon could be a source of mistyping in the PGM system. For example, if the blood from a victim of type PGM(1+) became mixed with saliva from an assailant of type PGM(2+), typing of the stain could give a PGM(2+1+) result and thus lead to an incorrect conclusion about the source of the blood. Secondly, characterization and purification of the enhancing factor may provide a means of typing saliva stains in the PGM system. With this possibility in mind a number of experiments were performed in order to characterize the PGM enhancing factor.

Properties of the PGM Enhancing Factor

1. The enhancing ability of blood is a property of the red cell and not the serum.

to be stronger than the control blood samples but the most noticeable effect was that in some mixtures, extra bands of PGM activity were clearly visible (Fig. 1).

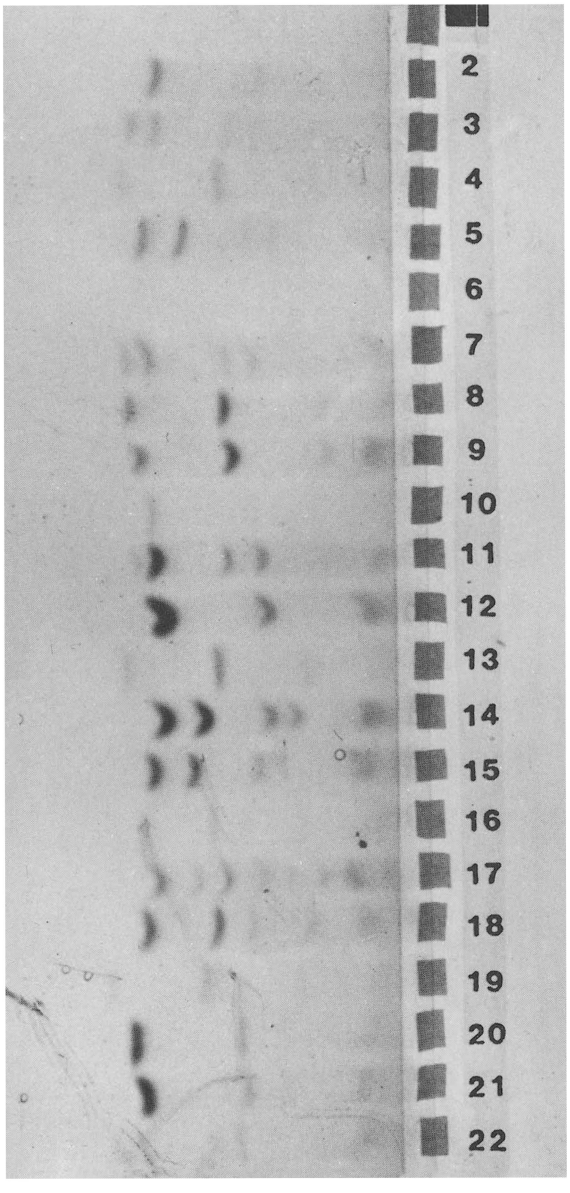

Fig. 1. PGM typing of blood / saliva mixtures

PGM ACTIVITY OF SALIVA AND ITS FORENSIC SIGNFICANCE

Graham B. Divall
Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road,
London SE1 7LP, England

INTRODUCTION

One of the problems encountered in the analysis of dried stains is interpretation of grouping results obtained from mixtures of body fluids whether they be blood and saliva, semen and blood or semen and vaginal secretions. With this problem in mind, one of the questions we have studied is the effect of saliva on the phosphoglucomutase (PGM_1) typing of blood.

MATERIALS AND METHODS

All whole blood and saliva samples were collected in plain glass bottles from donors of known PGM_1 phenotype.

Samples were analysed for PGM avtivity by non-equilibrium focusing in ultra-thin polyacrylamide gels as previously described. (1)

RESULTS AND DISCUSSION

Whole Saliva and Saliva Stains:

Out of 402 saliva samples tested, weak PGM activity was detected in only 51 or 13% of them. Furthermore, the activity was never of sufficient strength to ascertain the PGM phenotype of the donor. PGM activity was not detected in any of the stains made from the saliva samples.

Blood/Saliva Mixtures:

From the results described above it appeared that saliva would have very little effect on the PGM typing of a bloodstain. To test this prediction, lysed blood samples were mixed with whole saliva and the mixture analysed for PGM activity. In some instances the PGM bands appeared

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DNA isolated from all sperm samples was found to be high molecular weight. Each undigested DNA had a size larger than a 23 kb DNA size marker in agarose gels. This property is essential for RFLP analysis because degraded DNA consists of randomly cleaved DNA fragments that, after digestion and electrophoresis, do not migrate as discreet bands in a gel. As a result, degraded DNAs lose the radioactive bands normally seen after hybridization. The presence of bands larger than expected could result from incomplete digestion of DNA with restriction endonucleases. Thus, all DNAs were digested twice with at least a five fold excess of enzyme.

DNAs prepared from peripheral blood from the male and female volunteer sexual partners and from dried sperm recovered post-coitus, were used to prepare filters containing Eco Rl or Taq 1 digested DNA. These filters were hybridized with radioactively labelled DNA probes pAW101 or pLM0.8. In nine cases examined, the pattern of bands seen in the DNA lane from sperm matched the pattern of the DNA from the male peripheral blood. Thus, DNA purified from semen samples are not contaminated with detectable amounts of female specific DNA.

As with any polymorphic system, the ultimate power to assign identity depends on the number and frequency of alleles. We have collected a data base of over 1000 chromosomes for both probes from random blood donors in the New York City area. Information concerning race was used to assemble separate allele frequency tables. Presently, with the two probes used, the power of identity an individual is 1 in 1,000. The addition of more probes which recognize highly polymorphic DNA regions will increase the power of the DNA test.

Conclusions

Male specific high molecular weight DNA was isolated from dried semen stains up to 8 weeks old. The pattern of RFLPs seen with Eco Rl and Taq 1 digested DNAs hybridized with different probes which detect polymorphisms indicated the pattern seen with the semen DNA matched the male sexual partner. This procedure could prove useful in identification of assailants in rape cases.

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or Taq 1 (Bethesda Research Laboratories) using conditions recommended by the manufacturer. Samples were routinely precipitated with ethanol after the first digestion, resuspended in 20 microliters, and loaded on agarose gels. Eco Rl digested samples were loaded in 0.4% agarose gels and electrophoresed 65 hours at 0.6 volts/cm while the Taq 1 digested samples were loaded in 1.2% agarose gels and electrophoresed at 1.8 volts/cm for 20 hours. The length of gels varied from 15 cm to 22 cm. After size fractionation by electrophoresis, DNA was transferred to nylon membranes, and hybridized with ^{32}P-labelled probes using standard procedures (4).

DNA Probes

Two probes were used in this investigation which recognize highly polymorphic regions in the human genome. pAW101 contains a 6.5 kilobase (kb) Eco Rl insert in pBR322 (kindly supplied by R. White). It hybridizes with the D14S1 locus located in chromosome 14 and is polymorphic with Eco Rl with over 30 alleles distributed from 14 to 32 kilobases (5). pLM0.8 contains a 879 base pair Cla 1 - Sph 1 insert in pBR322 and hybridizes to the 3'-flanking region of the HRAS-1 oncogene in chromosome 11 (6). It is polymorphic with Taq 1 with 18 distinct alleles from 1.8 to 4.5 kb.

Results and Discussion

DNA isolated from matched blood and semen samples was analysed using two probes, pAW101 and pLM0.8, which detect polymorphic regions. For each individual we compared the pattern of RFLP in both blood and semen DNA. We examined 106 Taq 1 digested matched DNA samples with pLM0.8 probe and 40 Eco Rl digested matched samples with pAW101 probe. No size differences between alleles were seen in the DNA isolated from the two tissues.

The number of sperm recovered from dried semen stains varied considerably, presumably due to natural variation in sperm count. The dried stains were from 1 to 7 days old before processing. Studies where a known amount of sperm was applied to cotton cloth and aged for one day to 8 weeks indicated that about 30% of the sperm are recovered by this procedure and was independent of sample age.

On average, the amount of DNA recovered from all samples was about 90% of the expected (number of sperm multiplied by 2.5×10^{-12} g). The average amount of DNA isolated from a dried semen stain was 40 µg.

Titration experiments indicate that the amount of DNA necessary for RFLP analysis varies from 1 to 4 micrograms. Thus the amount of DNA isolated from the dried stains was sufficient for multiple analyses in duplicate.

Materials and Methods

Sources of DNA

Matched blood and sperm samples were collected from volunteers. Blood was collected in Vacutainer tubes using potassium EDTA as anticoagulant and the semen was left untreated.

Nine dried semen samples deposited on women's undergarments or sanitary napkins following intercourse were obtained along with blood samples from the volunteer male and female sexual partners.

Isolation of DNA

Fabric containing dried semen was cut into small pieces and scrubbed with a small brush in 30 ml of phosphate-buffered saline (136 mM NaCl; 8 mM Na_2HPO_4; 17 mM NaH_2PO_4, pH 7.0), while sanitary napkins were processed by removing the plastic and cutting the absorbent material into small pieces. The pieces were soaked for 24 hours at 4° C in 100 ml of PBS containing 2.0% Sarkosyl. Solutions were filtered with nylon mesh to remove fabric, a sperm count taken, and the sperm heads pelleted by centrifugation at 5,000 x g for 10 minutes at 4° C. Fresh semen samples were resuspended in 20 ml of PBS containing 2.0% Sarkosyl, mixed briefly, and the sperm heads pelleted by centrifugation as above. Sperm heads were lysed by resuspending the pellet in 2.0 ml of PBS containing 2.0% Sarkosyl, 100 µg/ml Proteinase K, 10 mM DTT, and 25 mM EDTA, and incubated at 37° C for 20 hours with gentle rocking.

DNA was isolated from 5 to 10 cc of peripheral blood by lysing the red cells with 4 volumes of blood lysis buffer (0.32 M sucrose; 10 mM Tris-HCl, pH 7.6; 5 mM MgCl, 1.0% Triton X-100) followed by the pelleting of the white cells by centrifugation at 2,000 x g for 10 minutes at 4° C. The white cells were resuspended in 2 cc of DNA lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl, 10 mM EDTA), with Proteinase K and SDS added to 100 µg/ml and 1,0% respectively, and incubated 4 - 16 hours at 37° C with gentle mixing. Sodium perchlorate was added to 1.0 M to both blood and sperm DNA preps and the samples were either stored at 4° C or processed into DNA by extraction with phenol-chloroform (1:1) twice, chloroform twice, followed by dialysis against a 1000 fold excess of TE (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). All DNA concentrations were measured in a spectrophotometer at 260 nm.

Restriction Endonuclease Digestion, Electrophoresis, and Hybridization

Two to five micrograms of DNA were digested with a five fold excess of the restriction endonucleases Eco Rl

APPLICATION OF DNA POLYMORPHISMS TO THE FORENSIC EXAMINATION OF SEMEN

M. Baird, A. Giusti, I. Balazs, and J. Glassberg
(Lifecodes Corporation, Elmsford, New York 10523, U.S.A.)

Abstract

We have developed a procedure for the isolation of sperm DNA from semen samples collected after sexual activity. The use of a differential lysis procedure allows the recovery of sperm free of female cells. DNA was purified from these samples, digested with particular restriction endonucleases, fractionated by agarose gel electrophoresis, and hybridized with specific recombinant DNA probes that recognize two highly polymorphic DNA sequences. DNA was purified from nine semen samples, recovered from the female partners of volunteer couples, and the sizes of the polymorphic DNA fragments were measured. These sizes were compared with those obtained from DNA purified from the blood of the male and female sexual partners. In all cases, the pattern of DNA polymorphisms obtained from the semen samples was identical to that of DNA purified from blood of the male sexual partner. This technique is applicable to the identification of an assailant in rape cases.

Introduction

In instances of sexual assault, the isolation of spermatozoa or prostatic acid phosphatase is the most common test used to indicate the occurrence of sexual activity (1), but yields no information concerning identity of the assailant. The analysis of a limited number of polymorphic proteins from semem recovered intravaginally or from dried stains is difficult and makes it hard to establish identity of an assailant to a high degree of certainty (2).

Differences in DNA nucleotide sequence among individuals can be visualized as size polymorphisms in restriction endonuclease digested DNAs. Because these restriction fragment length polymorphisms (RFLPs) have been shown to be inherited as Mendelian traits, they can be used as markers for genetic studies (3). Some RFLPs display a large number of discreet DNA fragments which greatly increase the chances for an individual possessing a distinct set of alleles.

This report describes the recovery of spermatozoa from dried semen stains, the purification of male-specific DNA from these samples, and restriction fragment length polymorphism analysis of this DNA, and DNA isolated from the blood of the male and female sexual partners.

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Allele sizes remained unchanged as the samples aged. High molecular weight DNA isolated from two and three year old bloodstains produced bands with both probes within the size range of alleles observed in the general population.

Non-allelic bands can not arise by DNA degradation. Rather, breakage of DNA produces a dispersion of DNA fragments resulting in the loss of any specific autoradiographic signal. Incorrect bands larger than expected are obtained only if high molecular weight DNA is not digested to completion by the restriction endonuclease. This possibility is avoided by digestion with at least a five fold excess of enzyme.

The ultimate power of an RFLP for identification is dependent on the frequency of the alleles. Hundreds of non-related individuals from the New York City area have been analyzed to generate a database of allele frequency. Presently the average power of identification using the two probes is about 1 in 1,000. Other probes which detect multiple allele RFLPs are under investigation and will increase the power of the DNA test.

Conclusions

Enough high molecular weight DNA was recovered from blood stains as old as three years for restriction fragment length polymorphism (RFLP) analysis using two probes which recognize highly polymorphic regions. For identification purposes, analysis of DNA RFLPs should prove to be a powerful addition to the current protein techniques.

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Bands were visualized by autoradiography at -70° C using x-ray film (X-Omat, Kodak) and Dupont Lightning-Plus intensifying screens.

DNA Probes

Two DNA probes were prepared from cloned inserts in the plasmid pBR322. pAW101 (kindly provided by R. White) contains a 6.5 kilobase (kb) insert that hybridizes to the D14S1 region on chromosome 14 and is polymorphic with Eco R1 (7). pLM0.8 contains a 879 base pair Cla 1 - Sph 1 insert derived from the 3'-flanking region of the HRAS-1 oncogene on chromosome 11 (8) and is polymorphic with Taq 1. Both inserts were purified from their pBR322 vectors and used as probes.

Results and Discussion

The amount of DNA recovered from 1 ml dried blood stains (45 mm in diameter) varied from 27 µg to 73 µg with the average recovery of 40 µg, similar to the amount obtained from one ml of peripheral blood. Titration experiments indicate that 1 to 4 µg of DNA are sufficient for RFLP analysis using unique sequence probes. Thus, a 200 microliter (9 mm) bloodstain could yield enough DNA for analysis using two probes.

High molecular weight DNA is essential for RFLP analysis. A band on an autoradiograph is obtained only if enough intact copies of the desired fragment are present. Broken fragments will migrate faster and will not be concentrated in a single band after electrophoresis. To measure the size of DNA recovered from blood stains, we used agarose gel electrophoresis with appropriate size markers. Undigested DNA which ran slower than the largest size marker was considered to be high molecular weight (ie. >23 kb). High molecular weight DNA was isolated from all stain samples including the 2 and 3 year old specimens.

The RFLP patterns obtained from each probe rely on the binding (hybridization) of the probe only to complementary DNA sequences bound to the membrane. Autoradiography of a membrane after hybridization with a labelled probe reveals one (homozygote) or two (heterozygote) allelic bands for each digested DNA sample. Because the RFLPs display Mendelian inheritance patterns, they can be used to establish identity. More than 30 alleles can be distinguished for pAW101 with fragment sizes ranging from 14 to 32 kb. For pLM0.8, 18 distinct alleles are observed with fragment sizes ranging from 1.8 to 4.5 kb.

High molecular weight DNA isolated from bloodstains on cotton cloth and glass at time points of 0, 1, 3, 7, 14, and 28 days were examined for RFLPs using the pAW101 and pLM0.8 probes. Clearly defined bands of equal intensity were seen on autoradiographs for all samples.

Preparation of DNA

Peripheral blood samples were mixed with 4 volumes of blood lysis buffer (0.32 M sucrose; 10 mM Tris-HCl, pH 7.6; 5 mM MgCl; 1.0% Triton X-100). The white cells were pelleted by centrifugation at 2000 x g for 10 minutes at 4° C and resuspended in DNA lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 10 mM EDTA). Proteinase K and SDS were added to final concentrations of 100 µg/ml and 1.0% respectively. Samples were incubated at 37° C overnight with gentle mixing. Sodium perchlorate was added to 1.0 M and either stored at 4° C for processing later or extracted twice with phenol-chloroform (1:1), twice with chloroform, and dialysed against 3 changes of a 1000 fold excess of TE (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). Ammonium acetate was added to a final concentration of 0.3 M and DNA precipitated overnight at -20° C with 2.5 volumes of 95% ethanol. DNA was pelleted by centrifugation at 10,000 x g for 20 minutes at 4° C, dried, and resuspended in 1.0 ml of TE. Blood stained cotton cloth cut into small strips or dried blood samples from glass beakers was suspended directly in DNA lysis buffer and treated as above. DNA concentrations were determined by absorbancy at 260 nm in a spectrophotometer.

Restriction Endonuclease Digestion and Electrophoresis

One to ten micrograms of DNA was digested with a six fold excess of the restriction endonucleases Eco Rl or Taq 1 (Bethesda Research Laboratories) according to the conditions recommended by the manufacturer. Digested DNAs were size fractionated with appropriate size markers by agarose gel electrophoresis in TAN buffer (40 mM Tris-HCl, pH 7,9; 20 mM sodium acetate; 2 mM EDTA). Eco Rl digested DNA was electrophoresed in 0.4% agarose gels at 0.6 volts/cm for 3 days while Taq 1 digested DNA was electrophoresed in 1.2% agarose gels overnight at 1.8 volts/cm. The total length of each gel varied from 15 cm to 22 cm. DNAs were visualized post-electrophoresis by ethidium bromide staining and photographed under ultraviolet light.

DNA Transfer and Hybridization

DNA was denatured in gel and transferred to nylon membranes (Zetabind, AMF Cuno) using standard blotting techniques (5). DNA probes were radioactively labelled by nick translation (6) to specific activities $>10^8$ cpm/µg using all four alpha-^{32}P deoxyribonucleotide triphosphates (Amersham). Membranes were hybridized with labelled probes and washed to remove non-specifically bound radioactivity as described by the manufacturer.

APPLICATION OF DNA POLYMORPHISMS TO THE FORENSIC EXAMINATION OF DRIED BLOOD STAINS

M. Baird, E. Kanter, *R. Shaler, and I. Balazs
(Lifecodes Corporation, Elmsford, New York 10523,
*Office of the Chief Medical Examiner, 520 Fifth Avenue,
New York, New York 10016, U.S.A.)

Abstract

We have examined the possibility of using DNA purified from dried blood stains for the analysis of restriction fragment length polymorphisms. High molecular weight DNA was recovered from blood dried on cotton cloth, aged from one day to three years. These DNA samples were digested with restriction endonucleases, separated by agarose gel electrophoresis and hybridized to recombinant DNA probes that recognize two different highly polymorphic DNA sequences. A set of controlled samples was aged at room temperature for up to 28 days. The polymorphic DNA pattern observed with each probe remained the same at all time points. The patterns of DNA polymorphisms observed in the two and three year old dried blood stains was consistent with patterns observed in the general population. These results indicate that DNA recovered from dried blood stains can be used for identification purposes.

Introduction

The analysis of dried blood stains to help determine identity presently relies on the examination of polymorphic proteins and cellular antigens (1,2). Methods to detect nucleotide sequence polymorphisms within DNA are presently available. Using specific restriction endonucleases and defined probes, many heritable restriction fragment length polymorphisms (RFLPs) have been described (3,4). This report investigates the recovery and RFLP analysis of DNA from dried blood stains.

Materials and Methods

Sources of Dried Blood Samples

Dried bloodstains were prepared by applying 6 one cc aliquots of freshly drawn blood to sections of cotton cloth or to the bottoms of glass beakers. These stains were allowed to age at room temperature for up to 28 days before DNA was isolated. Three forensic blood stains taken post-mortem and stored 2 or 3 years at 4° C were also analyzed. All blood stains on cloth were about 45 mm in diameter.

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the salivary glands, the sweat glands and the epididymis suggest in the light of Szulman's results (1962) that this is not the case.

The studies, however, also showed that the PAP method is an effective technique for demonstrating blood group antigens, even in very small tissue specimens. Through improved preparation techniques it should be possible by using this method to demonstrate ABH blood groups in, for example, blood stains and vaginal epithelium.

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Table 1

Staining of different elements from salivary glands of secretors (Se) and nonsecretors (Ns) with the peroxidase-anti-peroxidase (PAP) and PAS staining method

	Staining	Parotis	Submandibularis	Sublingualis
mucous end-pieces and connective pieces	PAP Se	-	pos	**pos**/neg
	PAP Ns	-	neg	**neg**/pos
	PAS	-	pos	pos
epithelia of excretory ducts	PAP Se	**neg**/pos	pos	pos
	PAP Ns	**neg**/pos	pos	pos
	PAS	neg	neg	neg
capillary endothelia	PAP Se	pos	**neg**/pos	neg
	PAP Ns	pos	pos	pos
	PAS	neg	neg	neg

Discussion

The studies in the salivary glands confirmed the earlier findings that the Parotis has nearly no mucous ("water soluble") blood group substance, as opposed to the glandula sublingualis, which has extremely large amounts of the substance (Lötterle and Scheithauer, 1984). In salivary glands the antisera appear to have far greater affinity for the water soluble blood group substances than for the alcohol soluble blood group substances in blood vessel endothelia and erythrocytes. For that reason, the vessel endothelia remained unstained in the glandula sublingualis in secretors.

The conventional subdivision into water soluble and alcohol soluble blood group substances should be further investigated, keeping the PAS reaction in mind, in order to determin if the PAS negative water soluble blood group substance is controlled by the secretor gene. Our preliminary findings in

Several tissues, especially salivary glands and epididymitis, were stained in parallel, using the PAS (periodic acid schiff) reaction as described elsewhere (Romeis, 1968). Counterstaining was done with haemalaun.

Results

Blood-vessels. The blood groups were found in all preparations - regardless of the secretor status - on the endothelia of the blood vessels. The erythrocyte membranes did not generally react as strongly as the blood vessel endothelia. A negative staining of capillary endothelia was always observed in the salivary glands of secretors whenever the mucous glands reacted with a particularly strong positive stain.

Epididymis. In epididymis neither the efferent duct nor the epididymid duct are stainde by the PAS reaction. With the peroxidase-anti-peroxidase technique, blood group substances were distinctly stained in the secretion of the efferent ducts, but not in the epididymid duct. It cannot be said whether or not this would be the case in non-secretors too, owing to the narrow scope of the study. The sperms themselves were not stained, or if so, only very lightly: thus, the seminal plasma is responsible for the large amount of blood group substance in the seminal fluid and not the sperms themselves.

Sweat glands. The sweat glands of the skin are PAS negative and produce blood group substances. According to Szulman (1962) this production is not under the control of the secretor genes.

Hair. The blood groups of hair with intact roots were determined in the hair papillae, because these are supplied with blood by the capillaries. Yet, most attempts to determine the blood group in a single hair with this method have failed, since most teared out hairs do not contain vascular elements. The inner and outer parts of the hair shaft occasionally stain with a false positive stain and are therefore not suitable for demonstrating the ABO-bloodgroup. If the hair follicle has been preserved, the blood group can also be seen here in the capillaries.

Salivary glands. In the study of the salivary glands of 26 persons (19 A secretors, 3 A non-secretors, 3 B secretors, 1 B non-secretor; for glandula sublingualis, glandula submandibularis and Parotis), the results obtained for the mucous endpieces and connective pieces, the epithelia of the excretory ducts and the capillary endothelia can be seen in Table 1. The information **pos**/neg means that in the greater number of cases the reaction was positive, but was also negative in a small number of cases. Of particular note is that in the glandula submandibularis in some of the secretors, individual cells of the mucous end and connective pieces produce a strong reaction, whereas others produce practically no reaction at all with anti-A or anti-B. Similar behaviour was found by Kent (1961) in the Brunner's glands of the duodenum.

Placenta. Studies were carried out in five mature placentas. The fetal erythrocytes always stained weaker than the maternal ones. These results agree nicely with those of Pedal et al. (1985) and are due to the fact that the fetal erythrocytes have just a relatively small number of blood group antigens (Hakomori, 1981).

Demonstration of Blood Group A and B Antigens in Human Tissue Using an Immunoperoxidase Staining Method

Joachim Lötterle and Wolf-Dieter Heine (Institut of Pathology, Leopoldina Hospital, D-8720 Schweinfurt, Federal Republic of Germany)

The distribution of A and B blood group substances and antigens in secretions and tissues was extensively studied by Putkonen (1930) and Hartman (1941), using the agglutination inhibition method. It was found that the strongest blood group activity was associated with mucous secretions and that non-secretors also produce small amounts of A and B substances. Later the distribution of blood group substances in human tissues was explored in more detail, using immunofluorescence microscopy (review in Szulman, 1966). It became possible with this method to demonstrate the actual histological localization of the antigens.

With the introduction of the peroxidase-anti-peroxidase technique by Sternberger et al. (1970), a new and more versatile method was offered, which was easier to work with than the immunofluorescence technique. Recently the peroxidase-anti-peroxidase method has been used to demonstrate the distribution of the ABH antigens in fresh and decomposed human kidney and placental tissue (Pedal and Hülle, 1984; Pedal and Becker, 1985; Pedal et al., 1985).

In our study the peroxidase-anti-peroxidase method was used to show the distribution of blood group antigens in human salivary glands and other tissues of particular interest for forensic medicine.

Materials and Methods

Tissue specimens were taken from the autoptic material of 40 corpses with blood groups A_1, A_1B and B. The tissue was fixed in unbuffered 10% formalin and routinely dehydrated, cleared and embedded. Serum glycerine fixative was used as an adhesive for the 4-5µ sections. Deparaffinization took place in a 60° C oven for at least one hour, followed by routine hydration. After rehydration the sections were submerged in PBS for only a few minutes and stained immediately afterwards, using the peroxidase-anti-peroxidase technique. All the steps were done at room temperature except for incubation with the primary antibody.

The slides were laid flat and incubated with 3% hydrogen peroxide for five minutes. After washing with PBS the tissue was incubated with normal swine serum (DAKO X 960) diluted 1:10 for 20 min. After tapping off the excess serum, an undiluted monoclonal anti-A or anti-B serum (Biotest, Seraclone) was applied and the slides were incubated at +4° C for 4 to 6 h. This method yieldet an intense specific stain with practically no background. Then the slides were gently rinsed with PBS and incubated for 20 min with peroxidase-conjugated rabbit antibody to mouse immunoglobulin (DAKO P 161) diluted 1:100. The slides were again gently rinsed with PBS and incubated for 20 min with peroxidase-conjugated swine antibody to rabbit immunoglobulin (DAKO P 217) diluted 1:100. After rinsing with PBS, a commercially available AEC (3-amino-9-ethylcarbazole) substrate solution was applied and the slides were incubated for 20 to 40 min. After a final washing in PBS the slides were counterstained with haematoxilin for 3 min.

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Conclusions

In forensic case-work problems may arise by an uneven concentration of the secretion in a stain, when parts of it are used for different grouping procedures. Therefore we prepare one extract, that is used for A, B, H- and Lewis-determination in an absorption inhibition test and for A, B, H-determination in an absorption elution test. By using two different methods, we are able to control the results of each test.

Correct A, B, H-grouping results were obtained in 85 % of all semen stains and in 95 % of vaginal secretion stains (Fig. 1). The high rate of incorrect results in semen stains is due to false B-positive reactions in A-secretors (Fig. 4). This aberrant blood grouping result did not appear in H-secretors and disappeared when a monoclonal anti-B serum was used (Fig. 5). The false B-positive reaction appeared in semen already after one day and was also found in 16 days old samples. It did not occur in this semen if it was dried up immediately.

Typing of saliva stains on cigarette tips (Fig. 1) was carried out correctly in 74 % of all cases, on prepared saliva stains in 87 % (Fig. 2) and on stamps in approximately 100 %. Incorrect results in prepared saliva stains of nonsecretors are mostly due to false negative reactions (Fig. 2).

In sweat and urine the rate of positive grouping results is insufficient. Investigations should therefore be restricted to the absorption elution test or Lewis grouping so that less water is needed for extraction resulting in higher antigene concentrations. Another possibility may be the concentration of large stains by ultrafiltration.

Lewis typing is also promising. Lewis substances could be successfully determined in 87 % of all semen stains and in 96 % of all saliva stains. A, B, H-secretor or nonsecretor results were confirmed by the typing of Lewis substances. The method has been proved to be successful in forensic case-work.

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RESULTS

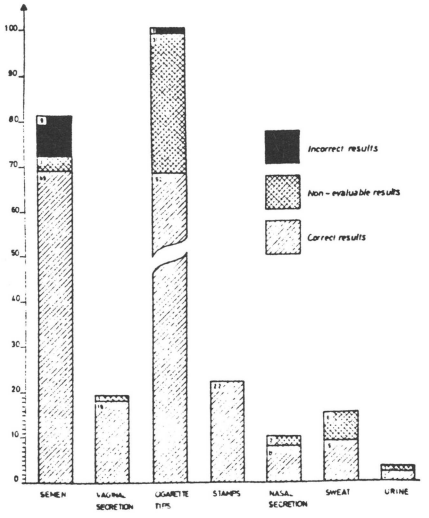


Fig. 1: ABO-grouping in secretion stains. Absorption inhibition and absorption elution tests are evaluated as a whole. Agglutinations (or inhibition of agglutination) including at least 3 dilution steps were considered as a positive indication of the concerning blood group factor. Weak reactions (below 3 dilution steps) or conflicting results between absorption inhibition and absorption elution tests are classified as non evaluable.

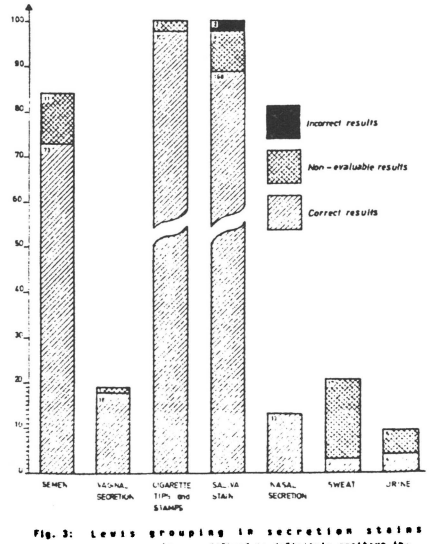


Fig. 3: Lewis grouping in secretion stains Correct results are defined as definitely positive inhibition reactions, weak and negative inhibition reactions as well as contradictory results of simultaneous tests with two different antisera were summarized as non-evaluable results and false positive inhibition reactions were considered as incorrect results.

ABSORPTION INHIBITION + ABSORPTION ELUTION	ASe	BSe	ABSe	OSe	Σ	Ase	Bse	ABse	Ose	Σ
Correct results	64	19	5	60	148	15	4	3	6	28
Non-evaluable results	1	3	3	6	13	2	1	1	3	7
Incorrect results	0	0	0	0	0	1	0	2	3	6
Number of stains	65	22	8	66	161	18	5	6	12	41

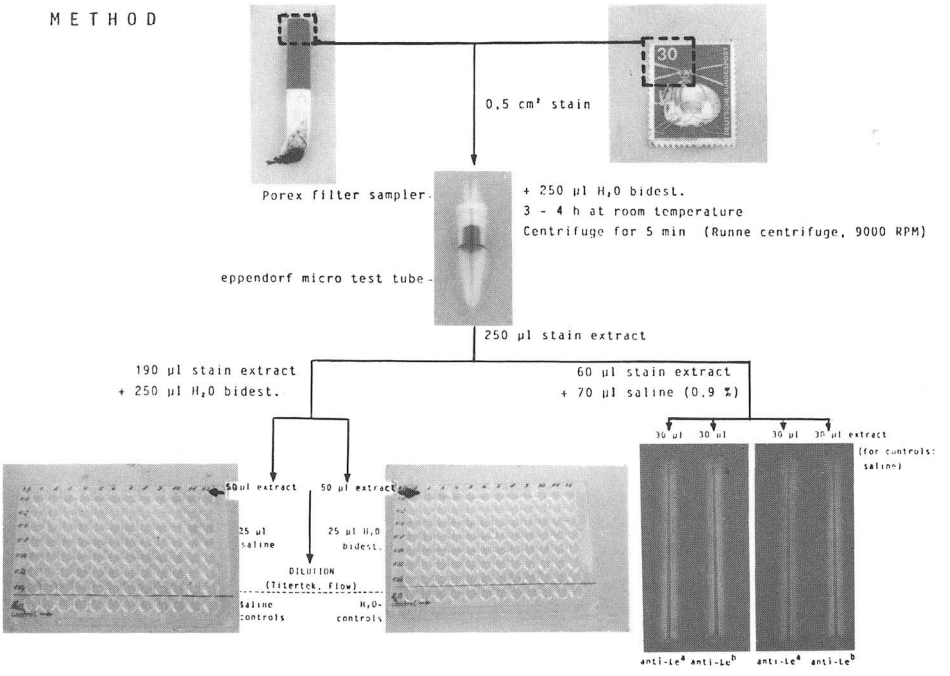
Fig. 2: Analysis of ABO-grouping results in saliva stains. Classification of results as in Fig. 1. Grouping results in nonsecretors are only related to absorption elution tests

		ABSORPTION INHIBITION + ABSORPTION ELUTION			HOLZER-TEST		
		A	B	0	A	B	0
I	AI	5	2	5	6,5	2	8
	AE	6,5	6,5	6,5			
II	AI	5	0	5	7	1,5	7
	AE	6	5	5			
III	AI	5	0,5	5	7	0	8
	AE	6,5	6,5	6,5			
IV	AI	5	0	5	5,5	0	6,5
	AE	6	5,5	6,5			
V	AI	5	0	5	6,5	0	7
	AE	4,5	1	6,5			

Fig. 4: False B-positive reactions in liquid semen of A-secretors. Absorption inhibition (AI) and absorption elution (AE) results are compared with HOLZER-test. The number of inhibited or agglutinated dilution steps is indicated.

	time	1d			2d			8d			16d		
		AI,	AEp,	AEm	AI,	AEp,	AEm	AI,	AEp,	AEm	AI,	AEp,	AEm
I	A	5	5	5	5	6,5	6	5	5	6,5	5	6	6
	B	0,5	6,5	0	0	6,5	0	0	6,5	0	0	6,5	0
	0	5	6		5	6,5		5	6,5		5	6,5	
II	A	5	6	6	5	6,5	6,5	5	5	6,5	5	5	6,5
	B	0	3	0	0	6,5	0	0	2	0	0	3	0
	0	5	6,5		5	6,5		5	6,5		5	6,5	

Fig.5: False B-positive reactions in liquid semen from two A-secretors using polyclonal (p) and monoclonal (m) antisera for absorption elution tests (AE). Semen was stored a certain period at room temperature.



- Dilution of stain extract up to 1:64
- Add 25 µl of diluted antisera
- Incubation overnight in a humid chamber at 4°C
- Add 25 µl of 0,5 % erythrocytes
- Read agglutination/inhibition of agglutination microscopically after 30 - 60 min

- Dilution of stain extract up to 1:64
- Fixation of stains onto microtiter plates by drying
- Add 25 µl of diluted antisera
- Incubation overnight in a humid chamber at 4°C
- Wash 5 times with cold saline
- Add 25 µl of 0,2 % erythrocytes
- Elution of antibodies (56°C,15 min)
- Read agglutination microscopically after 40 - 60 min

- Add 30 µl of diluted antisera
- Incubation overnight at 4°C (vials covered with a plastic film)
- Add 30 µl of 1,5 % Le^a-positive or Le^b-positive test erythrocytes
- Centrifugation at low speed (Molter centrifuge, 1500 RPM) after 30 min
- Read agglutination by eye

A, B, H- and Lewis grouping of body secretions from a common stain extract

G. Bäßler
Landeskriminalamt Baden-Württemberg, Stuttgart, FRG

MATERIALS

Antisera. A, B, H-antisera (Molter, Merz u. Dade) and Lewis-antisera were used for absorption-inhibition tests in dilutions that still gave fairly strong agglutination of NaCl-controls with test erythrocytes. Anti-Le^a and anti-Le^b were employed in parallel from two different manufacturers (Merz u. Dade/Behring).
Since slight titre variations may occur, the titre values of antisera have to be checked occasionally or if new antisera charges are to be used. The following dilutions are generally used:

anti-A	(Molter) :	1:200
anti-B	(Molter) :	1:200
Lectin-H	(Merz u. Dade):	1:20
anti-Le^a	(Merz u. Dade):	1:15
anti-Le^b	(Merz u. Dade):	1:25
anti-Le^a	(Behring) :	1:6
anti-Le^b	(Behring) :	1:8

A, B, H-antisera for absorption elution tests are diluted 1:8 (Anti-A, anti-B) and 1:2 (Lectin-H) to save material. A monoclonal anti-B was purchased by Biotest and diluted 1:4.

Indicator cells. For agglutination reactions in absorption inhibition tests we used 0,5 % test erythrocytes (Affirmagen, Ortho, H cells were papain-treated), in absorption elution tests 0,2 % test erythrocytes (Affirmagen, Ortho, papain-treated) and for Lewis testing 1,5 % test erythrocytes (Serocyte, Merz u. Dade), being Le^a or Le^b positive.

Secretion stains. All stains were collected from the laboratory staff and stored at room temperature or in the case of semen and vaginal secretion at -20°C. Cigarette tips and stamps were kept in original, semen was provided on squares of viscose material, vaginal secretion, being free of semen contamination was collected on sterile cotton swabs and nasal secretion, sweat and urine stains were made on filter paper.

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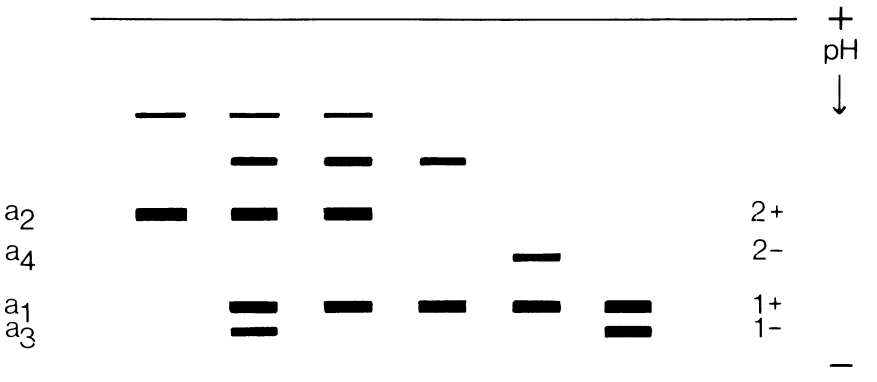
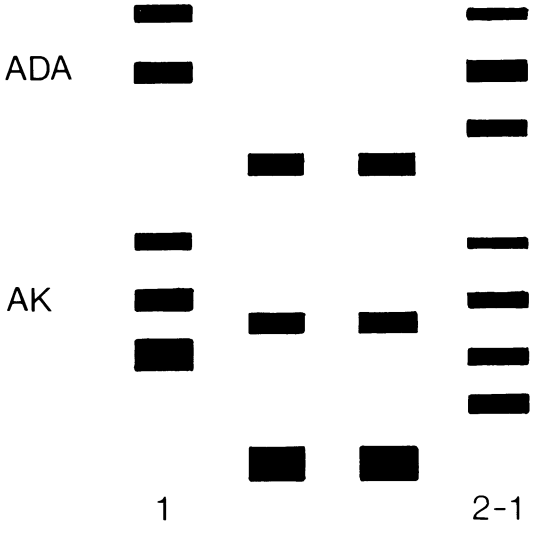


Schematic representation of PGM subtypes

Fig.1



Schematic representation

Fig.2

can be made, the maternal phenotype must be taken into consideration.

From these results, blood grouping on abortion material taken from the 6th to the 13th weeks of pregnancy can be recommended in the following systems: ABO, PGM-Subtyping, AK and possibly ADA.

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The intermediate group consists of those systems which often showed weak activity. Activity in the SEP (EAP) system was often observed but only in the B region and in these cases the maternal blood was typed as either B or BA. At present we are not able to prove whether this B band activity is a primitive fetal isozyme or only a low concentration of the enzyme, resulting in the absence of the weaker bands.

Similarly deficient was EsD and where activity was observed, it consisted of only one band where normally two would be found after isoelectric focusing. In these samples the band could also be found in the maternal phenotype. Here again we cannot say if this is a precursor or a low concentration of enzyme.

GPT is also included in this group although a weak 2-1 phenotype could be seen in only 2 samples.

The third group consists of those systems where no activity could be seen and includes Gc, GLO, C'3 and Pi.

From this relatively small number of samples it would be unreasonable to make any statistical calculations as to the frequency of fetal groups in comparison to the adult population. Furthermore, in only approximately one half of the samples could a maternal blood sample be obtained and as placental tissue was normally present and the decidua and intervillous areas are known to possess maternal individuality (Pedal 1985), the results obtained cannot be definitely attributed to the fetus.

From a forensic point of view it can be stated that fetal phenotypes are detectable in some blood group systems.

In practice, a mixture of maternal and fetal material cannot be avoided and therefore before any conclusions

tion and Absorption-Inhibition tests, ADA and AK using cellulose acetate membrane electrophoresis and for most other systems isoelectric focusing was used.

The abortion material from 25 terminations was prepared by washing several times in physiological saline followed by homogenisation and centrifugation of the extract. The supernatent was then used for all tests.

The results can best be described by separating them into 3 groups.

The first group consists of those systems in which blood group activity could be regularly identified and in some cases differed from the maternal phenotype.

In the ABO system, the antigens A, B and H could always be identified and the H antigen was found in combination with all other groups in the ABO system. This agrees with the findings of Constantoulakis et al. (1963). It was common in these samples to find additional antigens which the mother lacked but H substance was seldom found alone. The frequency of the B antigen was approximately in agreement with the population frequencies.

Subtyping in the PGM system also produced band patterns which differed from the maternal phenotype. As evidence for the presence of fetal blood group markers in the abortion material, 3 bands were observed in some samples which could normally only be attributed to the products of 3 alleles (Fig. 1). The AK 1 phenotype was identified in most samples but in many cases there occurred an additional atypical cathodal band (Fig. 2).

In approximately one half of the samples ADA activity was observed but the bands were often atypical and more cathodic than the normal pattern. Similar findings on placental material were reported by Edwards in 1971.

Blood group investigations on fetal tissue

B. Madea[1], S. Rand[2], K. Püschel[3], C. Henßge[1]

1. Institut für Rechtsmedizin, Münster
2. On loan from Metropoliton Police Laboratory, London
3. Institut für Rechtsmedizin, Hamburg

In contrast to laboratory experiments on fetal material, one often finds in cases of forensic interest fetal fragments mixed with placental tissue and maternal blood.

In 1983 Eriksen reported a case where the identification of a rape suspect depended on blood group determination of placental tissue from an abortion.

Another aspect to the examination of blood group systems from fetal material, which mostly involves older or new born fetuses, is the identification of the mother in cases of illegal abortion and infanticide.

The literature on blood group identification from fetuses is extensive (1,2,3,5,6,7,8,9) but most authors have confined their investigations to the red cell antigens.

In our investigations we have concentrated on determining which blood group systems can be detected in material from abortions between the 6^{th} and 13^{th} week of pregnancy and how they are expressed.

After a vacuum extraction there is not only fetal material present in the sample but often also placental tissue and maternal blood. For this reason a blood sample from the mother was obtained when possible and tested in the same systems for comparative purposes.

In this investigation the following systems were tested: ABO, PGM, SEP, EsD, ADA, AK, GLO, GPT, Gc, Tf, C'3 and Pi. The ABO antigens were detected using Absorption-Elu-

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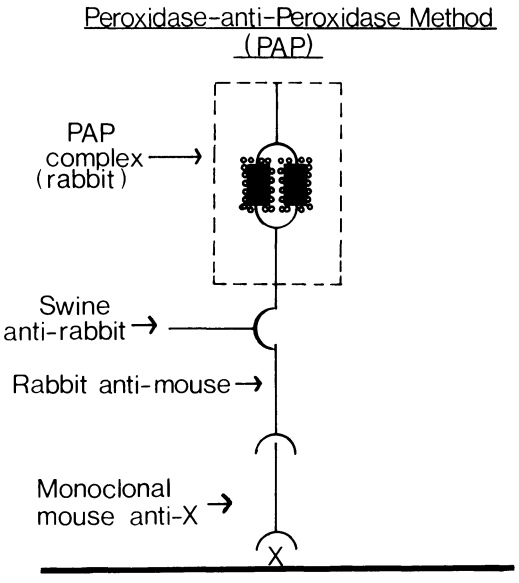

Fig. 1: Diagrammatic representation of the PAP-method

PAP blind trial n=50

blood	n	%	correct
A_1	15	30	15
A_2	4	8	4
B	7	14	7
O	23	46	23
AB	1	2	1

PAP blind trial - Lewis

blood	n	strong +	weak +
a-b+	37	37	-
a+b-	7	2	5
a-b-	4	4	-
unknown	2	2	-

Fig. 2: ABO and Lewis results from blind trial

2. It would seem to have an advantage over serological methods since the reaction can be directly attributed to cellular structures.

3. ABH detection is apparently independent of secretor status.

4. It seems to be possible to discriminate between 2 different cell populations of different ABO types.

5. Identification can be made from very small numbers of cells.

6. Lastly, definite results have been obtained from buccal and vaginal cells and recently also encouraging results from spermatozoa have been obtained.

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Some samples showed a weak red colouration but only as a background staining. The cause for this is not clear but so long as only a red staining on the cell membrane was counted as positive no errors were made.

Since the reaction is specific for A, B and H terminal sugars and as bacteria are known to possess similar structures this could be a reason for this phenomenon. If this is so, then the PAP method could distinguish between ABO antigens from cells and any false positives from bacterial contamination, assuming that bacteria have not absorbed onto the cell membrane.

After the success of this survey we have made limited investigations on vaginals swabs with encouraging results.
Here again background staining was observed and only when the cells were stained should a positive reaction be recorded.

Experiments on buccal cells taken from smoked cigarettes have also shown some success.
Our first experiments using spermatozoa only yielded limited success but later attempts after freezing and thawing of samples gave encouraging results. It was noticed that sometimes only approximately 50 percent of cells were stained in the positive sample which might reflect the heterozygote condition.

In conclusion we may state that the situation at present is as follows:

1. The immunohistochemical technique for detection of ABH antigens is a reliable technique.

As the first stage in this investigation, buccal cells were used as they are readily available from donors, and are essentially the same as vaginal cells. Buccal cells from mouth swabs, were collected from 50 donors on sterile cotton wool swabs and smears made on glass microscope slides. Blood samples were also collected and typed in the ABO and Lewis systems. After drying, the slides were fixed by carefully passing over a flame.

The 3-stage PAP method for the identification of the ABO antigens was carried out as follows (fig. 1):

1. Monoclonal mouse anti-A, -B and Ulex anti-H.
2. Rabbit anti-mouse IgM.
3. Swine anti-rabbit immunoglobin.
4. PAP complex from rabbit.

Visualisation of the PAP complex was performed using hydrogen peroxide and 3-amino-9-Ethylcarbazole resulting in a red colouration for a positive reaction.

Examination of the samples was carried out blind and independantly compared to the ABO and Lewis grouping. In recent experiments we have looked at other types of cells and mixtures of cells of different blood groups. A preliminary report of the findings is included.

Results

No false results were obtained, i.e. the results from PAP typing corresponded to the ABO blood type in each case. No negative results were obtained although some donors were non-secretors, from the Lewis type, suggesting an independance from secretor status (fig. 2).

IMMUNOHISTOCHEMICAL DETECTION OF ABH-ANTIGENS IN HUMAN SALIVA

Bernd Brinkmann, Beate Annuß, Steven Rand[1] (Institut für Rechtsmedizin, Münster/Germany. [1]On loan from Metropolitan Police Laboratory, London)

The detection of ABH antigens in bodyfluids and subsequent interpretation of results has four main drawbacks:
The amount of sample is often limited: The results are dependant on secretor status: In casework, body fluids are often found in combination with other body fluids: The possible presence of artifacts due to bacterial contamination.

In many cases the translation of ABO results obtained can only be made by assumption especially when one blood group masks another.

In principle, the use of an immunohistochemical technique allows identification of a particular antigen from this mixture and an association to a known morphological structure. For example cells from a group A person could be distinguished from cells from a group O person.

The 3-stage-peroxidase-anti-peroxidase technique (PAP) has been used extensively especially in clinical pathology for identification of cellular antigens and recently Pedal reported successful identification of ABH antigens from paraffin sections of decomposed kidney (1985) and placental tissue (1985).

If the method could be used to identify cellular ABH antigens from a mixture, it would be of immense value in cases of sexual assault.

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Table 3

Inhibition Score Conversion Chart

Dilution	Score								
Of Extract	-	+	1	1+	2	2+	3	3+	4
1/2	8	7	6	5	4	3	2	1	0
1/5	16	14	12	10	8	6	4	2	0
1/10	24	21	18	15	12	9	6	3	0

Table 4

Elution Score Conversion Chart

Dilution	Score								
Of Extract	-	+	1	1+	2	2+	3	3+	4
1/2	0	1	2	3	4	5	6	7	8
1/5	0	2	4	6	8	10	12	14	16
1/10	0	3	6	9	12	15	18	21	24
1/20	0	4	8	12	16	20	24	28	32
1/40	0	5	10	15	20	25	30	35	40

Table 5

Example of an Inhibition & an Elution result with converted score totals for an AB sec. saliva stain.

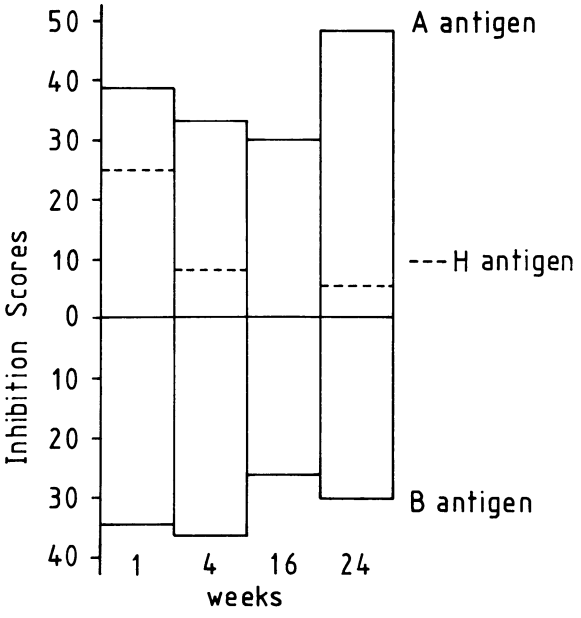
	INHIBITION			ELUTION		
	A	B	H	A	B	H
Agglutination	-	-	2+	3	3+	3+
scores	-	-	3	3+	3+	3
	-	+	3+	3+	3+	2+
				3+	3+	2
				3	3+	-
Converted						
scores	48	45	10	99	105	50

Consider a result from a group AB saliva stain, such as is given in table 5.

Each agglutination score is converted and then the total for each antigen represented by a single score, which can then be graphically represented.

The five group AB saliva stains used in this preliminary study did not show significant differential levels of A and B antigen. The H antigen, however, was greatly reduced with age. The results from one of these stains which was typical is given in figure 1.

Figure 1. An example of the levels of A, B and H antigen from a group AB secretor saliva stain, represented by converted scores.



The agglutination scores have been converted and the levels of A, B and H antigens detected by the inhibition method are represented over a period of six months.

Conclusions

1) There was a high incidence of loss of A antigen from A_2B blood stains stored at room temperature for over five years.

2) Most group A_1B stains retained their A and B antigenicity for eight years at room temperature. The only exception was one out of forty-six A_1B stains of seven to eight years old which had lost group B antigenicity.

3) In stains stored for ten weeks, loss of A antigenicity was observed only from A_2B stains, and then only when they were stored moist for over six weeks at the more adverse conditions of 37 C and 52 C.

4) The saliva stains used in this study did not show any loss in A or B antigenicity, but only a very small number of stains were typed.

Of the forty group AB blood stains stored at room temperature for one year, one stain repeatedly typed as a group B. This blood had not been subtyped.

Of the five to six year old stains three out of six A_2B blood stains had lost group A antigenicity and were typed as group B.

Of the seven to eight year old stains, twelve of a total of twenty-two A_2B stains were incorrectly typed as group B.

One of forty-six A_1B stains in the seven to eight year old category had lost B antigenicity and was typed as a group A.

None of the stains that had been typed after storage at room temperature for one to eight years had entirely lost antigenicity.

The results of the stains stored either dry or moist at varying temperatures are shown in table 2.

Three of the stains used in this study were A_2B, one was A_1B and the other was not subtyped as it was received in a lysed condition.All the stains stored dry at temperatures from -20 C to 52 C were typed correctly, with no loss of antigenicity, as shown on the top line of results in table 2. The stains that were stored moist at -20 C, 4 C and at room temperature were also typed correctly with no loss in antigenicity over the ten week period. At the higher temperatures (of 37 C and 52 C), the stains kept moist typed correctly for the first two weeks, but after six weeks there was noticeable loss of A antigenicity in the three A_2B stains. The A_1B and AB (sample not subtyped) were grouped correctly when kept moist at all the temperatures tested.

The three group O blood stains that were similarly stored over a ten week period did not give any cross-reactions. But H antigenicity was lost at elevated temperatures, particularly when kept moist at 52 C when no reactions were obtained even after one day.

In our laboratory saliva stains are grouped by testing dilutions of the stain extract by both absorption inhibition and absorption elution techniques. Thereby a series of agglutination scores are obtained for each of the three, A, B and H antigens.

A simple mathematical method has been devised, such that the amount of each antigen detected is represented by a single number. Direct comparison of the level of the three antigens is then made possible.

Agglutination is scored in our laboratory using a nine point scheme, -, +, 1 onwards to 4 (total agglutination). For the inhibition tests each antigen is tested on three dilutions of the original extract. Table 3 shows conversion figures for agglutination scores obtained for each dilution. The score at each dilution is weighted such that if inhibition is obtained at the third dilution the score will be greater than if inhibition is only obtained from the first dilution.

Table 4 shows the conversion chart for the absorption elution method. Here again the scores are weighted such that positive reactions at greater dilutions score more highly.

Table 1
Grouping results from blood stains stored from 1 to 8 years at room temperature.

Age in Years	Type	Total No.	No. incorrectly grouped as A	or	B
1	AB	40	0		1
2	A_1B	8	0		0
	A_2B	3	0		0
	AB	30	0		0
5 to 6	A_1B	30	0		0
	A_2B	6	0		3
	AB	3	0		0
7 to 8	A_1B	46	1		0
	A_2B	22	0		12
	AB	11	0		0

Table 2
Grouping results from blood stains stored under various conditions

	Type of Stain A_1B	A_2B	A_2B	A_2B	AB
Dry -20 C to 52 C 1 to 10 weeks	✓	✓	✓	✓	✓
Moist -20 C, 4 C, RT 1 to 10 weeks	✓	✓	✓	✓	✓
Moist 37 C, 52 C 1 to 2 weeks	✓	✓	✓	✓	✓
Moist 37 C, 52 C 6 weeks	✓	B	✓	*	✓
Moist 37 C, 52 C 10 weeks	✓	B	B	*	✓

* denotes low levels of A antigen
✓denotes correct grouping.

PRELIMINARY INVESTIGATIONS INTO THE DETECTION OF THE ANTIGENS IN GROUP AB BLOOD AND SALIVA STAINS

Kamala De Soyza (Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE1 7LP, England).

Introduction

The loss or decrease of A or B antigen from group AB blood and saliva stains has been occasionally observed in forensic case work. This study was undertaken to provide information on the frequency of this occurring.

The blood stains used in this study fell into two categories.

1) Stains varying in age from one to eight years. All these stains had been stored at room temperature.

2) Stains stored dry or moist at various temperatures and grouped at intervals over a period of two and a half months.

The saliva stains used in this study were stored at room temperature and grouped at intervals over six months.

Materials and Methods

The samples used in this study were liquid blood or salivas that had been sent to the laboratory in connection with criminal cases. The blood cells had been typed in the ABO system at the time of receipt, and A_1 and A_2 subtyping performed on some.

The stains that were over one year old had been made on cotton cloth and stored dry at room temperature and grouped by absorption elution.

In the study involving various conditions of storage five group AB blood samples were used. Ten stains were made from each sample. Five of these were allowed to dry overnight at room temperature and the other five were stored individually in polythene bags which were then enclosed in air-tight polystyrene boxes and stored in a moist condition.

A dry and moist stain was stored at each of the following temperatures: -20 C, 4 C, room temperature, 37 C and 52 C.

Therefore there were ten conditions of storage for the stains made from each blood sample.

For comparative purposes three group O blood samples were similarly made into stains and stored.

All the stains were grouped at intervals, starting when they were one day old and then after two, six and ten weeks.

Results

The results of typing group AB blood stains of one and two, five to six, and seven to eight years are given in table 1.

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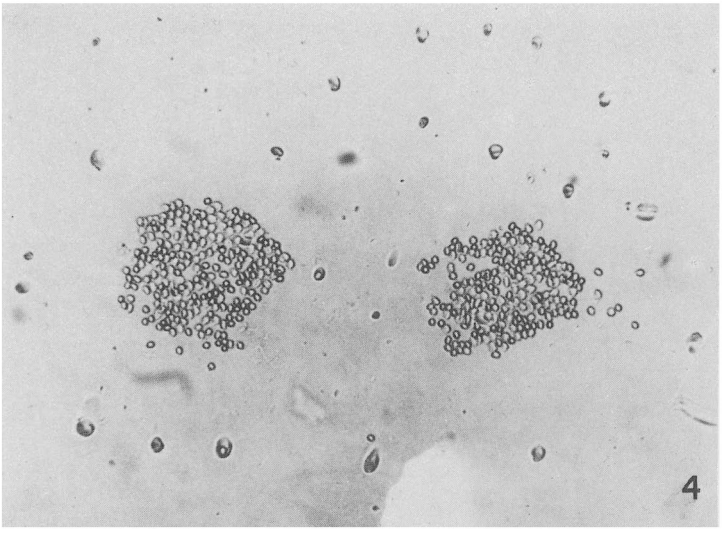

Plate 1 : Fresh latent print lifted from the glass surface; +ve agglutination, blood type A.

Plate 2 : Latent print a month old, lifted from plastic surface, +ve agglutination, Blood type A.

Plate 3 : Latent print 3½ months old, lifted from glass bottle, +ve agglutination, Blood type A.

Plate 4 : Fresh latent print subjected to constant temperature (47°C) for about 6 hours, +ve agglutination, Blood type B.

This study is of some relevance, as a large percentage of the Indian population are of group B(approx.40%) and the percentage of non-secretors is approximately 18-20%. So this can be of some use as a fingerprint detection system.

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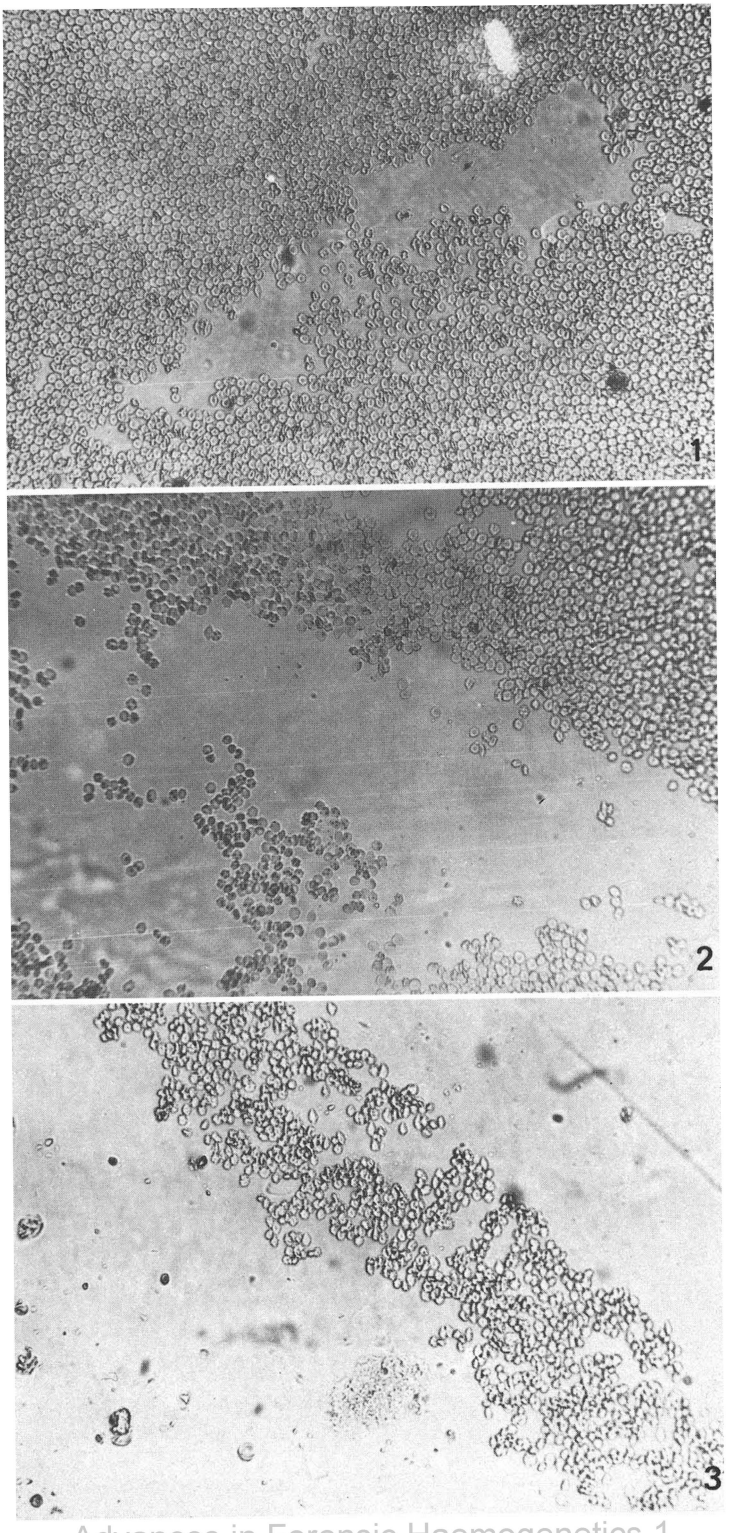

six months of age.

Results

To see the effect of age, latent prints were divided into 15 batches (of 17 prints each) and analysed after different intervals of time period viz.fresh, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 7 days, 15 days, 30 days, 60 days, 90 days, 120 days, 150 days and 180 days. It was observed that with increase in age of the latent print, the agglutination reaction became weak. There was a clear cut distinction between the results shown by fresher slides and the older one(Plates 1-3).

Effect of temperature was seen by putting the objects bearing latent prints in thermostat at varying constant temperature viz. 30°C, 35°C, 37°C, 40°C, 44°C, 47°C, 50°C and 52°C usually did not affect much the antigenic activity but high temperature exposure to the latent prints effected the ABH antigenic activity. Fresh prints exposed above 52°C even for one hour did not give any agglutination (plate 4).

Discussion:

Complete latent fingerprint is rarely found at the crime scene and for inconvenience, it has to be analysed. The modified mixed cell agglutination reaction technique on adhesive cellophane tape makes it possible to determine ABH isoantigens from latent fingerprints residue. It is not only the fingerball impressions, but any ridge portion from the palm made part of the present study. Adhesive cellophane tape material did not find to interfere the antigen-antibody reaction performed on its surface as far detection of antigens A and B in secretors is concerned. Anti-H(lectin) prepared from ulex europeus, gave non-specific reactions, and is believed to be due to the absorption of lectin onto the substrate resulting in background adhesion of cells, thus obscuring the results. This difficulty was removed to some extent by treating the adhesive tape surface with a .01% solution of tween-80 in saline, before sensitization with antibody. But even then it was only possible to detect antigen H on fresh samples,taken directly on cellophane tape.

Half life period of the ABH antigens is of the order of few months only. This phenomenon also proved true in the present study as with age the antigenic activity of the latent prints weaken and high temperature exposure seems to hasten this phenomenon.

It is extremely possible that a fingerprint impression may last for weeks at rather extreme storage conditions and still be easily detectable(Barneth and Berger,1977). This fact was confirmed, as the prints were stored at room temperature for six months and storage conditions do not found to effect much the original print quality. It is the antigenic activity which become weak with the passage of time.

IDENTIFICATION OF ABH BLOOD GROUP SPECIFIC SUBSTANCES FROM LATENT FINGERPRINTS

P.J.Singh, I.J.S.Bansal and I.J.Kaur
Department of Human Biology,
Punjabi University,Patiala-147002, INDIA.

SUMMARY:

Serological sensitivity of the modified mixed cell agglutination reaction on adhesive cellophane tape has proved to be a very useful method for typing ABH isoantigens from latent fingerprints. Its sensitivity is found to be several thousand times higher than that of absorption elution reaction, when the isoantigens are localized on the surface of latent fingerprints. Besides analysing fresh samples, effect of age and temperature conditions which are found to effect the isoantigenic activity has been discussed.

Introduction:

Latent fingerprints are, potentially, one of the most valuable source of trace evidence in crime cases. Recently progress has been made to detect latent fingerprints by biological techniques and the most useful technique for this is mixed cell agglutination. The mixed cell agglutination technique is especially recommended by many workers when blood group specific substances, which are localized on the surface of microscopic objects including fingerprints hair, skin, epidermal tissues, biological stains and histological sections, have to be determined (Ishiyama,1975; Ishiyama and Okada,1975; Okada and Ohrui,1978; Lincoln and Dodd,1960; Swinburne,1962; Poon and Dodd,1964; Coombs and Dodd,1961; Pereira et al.1969; Nickolis and Pereira,1962; Ishiyama,1979; Davidsohn,1972; Kouvarik et al. 1968 and Toender et al.1964). The present investigation has been done from view point of detecting ABH isoantigens from latent fingerprints.

Material and Method:

A total of 407 individuals of unknown ABO blood group and secretor status were asked to leave their fingerprints (both finger and palmar parts) on various surfaces viz., adhesive tape, glass, certain plastics, aluminium foil, and steel. While taking prints, each individual was asked to exert some pressure so that a good quality dermal ridge impression left at the surface.

The study was divided into stages: one to see the effect of age(255 prints) and second to see the effect of temperature conditions (152 prints) on the ABH antigenic activity. The prints were kept at room temperature.

Mixed cell agglutination reaction described by Ishiyama (1975) with some modifications was applied for analysis. To confirm the experimental results, individual's blood group and secretor status was tested later on.

The study was extended to analyse latent prints upto

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TABLE 1

LEVELS OF LE^A AND LE^B ANTIGENS IN SALIVA [1]

I. SECRETOR

	RECIPROCAL TITER	
	LE^A	LE^B
RANGE:	100-3200	400-51200
MEAN[2]:	478[3]	4240

II. NON-SECRETOR

	RECIPROCAL TITER	
	LE^A	LE^B
RANGE:	400-51200	100-400
MEAN:	4025	200[4]

[1] MINIMUM SALIVA DILUTION TESTED WAS 1/100
[2] GEOMETRIC MEAN
[3] 17/20 SPECIMENS EXHIBITED LE^A ACTIVITY
[4] 7/24 SPECIMENS EXHIBITED LE^B ACTIVITY

TABLE 2

LEVELS OF LE^A AND LE^B ANTIGENS IN SEMEN[1]

I. SECRETOR

	RECIPROCAL TITER	
	LE^A	LE^B
RANGE:	40-160[3]	20-5120
MEAN[2]:	63	288

II. NON-SECRETOR

	RECIPROCAL TITER	
	LE^A	LE^B
RANGE:	20-10240	20-40[4]
MEAN:	150	23

[1] MINIMUM SEMEN DILUTION TESTED WAS 1/20
[2] GEOMETRIC MEAN
[3] 6/24 SPECIMENS EXHIBITED LE^A ACTIVITY
[4] 5/26 SPECIMENS EXHIBITED LE^B ACTIVITY

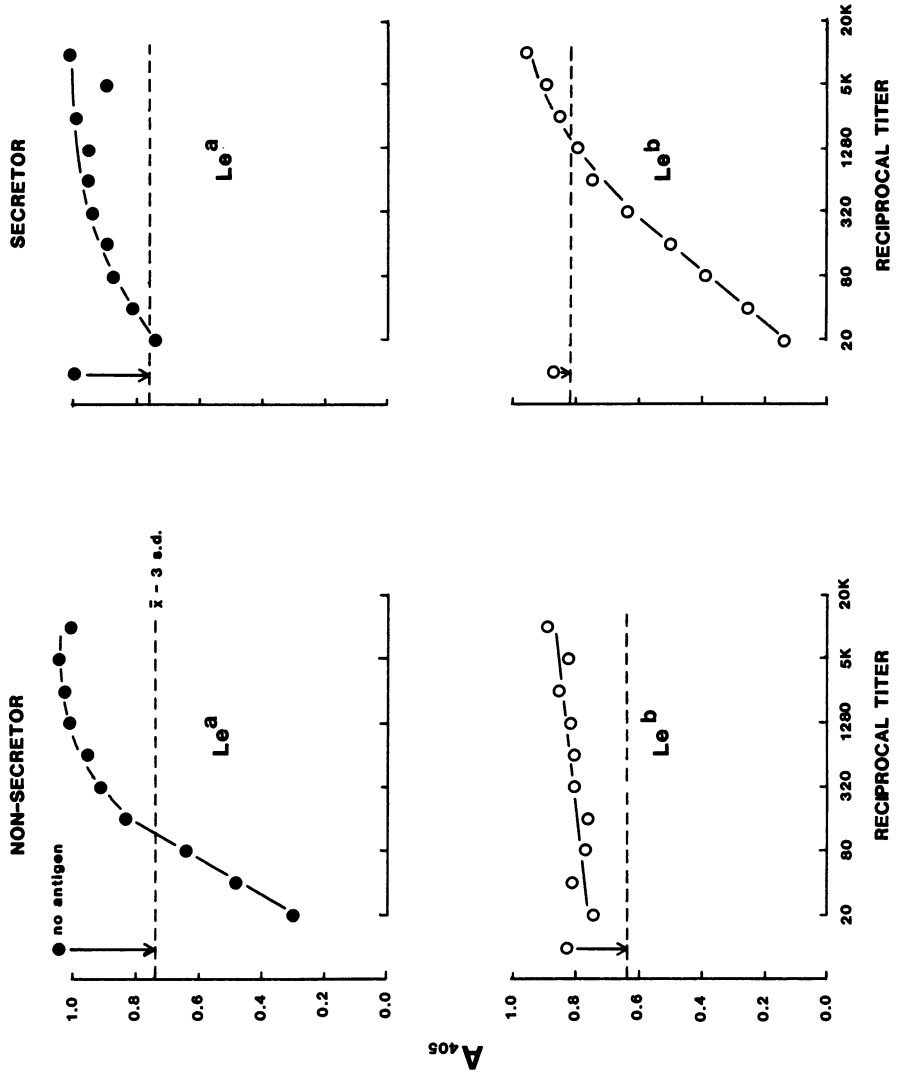

FIGURE 1. INHIBITION OF BINDING OF MONOCLONAL ANTI-LEWIS ^{A} AND ANTI-LEWIS ^{B} BY SECRETOR AND NONSECRETOR SEMEN.

Given the level of sensitivity of this ELISA method, it is important to consider its potential utility for the routine examination of body fluid stains associated with forensic evidence. Considering the magnitude of the Lewis antigen titers in saliva, one would not anticipate difficulty in demonstrating Le^b from secretors or Le^a from nonsecretors, assuming the specimen had sustained an overall dilution of no more than 50- to 100-fold. On the other hand, the much lower Lewis titers observed for semen suggest that these antigens will be detectable only in stain extracts which contain high concentrations of semen.

Despite the modest sensitivity exhibited by this ELISA method it does possess two salient features: (1) it enables the concurrent and expeditious analysis of a larger number of specimens than the conventional hemagglutination-inhibition method; and (2) it utilizes monoclonal antibodies to the Lewis antigens which are of defined specificity and affinity, insuring that this aspect of the assay is invariant. The latter features are unattainable with polyclonal antisera to these antigens.

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would be 80 (reciprocal titer). No Le^b activity was demonstrable in this specimen at the minimum diluton tested of 1:20. Semen from a secretor individual significantly inhibited the binding of monoclonal anti-Le^b up to a specimen dilution of 1:1280. The Le^a level in this specimen was low, yielding a reciprocal titer of only 20.

Using this approach, the levels of Le^a and Le^b were determined for saliva specimens from 44 individuals, 20 secretors and 24 nonsecretors. The results are shown in Table 1. The Le^b levels in saliva from secretors varied over more than a 100-fold range, with the mean titer being 4240. The mean level of Le^a in this group was 478, with the actual titers varying between 100 and 3200. Nonsecretor saliva specimens demonstrated the same range of Le^a titers as was seen for Le^b in secretors, with almost the same mean titer. For 7 of the 24 nonsecretor specimens, an apparent Le^b activity was present, although of low titer. Repetitive analyses of these specimens failed to consistently demonstrate Le^b presence. It should be pointed out that the minimum dilution of saliva tested was 1:100. Thus, the presence of either Le^a or Le^b at titers less than 100 was not established.

Table 2 displays the levels of Le^a and Le^b in 68 semen specimens. The magnitude of seminal fluid Lewis titers, for secretors and nonsecretors, was considerably less than that seen for saliva. The mean titer of Le^b in secretor semen was 15-fold less than that seen in secretor saliva. The mean titer of Le^a in nonsecretor semen was almost 30-fold less than nonsecretor saliva. Five of 26 nonsecretors exhibited very low titers of an apparent Le activity. As seen with saliva, the appearance of low titer Le^b activity in nonsecretor semen was unpredictable upon replication. Since the minimum dilution of semen examined was 1:20, the occurrence of Lewis titers less than 20 cannot be ruled out.

This technique was used to test for the presence of the Lewis antigens in vaginal fluid stains from eight donors. The Lewis phenotype of each donor was established by direct test upon the donor's red blood cells. Five of the donors were Le^{a-b+} and the Le^b antigen was demonstrable in stains from each. Two individuals were Le^{a+b-}, but stains from only one donor exhibited Le^a. Neither Le^a or Le^b was demonstrable in one Le^{a+b-} individual. The remaining donor was Le^{a-b-}.

DISCUSSION

The ELISA procedure described in this paper is capable of detecting the Le^a and Le^b antigens in body fluid samples in a relatively short period of time. The test can be completed in fewer than four hours and enables a large number of specimens to be analyzed concurrently. The major portion of the assay time is consumed by the incubation phases.

A comparison of the Le^a and Le^b titer ranges determined by this ELISA, with similar studies reported by others (7-8), indicates that the sensitivity of the current technique equals, but does not exceed, that of hemagglutination-inhibition.

the plate was placed at 37° C for 30 minutes.

After three washes with 1% GHBS, each well received 200 ul of alkaline phosphatase conjugated goat antimouse IgM (GAMIGMAP) (Sigma Chemical Company, St. Louis, Missouri) diluted 1:1000 with 1% GHBS. The plate was incubated one hour at 37° C followed by three washes with 1% GHBS.

The residual alkaline phosphatase activity in the wells was assayed by adding 200 uL p-nitrophenyl phosphate (6.0 mg/ml 0.1 M glycine buffer, pH 10.4, containing 0.001 M MgCl and 0.001 M ZnCl) and incubating at 37° C for 30 minutes. The absorbance in each well at 405 nm was determined in an automated microplate reader.

The mean and standard deviation of the mean were calculated for the absorbancies in the quadruplicate wells which received anti-Le^a or anti-Le^b but no test specimen. Three standard deviations were subtracted from the mean absorbance for each antibody to yield a corrected no antigen control value for each antigen. The reduction of binding of either monoclonal anti-Le^a or anti-Le^b, due to inhibition by antigen, was considered significant if the mean test well absorbance was equal to or less than the appropriate no antigen control value. The titer of Le^a or Le^b in a body fluid specimen was defined as the reciprocal of the greatest dilution that was capable of significant inhibition.

Saliva was obtained from personnel at the FBI Academy. Immediately after collection, each saliva sample was placed in boiling water for five minutes and stored at -70° C until examined for Le^a and Le^b presence. Semen specimens were obtained by regional fertility clinics from normal individuals with no history of genitourinary pathology. Semen specimens were stored at -70° C until tested. Vaginal fluid specimens were obtained on tampons inserted for six hours by donors who refrained from sexual activity for 72 hours prior to sample collection. The tampons were air-dried. Crusted areas were cut and extracted in buffered saline for tests.

RESULTS

For this study, it was desired that an absorbance at 405 nm of about 1.0 be obtained in the no antigen control wells for both Le^a and Le^b after 30 minutes of incubation with substrate. The concentrations of LBGS, the monoclonals to Le^a and Le^b, and the antimouse immunoglobulin conjugate necessary to achieve this final absorbancy were determined by titration. LBGS, diluted 1:3200 was optimal for detection of Le^a antigen; whereas a dilution of 1:1600 was necessary for Le^b detection. The optimal dilution of monoclonal anti-Le^a was shown to be 1:500, and the optimal dilution for the monoclonal anti-Le^b was found to be 1:1000. The GAMIGMAP was used at a dilution of 1:1000.

Figure 1 illustrates the typical inhibition patterns seen when serial dilutions of semen specimens from secretor and nonsecretor individuals were tested for their ability to inhibit the binding of the monoclonal antibodies. This figure points out also the method by which the Le^a and Le^b titers were derived for a given body fluid specimen. Note that nonsecretor semen was capable of significantly inhibiting the binding of monoclonal anti-Le^a up to a 1:80 dilution of the sample. The Le^a titer of this specimen

AN ELISA METHOD FOR DETECTING LEWIS ANTIGENS IN BODY FLUIDS

F. Samuel Baechtel and Frances C. Henning
Forensic Science Research Group, FBI Academy, Quantico, Virginia, U.S.A.

Detection of Lewis^{a} (Le^{a}) or Lewis^{b} (Le^{b}) antigens in body fluids enables the prediction or corroboration of the ability of the fluid depositor to secrete soluble ABH substances (1-4). The hemagglutination-inhibition technique, usually employed for the detection of the Lewis antigens, is not well suited for processing large numbers of samples on a routine basis. The genesis of monoclonal antibodies, specific for Le^{a} and Le^{b}, has promoted the development of enzyme-linked immunosorbent assays (ELISA) for the detection of these antigens (5-6). The ELISA described in this paper incorporates these monoclonal reagents into a technique that can be used to efficiently analyze a large number of samples for the presence of Le^{a} and Le^{b} in body fluids.

METHODS AND MATERIALS

The ELISA was configured such that the presence of Le^{a} or Le^{b} in a test specimen could be detected by its ability to inhibit the binding of the appropriate monoclonal antibody to immobilized homologous antigen. The assay was carried out in 96-well, polystyrene microplates (Dynatech Laboratories, Alexandria, Virginia). Lewis blood group substance standard (LBGS), containing both Le^{a} and Le^{b} activity (Ortho Diagnostics, Raritan, New Jersey), was diluted with 0.05 M carbonate/bicarbonate buffer, pH 9.6. The LBGS was diluted 1:3200 and dispensed in 200 uL portions to wells in the upper half of each plate, which were devoted to tests for Le^{a} antigen. Wells in the lower half of the plate received 200 uL LBGS diluted 1:1600. These were used to test for the presence of Le^{b}. After addition of the LBGS, the plate was maintained at 4^{o} C overnight to permit binding of the antigens to the plate.

After antigen immobilization, the plate was washed three times with buffered saline that contained 1% liquid gelatin (GHBS) (Hypure, New Brunswick, New Jersey). Unoccupied binding sites in the plate were blocked by filling all wells with 3% GHBS and incubating for one hour at 37^{o} C. Plates can be sensitized with LGBS, blocked, and stored up to four days in advance of their use.

Body fluid specimens were diluted with 1% GHBS and tested for Le^{a} and Le^{b} contemporaneously on the same plate. One-hundred uL portions of the specimens were added, in duplicate, to wells devoted to tests for Le^{a}; and duplicate 100 uL aliquots added to Le^{b} test wells. Immediately following addition of test specimens, 100 uL monoclonal anti-Le^{a} (1:500 dilution with 1% GHBS) were added to the Le^{a} test wells; and 100 uL monoclonal anti-Le^{b} (1:1000 dilution with 1% GHBS) placed in each of the Le^{b} test wells. Both monoclonal antibodies were obtained from the Genetic Testing Institute, Atlanta, Georgia. Four wells in the Le^{a} test area and four in the Le^{b} area received 100 uL 1% GHBS instead of test specimens. These wells served as no antigen controls in which maximum binding of monoclonal antibody could be assessed. After addition of reagents,

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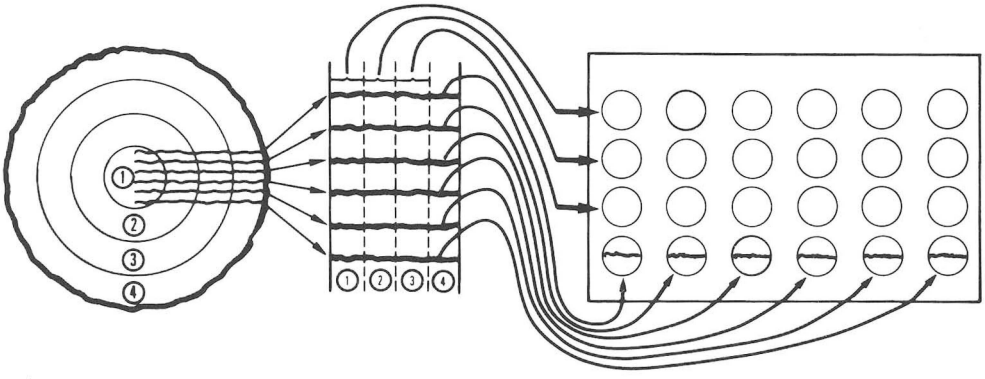
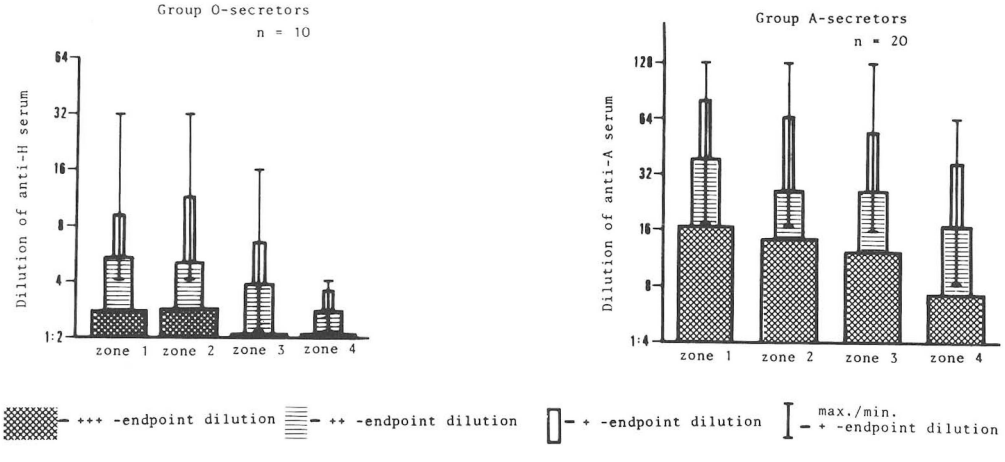
Picture 1, MATERIAL AND METHOD



Picture 2, RESULTS: Average endpoint dilution



diameter of the stain.

2.3. Inhibition

The segments of the threads were placed separately in the wells of microtitre plates and one drop of the corresponding antiserum was added in a series of 6 geometrical dilutions ("master titration"). The specimens were incubated for 20 hours at 4° C, then the antiserum was transfered to the test tubes.

2.4. Tube test

One drop of 0.1 erythrocyte suspension of the corresponding bloodgroup in 2% bovine albumine was added. After a further incubation of 2 hours at 4° C and 30 minutes at a room temperature the tubes were centrifuged for one minute with 1000 g. The reactions were read through the microscope.

3. RESULTS (picture 2)

3.1. A-substance

The 1+ -endpoint dilution differed from stain to stain just as inside the stains up to 3 degrees of dilution. 15 out of 20 stains proved to have the highest absorption in the peripheral section, 5 of the stains had a constant distribution, in one case the highest absorption was found in the second zone.

3.2. H-substance

In 8 out of 10 stains the highest absorption was found in zone 4, 2 stains showed a constant distribution.

3.3. Age of the stains

The age of the stains had no influence of the investigation.

4. DISCUSSION

The acquired test method, a master titration, has turned out to be appropriate for the investigation of small differences in the concentration of ABH-bloodgroup substances in seminal stains. For the routine laboratory the standard inhibition or inhibition-elution test remains the method of choice.

Our artificially produced stains should serve to be a model for e.g. underwear or bed-linen made of cotton, that are often to be investigated in the routine.

According to our results presented we suggest to use peripheral zone of seminal stains to test in the ABH-system.

THE DISTRIBUTION OF ABH-BLOODGROUP SUBSTANCE IN SEMINAL STAINS

Richard SCHEITHAUER, Joachim LÖTTERLE
Institute for Forensic Medicine, University Erlangen-Nürnberg, 8520 Erlangen, West Germany
(Director: Prof.Dr.med.H.-B. Wuermeling)

1.1. SUMMARY

To guarantee an economical utilization in bloodgrouping of seminal stains the various bloodgroup systems should be investigated in the region of their maximum concentration. The distribution for the Gm and PGM_1 system is already known. In the present study the distribution of the ABH-substance was investigated using artificially made seminal stains and an inhibition test ("master titration"). The results demonstrate that in samples of equal size the absorption and therefore the concentration of bloodgroup substances is higher in peripheral sections, or at least not lower than in other regions of the seminal stain.

1.2. ZUSAMMENFASSUNG

Um eine ökonomische Verwertung der oft geringen Spermaspuren zu gewährleisten, sollten die verschiedenen Blutgruppensubstanzen am Fleck dort untersucht werden, wo sie am höchsten konzentriert sind. Für das Gm- und PGM_1-System ist die Verteilung bereits bekannt. In der vorgelegten Arbeit wurde die Verteilung der ABH-Substanz an künstlich angelegten Spermaflecken mit Hilfe eines standardisierten Absorptionstests untersucht.
Es konnte gezeigt werden, daß die Absorption und damit die Konzentration der ABH-Substanz in den äußeren Abschnitten des Spermaflecks am höchsten, wenigstens aber nicht geringer als in anderen Abschnitten ist.

2. MATERIAL AND METHOD (Picture 1)

2.1. Stains

20 stains of A-secretors and 10 stains of group O-secretors were produced by dropping 300 µl of liquified human sperm on one point of fine woven cotton. At the time of examination the age for the stains was between one day and one year.

2.2. Preparation

The stains were divided in 4 concentric zones of the same width (Zone 1 = central; ... zone 4 = peripheral).
6 threads lying side by side were taken out reaching from the centre to the periphery. The threads were cut according to their sectional distribution; the particular segments had a length of 5 to 7 mm, dependend of the

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BLOOD ABO	BLOOD LEWIS	CORRELATION	PLAQUE ABS.	PLAQUE MIN.	PLAQUE MOD.	PLAQUE SEV.	GINGIVITIS ABS.	GINGIVITIS MIN.	GINGIVITIS MOD.	GINGIVITIS SEV.	POCKETS ABS.	POCKETS MIN.	POCKETS MOD.	POCKETS SEV.
A1	a-b+	B=S	2	1	-	-	1	-	2	-	3	-	-	-
	a+b-		1	1	-	1	-	1	1	1	-	1	1	-
	a-b-		1	-	-	-	-	-	1	-	-	1	-	-
	a-b+	B≠S	8	15	2	-	9	1	15	-	19	3	-	-
	a+b-		1	-	-	-	-	1	-	-	1	-	-	-
	a-b-		-	-	-	-	-	-	-	-	-	-	-	-
A2	a-b+	B=S	2	4	-	-	1	3	2	-	6	1	-	-
	a+b-		-	-	2	-	-	1	1	-	-	-	-	-
	a-b-		-	-	-	-	-	-	-	-	-	-	-	-
	a-b+	B≠S	1	1	-	1	1	-	2	-	1	1	-	-
	a+b-		-	-	-	-	-	-	-	-	-	-	-	-
	a-b-		-	-	-	-	-	-	-	-	-	-	-	-
B	a-b+	B=S	3	3	-	1	4	1	1	1	5	-	-	-
	a+b-		3	2	-	-	1	2	1	1	3	2	-	-
	a-b-		-	-	1	-	-	-	-	1	-	1	-	-
	a-b+	B≠S	1	-	-	-	-	1	-	-	1	-	-	-
	a+b-		-	-	-	-	-	-	-	-	-	-	-	-
	a-b-		-	-	-	-	-	-	-	-	-	-	-	-
O	a-b+	B=S	10	6	1	-	6	1	9	1	11	3	-	-
	a+b-		2	8	1	-	3	-	6	2	9	1	1	-
	a-b-		2	-	1	1	1	-	2	1	3	-	1	-
	a-b+	B≠S	2	-	-	-	1	-	1	-	2	-	-	-
	a+b-		-	-	-	-	-	-	-	-	-	-	-	-
	a-b-		-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3. PERIODONTAL STATUS COMPARED TO BLOOD AND SALIVA (ELUTION) ABO GROUPINGS

B=S Blood and saliva groupings agree
B≠S Blood and saliva groupings do not agree

CLASSIFICATION	PLAQUE INDEX	GINGIVAL INDEX	POCKET DEPTH (mm)
Absent	No plaque	Normal gingiva	0-3
Minimal	Film of plaque recognizable by probe only	Mild inflammation, slight change in colour	3-4
Moderate	Visible accumulation of soft deposit in gingival region	Moderate inflammation, redness	4-7
Severe	Abundance of soft deposit	Severe inflammation, ulceration, spontaneous bleeding	7+

TABLE 1. INDICES OF PERIODONTAL DISEASE

BLOOD		SALIVA (ELUTION)			
ABO		A	B	O	AB
A1	33	7	-	-	26
A2	11	9	-	-	2
B	14	-	13	-	1
AB	1	-	-	-	1
O	34	1	1	32	-

TABLE 2. ABO GROUPING OF LIQUID BLOOD AND SALIVA STAINS USING POLYCLONAL ANTISERA

BLOOD		CORRELATION	PLAQUE			GINGIVITIS			POCKETS	
			NO. OF SEVERE SPOTS			NO. OF SEVERE SPOTS			NO. OF SEVERE SPOTS	
ABO	LEWIS		1	2-3	4+	1	2-3	4+	1	2-3
A1	a-b+	B=S	1	-	-	-	-	-	-	-
	a+b-		-	-	2	-	-	2	1	-
	a-b-		-	-	-	1	-	-	-	-
	a-b+	B≠S	6	2	1	-	3	3	2	1
	a+b-		-	-	-	-	-	-	-	-
	a-b-		-	-	-	-	-	-	-	-
A2	a-b+	B=S	1	-	-	-	-	1	-	-
	a+b-		-	-	1	-	-	1	-	-
	a-b-		-	-	-	-	-	-	-	-
	a-b+	B≠S	-	-	1	-	1	-	-	-
	a+b-		-	-	-	-	-	-	-	-
	a-b-		-	-	-	-	-	-	-	-

TABLE 4. BLOOD AND SALIVA GROUPING RESULTS FOR GROUP A PATIENTS WITH AT LEAST ONE SEVERELY INFECTED LOCATION

Preliminary attempts to test A and/or B substance of microbial origin were partially successful. Species which had yielded positive results and which were known to be present in saliva (2) were supplied by the Department of Health as active cultures in nutrient broth. As we wished to test soluble substance rather than cell wall or capsular antigen, the broth was centrifuged, the supernatant filtered at an exclusion pore size of 200,000 M.W., the filtrate dialyzed against distilled water for 24 hours, concentrated five times in a Minicon (Amicon) concentrator, and tested against both monclonal and polyclonal reagents at dilutions from neat to 1/200 by absorption-inhibition and absorption-elution. Staphylococcus aureus, strain #79 and strain #94-96, Citrobacter freundii, Micrococcus luteus and Candida albicans were tested. Soluble A substance was detected by both inhibition and elution with both polyclonal antisera & monoclonal antibodies in the Micrococcus luteus preparation.

CONCLUSION:

1) Discrepancies between blood and saliva groupings by elution using polyclonal antisera were found.

2) The most common discrepancy was additional B substance in group A Lewis a-b+ saliva.

3) The severity of bacterial oral infection did not influence the accuracy of the grouping results.

4) Neither monoclonal anti-A nor anti-B recognized discrepant blood group substances in the saliva stains but monoclonal anti-A recognized prepared bacterial A.

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A liquid blood sample by fingerprick into saline and a saliva stain on kleenex were supplied by each patient. The red blood cells were typed for Lewis and for ABO. The saliva stains were typed with polyclonal antisera (Ortho Diagnostics) and affinity-purified ascitic fluid monoclonal antibodies (Chembiomed, Alberta, Canada) by absorption-inhibition and absorption-elution in parallel. The absorption-inhibition technique was as specified for case material but the absorption-elution technique was deliberately sensitized in two ways. A neat saliva extract was tested as well as dilutions of 1/10, 1/50, 1/100 and 1/200 and the extract was not heated for 3 hours at 56°C as would routinely be done.

The ABO and Lewis results were within expected limits given the small sample size (Table 2) but the elution results with polyclonal antisera revealed a discrepancy rate of almost 79% for the saliva stains from group A_1 individuals. As well there was A substance in the saliva of one group B and one group O donor and B substance in the saliva of another group O person.

Only one A_1 Lewis a+b- sample showed B substance in the saliva sample and both O and the single B saliva samples with additional antigens were from individuals who were Lewis a-b+ by blood. When the periodontal status was combined with the blood-saliva groupings (Table 3) it could be seen that the majority of the discrepancies occurred in the patients who had minimal or no periodontal findings.

When the dental data for the group A individuals was re-examined, choosing patients with from one to four or more single severely infected spots and comparing the blood and saliva groupings, an increasing number of infected spots did not necessarily dictate lack of correlation between blood and saliva groupings (Table 4).

There are two potential sources of the additional B substance in the saliva of group A individuals. The acquired B phenomenon has been described (4) as resulting from deacetylation of the group A immunodominant sugar N-acetyl-D-galactosamine to produce D-galactosamine. The deacetylase may be bacterial in origin. Although the group B immunodominant sugar is D-galactose, both D-galactose and D-galactosamine are recognized by polyclonal anti-B antisera. Acquired B may have been observed in semen stains (5). As well, there are several reports of micro-organisms which share A and/or B blood group determinants with humans (2).

Although the manufacturers of the monoclonal anti-B antibodies found no reaction with acquired B, it is not known whether these antibodies recognize B substance produced by micro-organisms.

When the thirty-one saliva stains which gave discrepant results were grouped using monoclonal antibodies and the same techniques as had been used with polyclonal antisera, no unexpected blood group substances were detected.

THE EFFECT OF PERIODONTAL DISEASE ON THE RELIABILITY OF GROUPING SALIVA STAINS IN THE ABO SYSTEM. A COMPARISON OF POLYCLONAL ANTISERA AND MONOCLONAL ANTIBODIES

Pamela J. Newall, Al Marignani, Keith Kelder *

In our laboratory, saliva stains are routinely ABO grouped by absorption-inhibition and absorption-elution in parallel (1) and if the results do not agree they are not reported. Since a blood sample is rarely received from the accused person in criminal cases, the reliability of the saliva stain grouping is extremely important. Spurious A and or B reactions as a result of bacterial contamination of the saliva sample have been reported (2) but no investigation of state of health of the saliva donor has been published. Consequently the aims of this study were:

1) to investigate, under controlled conditions, the influence of oral infection on the accuracy of grouping saliva stains

2) to evaluate the potential application of one source of monoclonal antibodies to the grouping of these stains.

Staff members of the Department of Oral Pathology, Faculty of Dentistry at the University of Toronto advised us to study a series of patients with periodontal disease.

The severity of periodontal disease is measured by three indices, plaque, gingivitis and pocket depth. Each index is assessed and recorded according to World Health Organization Standards (3). Plaque is defined as a 300 to 500 cell-thick coating of metabolically active bacteria which may initiate caries and/or periodontal disease. Gingivitis is an inflammatory response in the gum tissue initiated directly by bacterial hydrolytic enzymes, endotoxins and metabolic end products or indirectly by an immediate or delayed response to plaque antigens. It leads to connective tissue destruction and eventually tooth loss. The connective tissue destruction surrounding the teeth leads to the formation of pockets which may contain active bacteria.

Ninety-three patients attending a dental clinic participated in this study. Each patient was examined by one of two attending dentists. Six teeth representing the four dental quadrants were chosen and the three periodontal indices (Table 1) were measured at four predetermined locations, three buccal and one lingual on each tooth. These 72 measurements were averaged for each patient to yield an individual definition of disease for each index.

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* Centre of Forensic Sciences, 25 Grosvenor Street, CDN-Toronto, Ontario, Canada M7A 2G8

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Table I

MEAN TIME OF DETECTABILITY OF ENZYMES IN BLOOD STAINS

t i m e i n m o n t h s

Temperature	Kind of ground	ENZYMES			
		PGM_1	AK	AcP	EsD
4°C	glass	17	70	15	0.7
	linen	16	70	13	0.2
	wood	18	70	13	0.6
20°C	glass	9	70	8	0.023
	linen	7	40	3	0.03
	wood	10	50	7	0.1
	plastic	5	40	4	0.03
	refined iron	5	40	6	0.02
	rusty iron	6	40	6	0.03
	paper	6	40	4	0.03

t i m e i n w e e k s

45°C	glass	5	36	0.6	0.1
	linen	4	28	0.4	0.1
	wood	5	32	0.7	0.1

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Adress 1. author's :

Institut of Forensic Medicine
ul. Skłodowskiej - Curie 9
85 - 094 Bydgoszcz /Poland/

In the group of examined enzymes the shortest time of detectability and the least precise results were obtained in the investigation of EsD. In the stains stored in the room temperature only after several days the activity of the enzyme decreased considerably and the first negative results appeared.

According to conducted experiments it is possible to state that application of the method of investigation on cellulose acetate foil allowes to determine the phenotypes of the adenylate kinase, phosphoglucomutase and acid phosphatase for a relatively long time ; this in turn allows to use those enzymes for identification of blood stains in practice ; moreover, a small quantity of blood is needed for such an experiment. However a little stability of the esterase D and relatively short time of detectability of this enzyme while applying the method in this experiment limits considerably the usefulness of this enzyme for the forensic medicine.

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Results

The average time of detectability of the examined enzymes in the blood stains stored in various conditions is presented in the table I.
A progressive vanishing of the activity of the particular isoenzymes and appearance of quantative changes were observed in the course of investigations. In the case of phosphoglucomutase /PGM/ the appearance of additional weak zone, located above fraction d, which didin't cause the problems with interpretation of the electrophoretic picture, could be observed. Remarkably disadvantageous influence of increased temperature was observed in the case of determining phenotype AcP. It influenced not only the time of detectability of the enzyme but also the appearance of some additional fractions of various, dependened on a phenotype, localization. The reconstruction of the AcP picture obtained after the usage of 0.05 M of Mercaptoethanol, in most cases allowed to determine the phenotype AcP to the time given in the table I, according to the temperature of storage and the background of the blood stain. The least questionable was determining the phenotypes of AK. Only in bloodstains kept on the moisty background the increase of activity of normally weak zones and appearance of additional fractions located on the anoda side was observed.

EXPERIMENTAL STUDY ON DETECTABILITY OF SOME CHOSEN ENZYMES IN BLOOD STAINS

Danuta Miścicka- Śliwka, Stefan Raszeja

Institut of Forensic Medicine, Bydgoszcz / Poland /

Institut of Forensic Medicine, Gdańsk / Poland /

The examination of human bloodstains belongs to one of the most important aims of the forensic medicine. The application of polymorphic red cell enzymes in examination of bloodstains has allowed to extend the possibilities of their identification. In this paper, the authors have presented the results of the research on application of chosen red cell enzymes in the identification of human bloodstains.

Material and methods

There have been investigated 584 human blood stains taken from subjects of known phenotypes PGM, AK, AcP, EsD, prepared on various grounds such as glass, linen, wood, rusted iron, refined iron, plastic and paper. The stains were stored at the temperature of 4^{o}, 22^{o}, 45^{o}C and examined periodically by means of electrophoresis on Sartorius cellulose acetate foil, on Cellogel and with the usage of Beckman Microzone Cell /1, 2, 3/. The isoenzymes were dyed in a typical way /1/.

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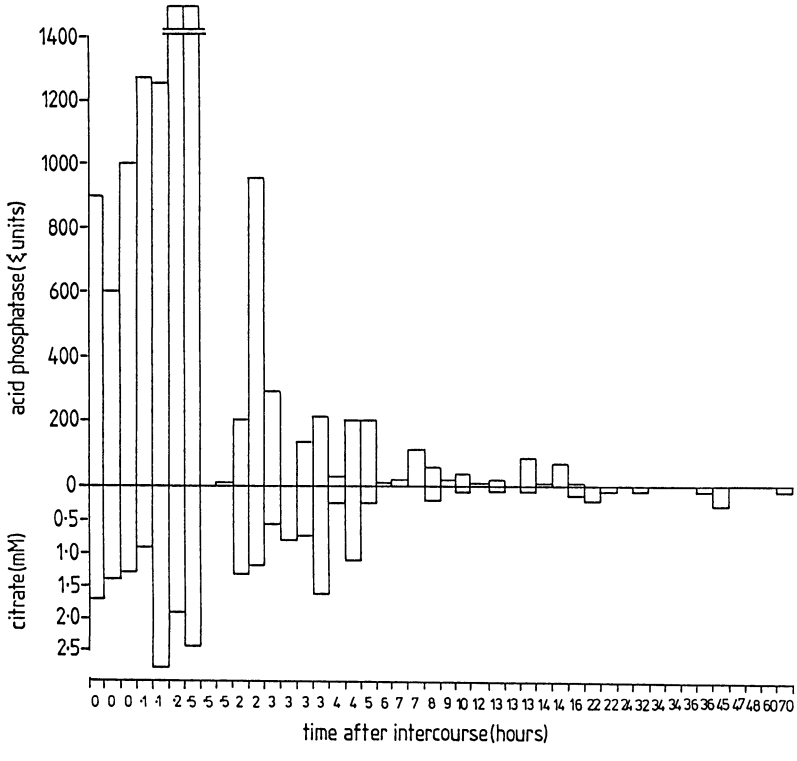

Fig 3 Decrease in the level of citrate ions and acid phosphatase activity from vaginal swabs taken at increasing times after sexual intercourse

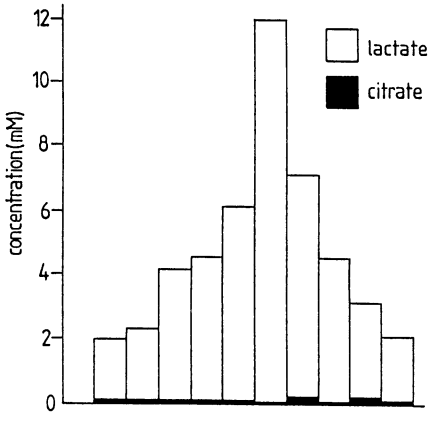

Fig 4 Distribution of citrate and lactate ions in extracts from 10 semen-free post-coital vaginal swabs

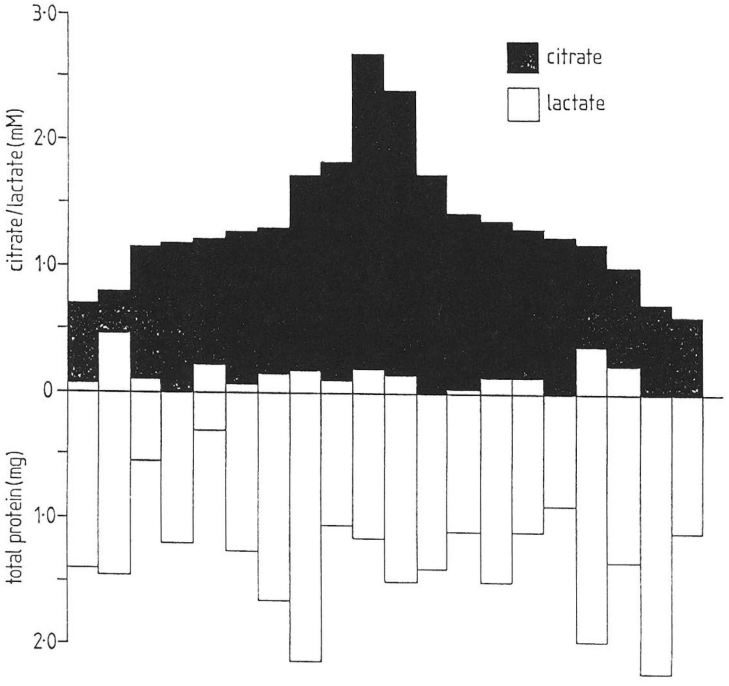

Fig 1 Distribution of citrate and lactate ions and protein concentrations in 20 semen samples

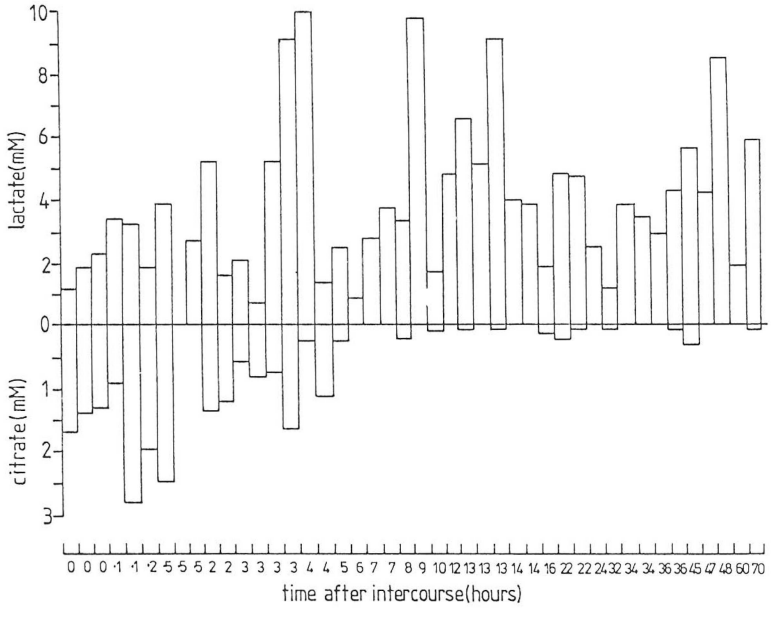

Fig 2 Concentration of citrate and lactate ions from vaginal swabs taken at various times after sexual intercourse

phosphatase and citrate concentrations in semen samples. This relationship was not apparent from the results obtained in this study when semen was extracted from vaginal swabs. This could be due to a variety of reasons; the most likely being a loss by drainage or denaturation. Citrate ions are unlikely to be affected by the vaginal environment but could also be lost by drainage. Vaginal secretion contains very low levels of citrate as shown in the results from post-coital semen-free vaginal swabs (Fig. 4) and from normal vaginal swabs taken more than six hours after intercourse.

Extracts from 15 oral swabs, obtained from donors and from case-work samples, showed levels of citrate which ranged from 0 - 1.0 mM with a mean of 0.34mM. Although these are not high concentrations, it may be necessary to determine whether saliva is present in order to avoid confusion with low levels of semen. Lactate determinations from these swabs showed much lower amounts than those found in vaginal secretion (0 - 0.35mM; mean, 0.08mM) and are unlikely to cause any confusion.

We found negligible levels of lactate and citrate in urine but an elevated level of lactate may be found in cases of bladder infections and after periods of physical activity. Although lactate is not specific for vaginal secretion and citrate is not specific for seminal plasma, quantitative estimations of these two carboxylic acids from dried stains may be of considerable help in the determination of these particular fluids and can also be used to confirm other qualitative assays.

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Stains were prepared from each of ten samples on clean cotton sheeting and allowed to dry at room temperature. Each complete stain was extracted with vortexing into 200µl of distilled water and centrifuged to remove any extraneous material.

Urine samples were prepared in the same way as the semen stains.

Oral swabs were extracted and stored in the same manner as the vaginal swabs.

All extracts were assayed for citrate and lactate ions using capillary isotachophoresis.

Total protein estimations were obtained by a modification of the Lowry method (7).

Acid phosphatase assays were kindly supplied by A. Davies and S. Wotherspoon of the Metropolitan Police Laboratory.

All estimations were made from 15µl injections of each sample. Separations were performed at a constant current of 150µA, with a chart speed of 0.5mm/sec and a 200mV input.

Leading Electrolyte: 10mM HCl adjusted to pH 2.5 with β-alanine.

Terminating Electrolyte: 10mM Propionic Acid adjusted to pH 4.0 with 1M NaOH.

Initially 15µl of ech sample were injected and assayed. This was followed by a second injection of 15µl of sample which was mixed with 5µl of a standard solution containing 4mM citric acid, 4mM lactic acid and 4mM trichloroacetic acid.

Trichloroacetic acid was chosen as an internal standard because it separates clearly using these electrolytes and does not form mixed zones with either citrate or lactate ions.

A full account of the theory of isotachophoresis has been provided by Holloway and Trautschold (8).

RESULTS AND DISCUSSION

The citrate and lactate concentrations obtained from seminal stain extracts are shown in Fig. 1. Both of the ions show variation from sample to sample but the citrate levels are always considerably higher than those of lactate.

There does not appear to be any relationship between the protein concentrations and either of the carboxylic acids; therefore the ratios of protein : citrate could not be used as an indication of the amount of semen in a particular stain.

Vaginal swabs taken at various times after intercourse were extracted and assayed for citrate, lactate and acid phosphatase. Lactate levels were extremely variable ranging from approximately 1.0 to 10mM with no apparent relationship to post-coital interval (Fig. 2). Citrate concentrations, however, although variable, showed a decrease with time after intercourse similar to that of acid phosphatase (Fig. 3). Gavella (9) has shown a direct correlation between acid

A COMPARATIVE STUDY OF THE CITRATE AND LACTATE CONCENTRATIONS IN STAINS FROM SEMEN, VAGINAL SECRETION AND MIXTURES OF THE TWO USING ISOTACHOPHORESIS

P.D. Martin and S.K. Cheshire

Metropolitan Police Laboratory, 109 Lambeth Road, London SE1 7LP, U.K.

INTRODUCTION

In the scientific examination of sexual assault cases the forensic scientist is continually faced with the inability to reliably determine the presence or absence of vaginal secretion particularly in mixtures with semen.

It has been established that vaginal secretion has a high level of lactic acid (1) and that citiric acid is a good indicator of androgenic stimulation (2).

The objective of this study was to determine whether these two carboxylic acids could be used to determine the presence of semen, vaginal secretion or mixtures of the two from a dried stain.

In the sexually mature woman there is always an excess of glycogen in the vaginal epithelial cells (3) and this is, in some way, metabolised by the lactobacilli and fermented to lactic acid. Lactic acid is also present in semen (4) but in much lower levels than that found in vaginal secretion.

One of the characteristic components of seminal plasma is citric acid (5). The specific function if the citrate ions is not known but they may be present as activators of prostatic acid phosphatase and may also be involved with the coagulation and liquefaction of semen (6). The concentration of citric acid in semen is much higher than in vaginal secretion.

We have attempted to determine quantitatively the levels of citrate and lactate in semen and vaginal secretion as an aid to the identification of these fluids when encountered in sexual assault cases.

We used capillary isotachophoresis because the samples require no prior treatment, very small quantities can be assayed, it is a relatively rapid technique, simultaneous estimations can be made and the results are reproducible.

MATERIALS AND METHODS

Forty-four vaginal swabs taken at various times after intercourse were obtained from seven donors within the Metropolitan Police Laboratory. Each swab was extracted with vortexing into 1 ml of distilled water. The extract was centrifuged to remove any debris and the supernatant stored at -15^{o}C until used.

Ten semen-free post-coital swabs were provided by donors who used a contraceptive sheath during sexual intercourse. These swabs were extracted and stored as above.

Twenty semen samples were obtained from a fertility clinic and stored at -15^{o}C until used.

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V. Stains

TABLE 2. Gm haplotype frequencies among the five populations in China

Gm haplotype	Inner Mongolia		Beijing		Anhui		Zhejiang		Guangzhou	
	Freq.	S.E.	Freq.	S.E.	Freq.	S.E.	Freq.	S.E.	Freq.	S.E.
a,z;..;g,u	0.4708	0.0271	0.4285	0.0252	0.4163	0.0305	0.3496	0.0274	0.1825	0.0222
a,x,z;..;g,u	0.2027	0.0218	0.2140	0.0209	0.1715	0.0233	0.1835	0.0223	0.0543	0.0130
a,z;..;b0,b3,b5,s,t	0.0971	0.0161	0.1166	0.0163	0.0840	0.0171	0.0795	0.0156	0.0329	0.0102
a,f;n;b0,b1,b3,b4,b5,u	0.2294	0.0228	0.2409	0.0218	0.3282	0.0290	0.3874	0.0280	0.7303	0.0255

TABLE 3. Km phenotype and allele frequencies among the five populations in China

Km phenotype	Inner Mongolia	Beijing	Anhui	Zhejiang	Guangzhou
I+	106	114	73	77	82
I-	67	81	58	78	70
Total	173	195	131	155	152
Km allele frequency					
Km^1	0.3777	0.3555	0.3346	0.2906	0.3214

TABLE 1. Gm phenotype frequencies among the five populations in China

Gm phenotype	Inner Mongolia		Beijing		Anhui		Zhejiang		Guangzhou	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
a,z;g,u	40	37.7	36	35.4	22	22.7	24	18.5	7	5.1
a,z;b0,b3,b5,s,t,g,u	19	15.5	21	19.3	8	9.2	7	8.4	1	1.8
a,x,z;g,u	39	39.4	43	44.2	23	22.6	26	24.5	4	3.5
a,f,z;n;b0,b1,b3,b4,b5,u	29	36.7	38	39.9	38	35.8	30	40.9	37	40.5
a,x,z;b0,b3,b5,s,t,g,u	4	6.7	7	9.6	5	3.8	4	4.4	0	0.5
a,f,z;n;b0,b1,b3,b4,b5,s,t,u	8	7.6	11	10.8	9	7.2	9	9.3	9	7.3
a,z;b0,b3,b5,s,t	1	1.6	3	2.6	0	0.9	2	1.0	0	0.2
a,x,f,z;n;b0,b1,b3,b4,b5,g,u	19	15.8	24	19.9	13	14.7	20	21.5	12	12.1
a,f;n;b0,b1,b3,b4,b5,u	11	8.9	10	11.2	13	14.1	29	22.7	82	81.1
Total	170	170.0	193	193.0	131	131.0	151	151.0	152	152.0
χ^2	*4.98*		*2.03*		*1.63*		*7.35*		*1.68*	
d.f.	*5*		*5*		*4*		*4*		*3*	
p	*0.416*		*0.844*		*0.802*		*0.118*		*0.641*	

As compared these results with the data of Chinese populations collected in Taiwan and in Ann Arbor, Michigan in the States and classified as to the province of origin by Schanfield (1972), heterogeneity was found between Beijing and north region (included Shantung, Hopei, Liaoning and Shansi) by Schanfield (χ^2 =12.45 for 3 d.f., p=0.00), whereas the result of Zhejiang is in accord with the central region by Schanfield (χ^2 =1.21 for 3 d.f., p=0.75) and also Guangzhou with south region by Schanfield (χ^2 =3.06 for 3 d.f., p=0.38), respectively. As shown in the results, clear genocline changing in a regular fashion is observed, i.e., a regular decrease from north to south in the frequencies of Gm a,z;..;g,u and Gm a,z;..;b0,b3,b5,s,t and on the contrary, a regular and remarkable increase from north to south in the frequency of Gm a,f;n;b0,b1,b3,b4, b5,u.

The haplotype frequencies determined for the 2,360 samples in the 11 Japanese populations from the various districts reported up to that time were tested for heterogeneity. The tests gave χ^2 =9.21 for 20 d.f., 0.97 > p > 0.95 (Matsumoto et al., 1977). Clearly the data showed Japanese to be homogenous. Pairwise comparison of the Gm phenotypes of Japanese from Osaka (haplotype frequencies: Gm a,z;..;g,u = 0.4503, Gm a,x,z; ..;g,u = 0.1590, Gm a,z;..;b0,b3,b5,s,t = 0.2609, and Gm a,f;n;b0,b1, b3,b4,b5,u = 0.1297) and each of the five populations in China revealed the differences to be statistically significant. Japanese samples differs significantly from all of the five regional populations in China (χ^2 =57.22 for 3 d.f., p=0.000 for Inner Mongolia; χ^2 =58.36 for 3 d.f., p=0.000 for Beijing; χ^2 =86.27 for 3 d.f., p=0.000 for Anhui; χ^2 =136.28 for 3 d.f., p=0.000 for Zhejiang; χ^2 =509.29 for 3 d.f., p=0.000 for Guangzhou, respectively). Thus, the differences between Japanese and each of the regional populations in China become greater from northern population to southern population.

In contrast to the significant and regular variation in the Gm haplotypes Km allele frequencies do not show any significant variation among the five Chinese populations as shown in table 3.

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DISTRIBUTION OF Gm AND Km ALLOTYPES AMONG THE FIVE POPULATIONS IN THE PEOPLE'S REPUBLIC OF CHINA.

Hideo Matsumoto, Tokiko Miyazaki, Kotoyo Yamazaki, and Xiping Xu
(Dept. of Legal Medicine, Osaka Medical School, Takatsuki, Osaka, JAPAN)

INTRODUCTION

Inherited structural differences in human immunoglobulins are referred to as allotypes or genetic markers. So far, genetic markers have been found for the IgG heavy (H) chain (Gm), the IgA chain (Am), the IgE chain (Em) described recently by van Loghem et al. (1984), and the kappa type light chain (Km) common to all classes of immunoglobulins. The Gm system provides genetic markers which are unique in studies of human genetics, particularly in the characterization of different populations and in studies of gene flow and genetic drift determined by the presence of either a unique haplotype in a particular race or by differences in the frequencies of the same haplotypes in a given ethnic group.

This study of the five populations in Mainland China forms part of an extensive survey aimed at investigating the distribution of Gm and Km alleles among the Mongoloid populations acattered from Southeast Asia through East Asia into South America.

MATERIALS AND METHODS

Serum samples from a total of 806 unrelated individuals from five distinct regional populations in mainland China (173 from Inner Mongolia, 195 from Beijing, 131 from Anhui, 153 from Zhejiang and 152 from Guangzhou) were tested for G1m (a,x,f and z), G2m(n), G3m(b0,b1,b3,b4,b5, s,t, and u), and Km(1) allotypes. The reagents used for these tests and the methods were described previously (Matsumoto et al., 1979).

RESULTS AND DISCUSSION

Data of Gm phenotypes in five distinct Chinese populations are presented in table 1 and estimated frequencies of Gm haplotypes are presented in table 2. Haplotype frequencies and degree of fit with the Hardy-Weinberg distribution were determined using the computer program MAXIM. Nine to seven Gm phenotypes which are explained by the presence of four haplotypes, Gm a,z;..;g,u, Gm a,x,z;..;g,u, Gm a,z;..;b0,b3,b5,s,t and Gm a,f;n;b0,b1,b3,b4,b5,u, characteristic of Mongoloid populations were observed among these populations. Agreement was obtained for all five populations between the observed and the expected frequencies on the basis of the Hardy-Weinberg equilibrium of phenotypes.

To determine if significant heterogeneity in haplotypic distributions exists among the five Chinese populations, haplotype frequencies were analyzed using contingency chi-square test according to the methods of Snedecor (1956). Heterogeneities were not found between Inner Mongolia and Beijing (χ^2 =2.30 for 3 d.f., p=0.51) and also between Anhui and Zhejiang (χ^2=4.95 for 3 d.f., p=0.17). On the other hand, heterogeneities were observed between Inner Mongolia and Anhui (χ^2 =10.32 for 3 d.f., p=0.01), Inner Mongolia and Zhejiang (χ^2 =29.55 for 3 d.f., p=0.00), Inner Mongolia and Guangzhou (χ^2 =263.02 for 3 d.f., p=0.00), Beijing and Anhui (χ^2=9.62 for 3 d.f., p=0.02), Beijing and Zhejiang (χ^2 =25.13 for 3 d.f., p=0.00), Beijing and Guangzhou (χ^2 =262.88 for 3 d.f., p= 0.00), and between Zhejiang and Guangzhou (χ^2 =124.30 for 3 d.f., p= 0.00), respectively.

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8.85 %, 4. 9.53 %, 5. 13.26 %.
RhD positive of BG AB are towards AB RhD negative in the proportion: 1. 85.86 % : 14.14 %, 2. 82.62 % : 17.38 %, 3. 78.17 % : 21.83 %, 4. 88,89 % : 11.11 %, 5. 82.50 % : 17.50 %.
A_1B RhD positive towards A_1B RhD negative are in Maribor in a proportion of 83.87 % : 16.13 %, in the surroundings 86.05 % : 13.95 %, the small number of tested persons in the remaining groups (3 - 5) as well as the comparison of A_2B RhD positive and A_2B Rh D negative allow no credible comparison.
However, the above establishments and calculations are well comprised in our long years of observations of a relatively larger need for blood of groups B and AB during the summer vacation period, in the months with denser traffic, especially through Maribor, since also a significantly larger % of BG AB is present in group 5 and BG B in group 2 than in group 1.
The above data are surely interesting also in paternity affairs, for thex can be used for comparison, just as the data of other researchers are compared. They also prove the specific independence and roundedness regarding the comparing of BG and Rh individuals who come from certain geographical regions. For Yugoslavia as a whole it is not possible to present any uniform frequency of BG nor of the RhD factor

SUMMARY
In 12091 presons arranged into 5 groups (geographical arrangement acc. to birthplace) blood group (BG) O was ascertained in 31.8 %, BG A in 37.25 %, BG B in 21.14 %, BG AB in 9.80 %. A significantly larger % of BG AB is present in the east of the country and BG B in the surroudings of Maribor. The results have a particular value for the supplying of transfusion institutions with blood of certain BG during the summer vacation period, in the months of denser traffic and the influx of traffic victims in Maribor, but they are also interesting in paternity affairs.

ZUSAMMENFASSUNG
Bei 12091 Personen, in 5 Gruppen eingeteilt (geographische Einteilung nach Geburtsort), wurde die Blutgruppe (BG) O bei 31.81 % festgestellt, BG A bei 37.25 %, BG B bei 21.14%, BG AB bei 9.80 %. Ein signifikant grösserer % von BG AB besteht im Osten des Landes und von der BG B in der Umgebung von Maribor. Die Resultate haben einen praktishen Wert für die Versorgung der Transfusionsinstitution mit Blut gewisser BG während der Sommerferien, in den Monaten mit stärkerem Verkehr und Zufluss von Verunglückten in Maribor, sie sind aber auch für Vaterschaftsangelegenheiten interessant.

while BG AB was present in 9.8 % (AB Rh D positive were 83.39 %, negative 16.61%).
In those born in Maribor (1) BG O was present in 32.23 %, in the surrounding inhabitants (Maribor-surroundings) (2) in 27.83 %, in the remaining Slovenia (3) in 43.83 %, among Croatians (4) in 32.84 % and in the rest of Yugoslavia (5) in 30.42 %.
BG O RhD positive is arranged in rubrics:
1. 80.59 %, 2. 81.24 %, 3. 80.64 %, 4. 83.57 %, 5. 83.51 %, RhD negative: 1. 19.41 %, 2. 18.76 %, 3. 19.36 %, 4. 16.13%, 5. 16.49%.
In BG O considerable percentage deviations were observed in comparison to Maribor (1), its surroundings (2), the rest of Slovenia (3) and Yugoslavia (5).
The nomber of O RhD positive is larger among Croatians and the remaining Yugoslavs, which is also expressed in the smaller percentage of O RhD negative in the test regions 4. and 5.
BG A is arranged from 1. to 5. as follows: 1. 39.56 %, 2. 39.54 %, 3. 27.72 %, 4. 31.36 %, and 5. 32.20 %.
A RhD positive are in group 1. 83.57 %, 2. 81.61 %, 3. 84.14 %, 4. 85.14 % and 5. 89.94 %, thus A Rh negative are from 1. to 5.: 1. 16.43 %, 2. 18.39 %, 3. 15.86 %, 4. 14.86% and 5. 10.06 %.
An evident fall of A RhD negative was established geographically from west to east, while more RhD positive are present in the opposite direction.
Subgroups A_1 and A_2 are represented in the proportion:
1. 86.41 % : 13.59 %, 2. 83.48 % : 16.52 %, 3. 90 % : 10 %, 4. 83.13 % : 16.67 %, 5. 83.33 % : 16.67 %.
The proportion A_1 Rh D positive towards A_2 Rh D positive is:
1. 87.25 % : 12,75 %, 2. 83.92 % : 16.08 %, 3. 91.50 % : 8.50 %, 4. 88.89 % : 11.11 %, 5. 83.33 % : 16.67 %.
A_1 Rh D negative towards A_2 Rh D negative are in proportion:
1. 82.86 % : 17.14 %, 2. 80.65 % : 19.35 %, 3. 84.62 % : 15.38 %, 4. 66.67 % : 33.33 %.
The relationship A_1 RhD positive towards A_1 RhD negative is:
1. 81.76 % : 18.24 %, 2. 86.98 % : 13.02 %, 3. 79.63 : 20.37 %, 4. 80 % : 20 %.
A_2 RhD positive towards A_2 RhD negative: 1. 76 % : 24 %, 2. 84.21 % : 15.79 %, 3. 66.67% : 33,33 %, 4. 50 % : 50 %.
Oscillations which appear in comparing Rh D positive and RhD negative among subgroups A_1 and A_2 are on account of an ever smaller nuber from 1 to 5.
Group B is present in Maribor (1) in 18.62 %, in the surroundings (2) in 22.77 %, in Slovenia (3) in 19.60 %, in Croatia (4) in 26.27 % and in the rest of Yugoslavia (5) in 24.11 %, thus there is a considerable increase of BG B from west to east.
B RhD positive towards B RhD negative are in the proportion:
1. 82 % 18 %, 2. 79 % : 21 %, 3. 78.82 % : 21.48 %, 4. 79.84 % : 20.16 %, 5. 79.87 % : 20.13 %.
There are less positive from 1 - 5, Rh D negative are more in the opposite order.
BG AB is present in group 1. in 9.60 %, 2. in 9.87 %, 3.

SUPPLEMENT TO THE OBSERVED ARRANGEMENT OF BLOOD GROUPS "ABO" IN MARIBOR, SLOVENIA AND THE REPUBLICS AND PROVINCES OF YUGOSLAVIA

GLASER EDVARD Oddelek za transfuziologijo in imunohematologijo, Splošna bolnišnica Maribor, Jugoslavija

The good old classical arrangements of nations according to geographic regions with regard to the appertenance to this or that blood group practically almost have no more value, but also those which were actually published several times up to now have no real usable value because of the continually larger population migration. This is practically noticeable aspecially in applicable transfusiology in larger traffic accidents, in our very region along the highway leading from western Europe through Maribor and from our parts toward the east, when in the hospital the corresponding quantity of stored blood of certain blood groups from donors in our region ist at our disposal and can be counted on. Already some years back we noticed that especially in the summer months the need for stored blood of blood groups (BG) B and AB was larger than in the previous years. This lead to the study of BG Rh and others in donors in Maribor with regard to their appertenance to various nations and nationalities.

In this study blood donors from Maribor and its surroundings were included, i. e. a large area which exceeds the municipal boundaries of the town and includes certain characteristic ethnographic districts, by chance selection as well. A broader study, not published, includes besides BG and Rh D also the Rh phenotype (resp. the most likely genotypes) and M, N, S, s, P, K, Fy and other systems.

BG and Rh D will be shown in blood donors divided into five groups according to place of birth:

1. Maribor
2. Maribor - surroundings, where we also count some places respectively blood withdrawal centers from the neighbouring communities of Lenart, Slovenska Bistrica and Radlje ob Dravi which geographically actually terminate our region and have for decades belonged in the blood donation district of Maribor.
3. the remaining Slovenia
4. Croatia, which could especially in its bordering regions resemble the bordering parts of Slovenia in many ways,
5. the remaining republics: Bosnia and Hercegovina, Montenegro, Macedonia. Our reason for deciding on a collection of these republics in one rubric lies mainly in the relatively small number of tested persons - citizens of this or that republic - in relation to the rest, but also in that we truly cover simultaneously a larger geographic and cultural historical region.

12091 persons from all over Yugoslavia were tested. The presence of BG O was established in 31.81 %, of these 81.12 % were Rh D positive and 18.88 % were Rh D negative. BG A was present in 37.25 %, of these 83.06 % were Rh D positive and 16.94 % were Rh D negative, BG B was established in 21.14 % (Rh D positive sere 63.23 %, negative 36.77%),

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Enzyme activity was revealed using the staining procedure described by Sutton and Burgess (5).

RESULTS AND DISCUSSION

All ten phenotypes were observed during our study; their distribution is in good agreement with Hardy-Weinberg law. We were not able to find any rare variants.

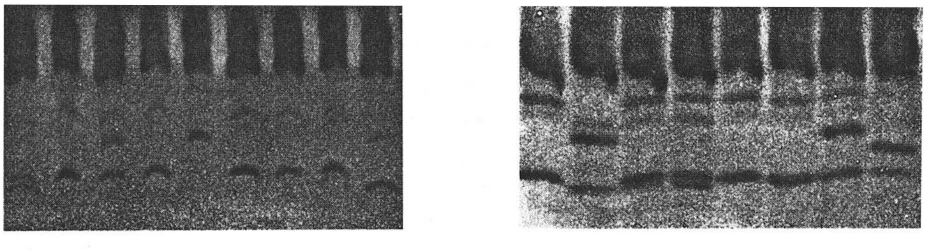
The distribution of phenotypes and the genic frequencies are shown in Table 1. The genic frequencies we have found are not different in the mean from those reported in former population samples of Italy (12) (15) (16).

In our experiment conditions we found the result that 0.3 mm thick gels are less expensive and more clearly defined than 1 mm thick ones but they present more frequently distorsions of the patterns (Fig. 1).

Table 1

Phenotypes	Observed n	Observed %	Expected n	Allele frequencies
1+1+	214	36.03	213.13	PGM_1^{1+} 0.599
1+1-	78	13.13	76.85	
1-1-	7	1.18	6.93	
1+2+	165	27.78	166.52	PGM_1^{1-} 0.108
1+2-	41	6.90	41.99	
1-2+	28	4.71	30.02	
1-2-	8	1.35	7.57	PGM_1^{2+} 0.234
2+2+	34	5.72	32.52	
2+2-	17	2.86	16.40	
2-2-	2	0.34	2.07	PGM_1^{2-} 0.059
Total	594	100.00	594.00	X^2 = 0.31066 df = 6; P > 0.99

Fig. 1



0.3 mm 1 mm

PGM_1 POLYMORPHISM IN THE POPULATION OF ANCONA BY ISOELECTRIC FOCUSING

A. Tagliabracci and R. Giorgetti

Istituto di Medicina Legale e delle Assicurazioni dell'Università di Ancona, Italy.

INTRODUCTION

The polymorphism of PGM_1 was first detected by Spencer et al. (1) in 1964 by conventional starch gel electrophoresis.

Using isoelectric focusing in polyacrylamide gel, Ishimoto and Kuwata (2) found more bands than are usually observed by conventional electrophoresis. Later studies of Bark et al. (3), Künhl et al. (4), Sutton and Burgess (5), Künhl and Spielmann (6), Welch et al. (7) showed that the more complex band patterns which were observed were due to the presence of four common alleles at the PGM_1 locus, called 1+, 1-, 2+, 2-, which have given rise to ten possible phenotypes.

Some rare variants have been found and studied recently by IEF (Scherz et al. (8), Sachs et al. (9), Dykes and Polesky (10), Dykes et al. (11), Bargagna and Abbagnale (12), Driesel et al. (13), Bär and Biedermann (14).

The high polymorphism of PGM_1 system displaied wide application in paternity testing.

The purpose of this work is to relate the distribution of PGM_1 genic frequencies in the population of Ancona.

MATERIALS AND METHODS

Hemolysates, prepared from packed red cells by freezing and thawing, were obtained from fresh blood samples of 594 unrelated healthy donors living in the district of Ancona.

IEF was performed in a multiphor LKB 2117 equipment connected to an LKB 2197 power supply. Polyacrylamide gels of various thickness (1 or 0.3 mm) were prepared with a concentration T = 5%, a degree of cross-linkage C = 3% and an ampholine concentration (pH range 4 - 6.5) (LKB) of 2.2% (w/v).Plate dimensions were 250 x 125 mm. 10 ul of hemolysate diluted 1:2 was applied on pieces of filter paper (5 x 7 mm) at 2 cm from the anode end. The electrode paper strips were soaked with 0.1 M glutamic acid/0.5 M H_3PO_4 for the anode and 0.1 M beta-alanine for the cathode, respectively.

Focusing was carried out for 190 min, removing paper after 75 min, with the following maximal conditions: 1200V, 35mA, 25W and T +8 ° C. For 0.3 mm slabs the conditions were 1500V, 35mA, and 5W.

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until recently for the study of this polymorphism. These methods include starch gel electrophoresis after protein purification or specific immunofixation and cross immunoelectrophoresis.

Using PAGIF followed by immunofixation, Arnaud and Gianazza(2) found that orosomucoid shows both microheterogeneity and polymorphism, the latter being under genetic control.

With the method described above we have observed that silver staining render the use of immunotechniques unnecessary.

The results of population and family studies are given in Tables 1 and 2.

The observed frequencies were 0.460 for the allele Or^1 and 0.540 for the allele Or^2. Good agreement was noted for the Hardy-Weinberg distribution, which is particularly important for the use of this marker in paternity testing since the Hardy-Weinberg equilibrium of the orosomucoid seems to be greatly disturbed in the Japanese group. Furthermore, investigation of 115 families with 203 children support the assumed autosomal codominant way of inheritance (Table 2).

With a theoretical chance of exclusion of non-fathers 18.7% in our population, Or polymorphism typed by PAGIF and silver staining becomes a useful marker in paternity testing.

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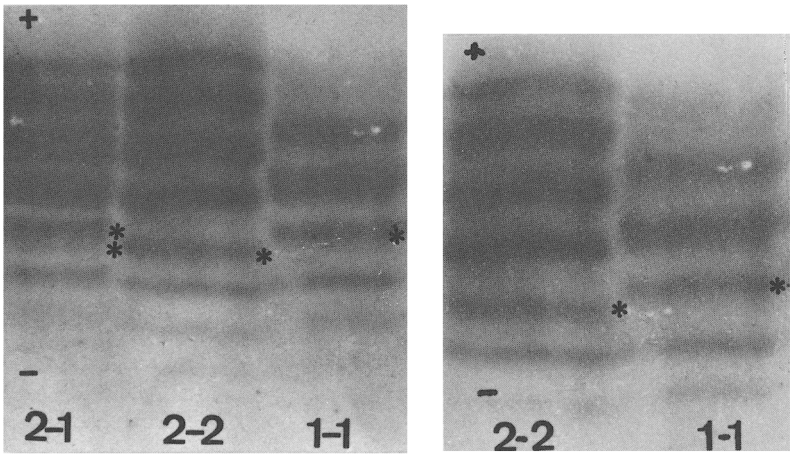

Fig. 1-2 Orosomucoid variants after PAGIF pH 2.5-4 and silver staining.

Table 1. Frequencies of Or phenotypes and Or alleles in Galicia

Phenotype	Observed	Expected	
1-1	134	137.5	
2-1	330	322.9	Or^1:0.460
2-2	186	189.5	Or^2:0.540
	650	649.9	χ^2=0.292
			p>0.50

Table 2. Segregation of Or phenotypes in 115 families with 203 children

	nF	1-1	2-1	2-2
1 X 1	4	7		
1 X 2-1	29	20(22)	24(22)	
2-1 X 2-1	30	13(14)	29(28)	14(14)
2 X 2-1	34		35(33)	31(33)
2 X 2	6			10
1 X 2	12		20	

3 MM filter papers (1 x 1 cm). Focusing was carried out at 15 W constant power, current unlimited. A maximum voltage of 2000 V and a cooling temperature of 10°C were used.

A focusing time of 3.5 hours was needed.

After isoelectric focusing the gels were stained with the silver staining method of Carracedo et al. (3) with some modifications:

First they were prefixed in 12% trichloroacetic acid for 15 min and washed three times for 20 min in 200 ml of 50% ethanol at 50°C in a shaking water bath to remove ampholytes, and then fixed in 100 ml of 10% glutaraldehyde for 20 min. The gels were then washed four times for 15 min in 200 ml of distilled water. They were then soaked in 100 ml of 0.01% dithiothreitol and the treated for 30 min in 100 ml of 0.1% silver nitrate. The gels were then given two rinses. First in 100 ml of distilled water and then with a small amount of developer (75 l of 37% formaldehyde in 150 ml of 3% sodium carbonate) until enough contrast in the bands was obtained.

The staining was stopped by adding 10 ml of 2M citric acid and shaken for 5 min. The gels can also be wrapped in protective celophane sheets, being stable the colours for several months.

Results and discussion

Figures 1 and 2 demonstrate the Or phenotypes as observed with the method described. A clear distinction between phenotypes was found.

The first studies about the electrophoretic pattern of serum orosomucoid were carried out by Schmid and Binette (4) in 1961 using starch gel electrophoresis.

Later (5) it was found that when the sialic acid residues had been removed from the protein, one of three alternative patterns (designated I, II and III) were observed. Once the Or polymorphism had been further elucidated by general population and family studies, the phenotypes were designated FF, SS and FF.

At present, orosomucoid is not habitually used in paternity testing, due to technical difficulties in the methods used

POLYMORPHISM OF SERUM OROSOMUCOID. FAMILY AND POPULATION STUDIES IN GALICIA.

Angel Carracedo, Luis Concheiro, Javier Estefanía and Carlos Suárez (Dep. of Legal Medicine, Faculty of Medicine. Santiago de Compostela. Spain).

Introduction

Alpha-1-acid glycoprotein or orosomucoid (Or) is one of the most extremely studied plasma proteins and it is characterized by an exceptionally high carbohydrate content and a large number of syalic residues (12%) together with an extremely acidic isoelectric point (around 2.5-3).

The current state of research into this protein has been reviewed by Schmid (1) and Arnaud and Gianazza (2).

At present this protein is not habitually used in paternity testing and bloodstain analysis due to the technical difficulties involved in the methods used until recently for the study of this polymorphism.

In this paper we present a simple method for Or typing by PAGIF followed silver staining together with the results of population and family studies in 650 individuals and 115 families from Galicia (NW Spain).

Material and Methods

Serum from freshly collected blood samples from 650 healthy donors and 115 families was used.

Samples were stored at -30°C and pretreated with fresnly Clelland's reagent (0.05 M dithiothreitol) before typing.

PAGIF was carried out in 0.4 mm thin-layer polyacrylamide gels at a gel concentration of T=5.5% and cross-linking of C=3%. Ampholine (pH 2.5-4) concentration was 5%.

Sucrose was added as a stabilizing agent at a final concentration of 12% (w/v).

Polymerization was carried out with ultraviolet light and a 0.02% riboflavin solution (20 mg in 100 ml of distilled water).

The electrode solutions were 0.7% ethanolamine for the cathode and 1M phosphoric acid for the anode.

Samples were applied 2 cm from the cathode using Whatman

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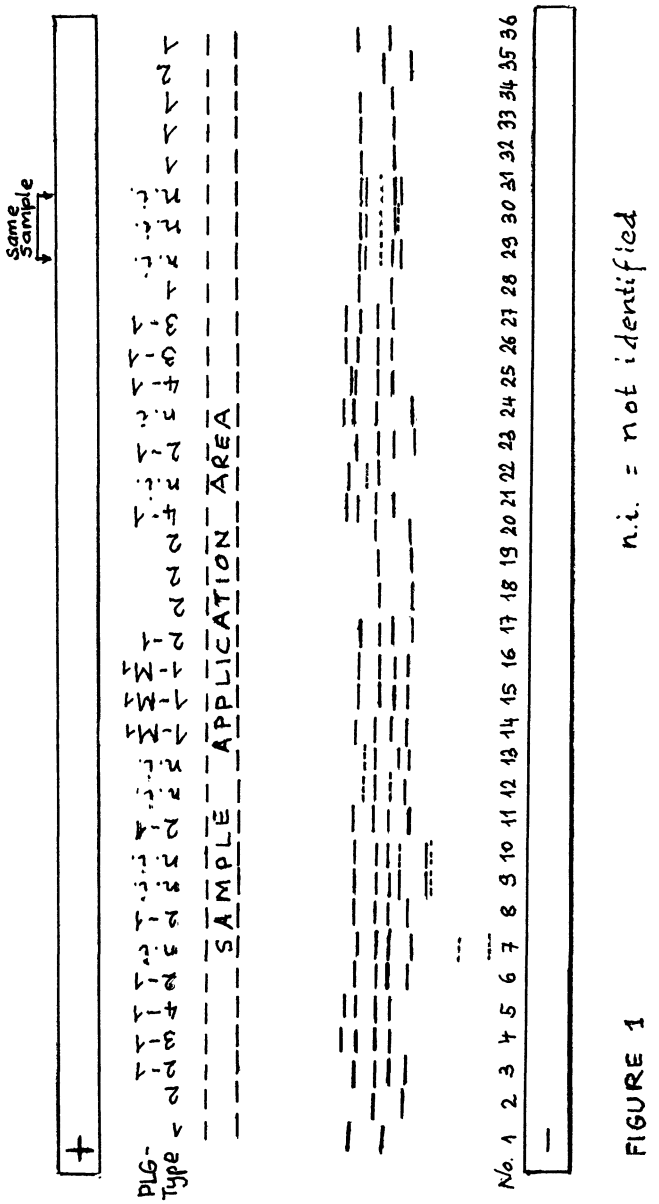

FIGURE 1

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ACKNOWLEDGEMENT

RARE PHENOTYPES WERE KINDLY IDENTIFIED BY DR. S. WEIDINGER, UNIVERSITY OF MUNICH

TABLE 1 DISTRIBUTION OF PLG PHENOTYPES AND ALLELES IN THE SWISS POPULATION

PHENOTYPE	OBSERVED N	OBSERVED %	EXPECTED N	EXPECTED %	χ^2
1	616	44.25	610.03	43.83	0.0548
2-1	544	39.08	554.76	39.85	0.2087
3-1	58	4.17	59.58	4.28	0.0419
2	131	9.41	126.12	9.06	0.1888
3-2	28	2.01	27.09	1.95	0.0306
3	2	0.14	1.45	0.10	0.2086
1-M1	9	0.65	8.61	0.62	0.0177
2-M1	4	0.29	3.91	0.28	0.0021
3-M1	0	0	0.42	0.03	-
M1	0	0	0.03	0.00	-
TOTAL	1392	100.00	1392.00	100.00	0.7568
RARE, NOT IDENTIFIED	6	0.43	-	-	-

DF = 7 $\quad 0.995 < P < 0.999$

ALLELE FREQUENCIES: $PLG^1 = 0.662$; $PLG^2 = 0.301$; $PLG^3 = 0.032$; $PLG^{M1} = 0.004$

TABLE 2 PLG CONCENTRATIONS IN CASES SUSPECT FOR THE PRESENCE OF A SILENT ALLELE

CASE		PHENOTYPE	CONCENTRATION (MG/L)
A	MOTHER	2	61
	CHILD	1	40
	PRESUMPTIVE FATHER	1	86
B	MOTHER	1	126
	CHILD	2	156
	PRESUMPTIVE FATHER	2-1	34
C	MOTHER	2	25
	CHILD	1	99
	PRESUMPTIVE FATHER	2-1	143
D	MOTHER	2	71
	TWIN CHILD 1	1	44
	TWIN CHILD 2	1	56
	PRESUMPTIVE FATHER	2-1	42
E	MOTHER	2	64
	CHILD	1	56
	PRESUMPTIVE FATHER	2-1	112
F	MOTHER	2-1	135
	CHILD	2	39
	PRESUMPTIVE FATHER	1	51
NORMAL RANGE: N = 154; 2 S LIMITS			80 - 150

GENETIC POLYMORPHISM OF PLASMINOGEN IN THE SWISS POPULATION

R.SCHERZ, R. ROHNER, R. PFLUGSHAUPT, AND R. BÜTLER
BLOOD TRANSFUSION SERVICE SRC, CENTRAL LABORATORY, BERN, SWITZERLAND

MATERIALS AND METHOD

SERUM OR EDTA-PLASMA CAME FROM BLODD DONORS, LABORATORY PERSONNEL AND PEOPLE ENGAGED IN CASES OF DISPUTED PATERNITY.

THE ISOELECTIC FOCUSING METHOD COMBINED WITH THE DETECTION OF FUNCTIONAL PLG ACTIVITY ACCORDING TO HOFFMANN (1) WAS USED WITH MINOR MODIFICATIONS.

IEF WAS PERFORMED ON 1 MM THICK POLYACRYLAMIDE GELS OF THE STANDARD LKB-SIZE (11x25 CM). ACRYLAMIDE CONCENTRATION WAS 5.5%, CROSS LINKER (BIS) WAS 3.1% REL.

CONCENTRATION OF AMPHOLINE pH RANGE 5-8 WAS 1.86%, AND AMPHOLINE pH RANGE 5-7 0,6%, RESPECTIVELY.

SAMPLES WERE PRETREATED WITH NEURAMINIDASE:
10 µL OF SERUM OR EDTA-PLASMA WERE MIXED WITH 10 µL OF NEURAMINIDASE TYPE V (FROM CLOSTRIDIUM PERFRINGENS, SIGMA NO. N-2876, 10 UNITS/ML IN SODIUM ACETATE BUFFER 0.2 M, pH 5.5). AFTER INCUBATION OVER NIGHT 12 µL OF THE MIXTURES WERE APPLIED ON WHATMAN NO. 1 FILTER PAPER (5x9 MM) 2 CM FROM THE ANODE ON THE PREFOCUSED GEL.

PREFOCUSING CONDITIONS: MAXIMAL SETTINGS ON POWER SUPPLY WERE AS FOLLOWS: 1200 V, 20 mA, 15 W. TIME: 30 MINUTES. FOCUSING WAS PERFORMED UNDER THE SAME CONDITIONS DURING 3 HOURS.

DETECTION OF PLG BANDS:
1.4 ML OF A SOLUTION OF UROKINASE (CALBIOCHEM NO. 672123) IN PBS CORRESPONDING TO 150 PLOUG UNITS PER PLATE WAS SPREAD OVER THE FOCUSED PLATE, USING A GLASS ROD. AFTER 5 MINUTES OF INCUBATION AT ROOM TEMPERATURE THE PLATE WAS OVERLAID WITH A PRECASTED GEL OF 0,6% AGAROSE (SEAKEM ME) CONTAINING 1% OF CASEIN (ACCORDING TO HAMMARSTEN, SIEGRIED NO. 119360).

AFTER INCUBATION FOR 2 1/2 HOURS AT 37°C THE OVERLAY WAS REMOVED, AND THE POLYACRYLAMIDE PLATE WAS FIXED IN A MIXTURE OF TRICHLOROACETIC ACID (11,5%)/SULPHOSALYCILIC ACID (5%) AND THEN STAINED WITH SERVA VIOLET 49 (0.07% SERVA VIOLET 49, 25% METHANOL, 10% ACETIC ACID).

AFTER DESTAINING WITH THE SAME SOLVENT, THE PLG BANDS APPEAR AS COLORLESS ZONES ON BLUE-VIOLET BACKGROUND.

DETERMINATION OF PLASMINOGEN CONTENTS WAS PERFORMED BY LASER NEPHELOMETRY ACCORDING TO THE HYLAND PROCEDURE. EITHER SERUM OR EDTA-PLASMA AFTER CLOTTING BY ADDITION OF THROMBINE WERE USED.

RESULTS AND DISCUSSION

OF A TOTAL OF 1398 SAMPLES ALL BUT 6 COULD READILY BE TYPED.

PHENOTYPE AND GENE FREQUENCIES ARE SHOWN IN TABLE 1. THE DISTRIBUTION IS IN EXCELLENT AGREEMENT WITH THE HARDY-WEINBERG LAW, AND IT COMPARES WELL WITH DATA OBSERVED IN OTHER WHITE POPULATIONS (2).

FROM THESE DATA WE CALCULATE AN AVERAGE CHANCE OF EXCLUSION (3) IN PATERNITY TESTING OF 21.9% (WITHOUT CONSIDERING THE RATHER RARE PLG^{M1} AND OTHER RARE ALLELES). THE PLG SYSTEM THEREFORE REPRESENTS A VERY INFORMATIVE PARAMETER FOR THE APPLICATION IN THIS FIELD. AMONG THE 6 SAMPLES WHICH COULD NOT YET BE IDENTIFIED, MAINLY DUE TO LACK OF REFERENCE SAMPLES OR PROBLEMS OF NOMENCLATURE, THERE MUST BE SOME RATHER RARE ALLELES. PATTERNS ARE SHOWN IN FIGURE 1, TOGETHER WITH REGULAR TYPES AND SOME RARE VARIANTS WE ENCOUNTERED BESIDE OF THIS POPULATION STUDY (NUMBERS 7, 9, 10, 12, 13, 22, 24, 29, 30, 31).

AMONG 385 MOTHER/CHILD PAIRS FROM OUR ROUTINE PATERNITY TESTING THERE WERE NO "IMPOSSIBLE" PHENOTYPE COMBINATIONS, EXCEPT FOR 5 PAIRS WITH OPPOSITE HOMOZYGOSITY WHICH WOULD SUGGEST THE PRESENCE OF A SILENT PLG ALLELE (TABLE 2). IN ADDITION WE OBSERVED THIS PHENOMENON IN A CHILD AND THE PRESUMPTIVE FATHER WHO COULD NOT BE EXCLUDED FROM PATERNITY BY ANY OF THE OTHER MARKERS, INCLUDED HLA (PROBABILITY OF PATERNITY = 99.86%).

IN THESE CASES WE DETERMINED THE PLASMINOGEN CONTENT IN ORDER TO SUPPORT THE EVIDENCE FOR THE PRESENCE OF A "NULL" ALLELE.

NO DIFFERENCES OF NORMAL VALUES BETWEEN THE COMMON PHENOTYPES COULD BE OBSERVED (N = 10 FOR EACH GROUP). DATA OF TABLE 2 ARE SOMEWHAT DIFFICULT TO INTERPRET. WHILE IN CASES A, E, AND F THE THEORY OF THE EXISTENCE OF A SILENT GENE IS SUPPORTED BY THE CONCENTRATION LEVEL, THIS IS HARDLY OR NOT AT ALL SO IN THE OTHER CASES. NO FURTHER FAMILY DATA ARE AVAILABLE UNTIL NOW.

UNDER THIS ASPECT A CERTAIN PRECAUTION SEEMS TO BE NECESSARY IN THE INTERPRETATION OF OPPOSITE HOMOZYGOSITIES IN CASES OF DISPUTED PATERNITIES.

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PHENOTYPES		OBSERVED	%	EXPECTED	X^2
PLG 1		936	70.64150	935.76026	0.000061
PLG 2		33	2.49057	33.12480	0.000470
PLG 2-1		352	26.56604	352.11797	0.000039
PLG 1-V	3		0.22642		
PLG 2-V	1	4	0.07547	3.99697	0.000002
PLG V-V	0		0.00000		
		1325	100.00000	1325.00000	0.000572

Gene frequencies PLG 1 = 0.84038

PLG 2 = 0.15811

PLG V = 0.00151

X^2 = 0.000572. For 3 degrees of freedom $0.99 < P$.

Distribution of PLG phenotypes in 35 mother-child pairs:

MOTHERS		CHILDRENS			
		1	2	2-1	n
1	12	11	-	1	12
2	15	-	11	4	15
2-1	8	1	3	4	8
n	35				35

PREFOCUSING	60 min
FOCUSING	60 min with samples
	60 min without samples

RESULTS :

The pattern of PLG phenotypes are shown in Figure 1.

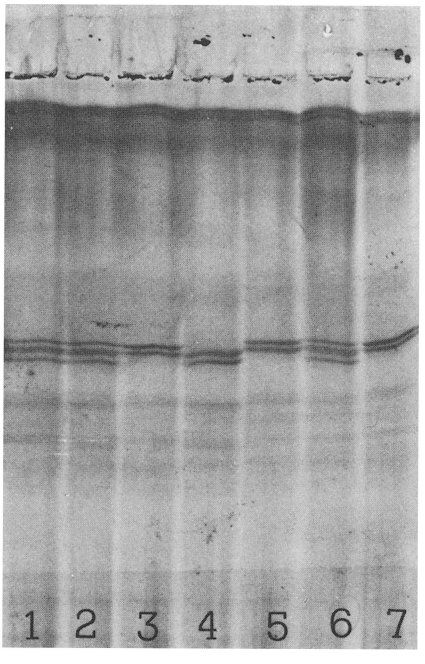

Fig. 1: (anode on the top) 1, 2 and 6 PLG 2-1
3, 5 and 7 PLG 1
4 PLG 2

The distribution of PLG phenotypes and gene frequencies in the Veneto population are reported as follows:

THE POLYMORPHISM OF PLASMINOGEN (PLG) IN THE POPULATION OF VENETO (ITALY) BY ISOELECTRIC FOCUSING.

P.Cortivo[1], L.Caenazzo[1], C.Crestani[1], C.Scorretti[1], P.Benciolini[1] and E.Pornaro[2].

1 Istituto di Medicina Legale dell'Università di Padova
Via Falloppio 50 35100 PADOVA (ITALY).

2 Servizio di Immunoematologia e Trasfusionale U.S.L. 21 PADOVA.

The distribution of PLG phenotypes in the Veneto population was investigated by ultrathin-layer isoelectric focusing.

MATERIAL: sera from 1325 unrelated subjects and 35 mother-child pairs provided by the Transfusion Centre of the Civil Hospital of Padua

METHOD:

Gel concentration T: 5%

Degree of cross-linkage C: 3.2%

Gel dimensions: 250x120x0.2 mm

Gel composition: Acrylamide 5% (w/v)
Ampholine pH 5-8 3%
Sucrose 12% (w/v)
Ammonium Persulfate 0.05% (w/v)

Electrodic solutios: 0.25 M HEPES (anolyte)
0.25 M Arginine + 0.25 M Histidine in Ethylendiamine 12% (catholyte)

Conditions: 1800 V 11mA 3W 6°C

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component of human complement (C8). J Clin Invest 72: 1526-1531
Kolb WP and Müller-Eberhard HJ (1976) The membrane attack mechanism of complement: The three polypeptide chain structure of the eighth component (C8). J Exp Med 143: 1131-1139
Nakamura S et al.(1984) Genetic polymorphism of the seventh component of complement in a Japanese population. Hum Genet 66: 279-281
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Steckel EW et al.(1980) The eighth component of human complement: Purification and physicochemical characterization of its unusual subunit structure. J Biol Chem 255: 11997-12005
Tedesco F et al.(1983) Two types of dysfunctional eighth component of complement (C8) molecules in C8 deficiency in man. J Clin Invest 71: 183-191

The treatment of EDTA plasma samples with neuraminidase reduced the heterogeneity of C81 types to one or two major and some minor components, and made it easy and clear to classify these types. Especially, it was easy to differentiate C81A1J component from C81A or C81B by using our method with neuraminidase treated plasma samples.

Family studies including 45 matings with 54 offspring were done to test the genetic hypothesis. It was confirmed that the C81 alleles were controlled by autosomal codominant Mendelian inheritance consisting of a single locus. No evidence of a null allele was found by studying these 45 families, and unexpected types were not observed in the children.

Table 1. Distribution of phenotypes and gene frequencies of human C81 among unrelated Japanese blood donors.

Phenotypes	Observed no.	Percent	Expected no.	x^2
A	174	38.8	173.77	0.000
AB	205	45.8	204.91	0.000
B	60	13.4	60.41	0.003
A1JA	4	0.9	4.35	0.029
A1JB	3	0.7	2.57	0.073
A2A	1	0.2	0.61	0.243
B1B	1	0.2	0.36	1.125
Others	0	0.0	1.02	1.022
Total	448	100.0	448.00	2.495

C81*A =0.6228 C81*A2=0.0011 0.5<P<0.7
C81*B =0.3672 C81*B1=0.0011 (df=4)
C81*A1J=0.0078

Distribution of phenotypes and gene frequencies of C81 in the Japanese population are presented in Table 1. The gene frequencies were estimated to be 0.6228, 0.3672, 0.0078 0.0011 and 0.0011 for C81*A, C81*B, C81*A1J, C81*A2 and C81*B1, respectively. The distribution of phenotypes fitted the Hardy-Weinberg equilibrium. The C81*A was the most common allele in the Japanese population, and the gene frequencies of two common alleles, C81*A and C81*B, agreed approximately with other ethnic groups.

A single exclusion chance for putative fathers in paternity cases was calculated as 19.3 %, so it was indicated that the C81 was the useful genetic marker in paternity test.

The presented data suggest the applicability of C81 polymorphism in forensic science, human genetics and anthropologic studies.

References

Alper CA et al.(1983) Genetic polymorphism in C8 β-chains: Evidence for two unlinked genetic loci for the eighth

types, the rabbit mono-specific anti human C81 serum which were a gift of Dr. K. Yamamoto, Kanazawa University School of Medicine and a peroxidase conjugated anti rabbit immunoglobulin.

Results and discussion

Phenotypes of C81 detected in the Japanese population were classified into three common and four rare patterns, and these were considered to be controlled by two common alleles, C81*A and C81*B, and three rare alleles which were tentatively designated C81*A1J, C81*A2 for acidic variants and C81*B1 for basic variant. So each typical type were designated to be C81A, C81AB, C81B, C81A1JA, C81A1JB, C81A2A and C81B1B, respectively. In this study, 3.1 M urea was added in the PAGIEF gels in order to obtain the clear-cut bands of C81 phenotypes (Alper et al.1983). C81A1JA and C81A1JB are similar to C81A and C81B, respectively, but these types have the same bands in the more anodal region than C81A. C81A2A has three major bands and one anodal and cathodal minor band, and C81B1B has C81B components and also some more cathodal bands than C81B and its major band corresponded in position to the minor cathodal band of C81B. C81 A2A and C81B1B were new rare variants detected in the Japanese population. Although C81A1JA and C81A1JB which were expressed by two common alleles and C81*A1J allele were also rare variants detected in the Japanese population, but these variants might correspond to C81A1A and C81A1B in the previous report (Raum et al.1979; Rittner et al.1984). Because the major cathodal band of C81A1J component roughly corresponded in position to the minor anodal band of C81A in the same manner as C81A1. Neuraminidase treated EDTA plasma samples from the same individuals with typical types were subjected to PAGIEF followed by an electroblotting with a mono-specific anti C81 serum, and a schematic diagram of these desialylized band patterns are shown in figure 1. But only major bands of each typical type were presented in this figure.

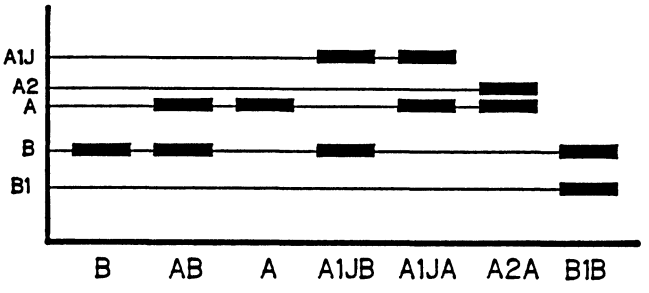

Figure 1. Schematic diagram of C81 phenotypes using PAGIEF of neuraminidase treated EDTA plasma samples followed by an electroblotting technique.

GENETIC POLYMORPHISM OF HUMAN COMPLEMENT COMPONENT C81 IN THE JAPANESE POPULATION.

Shigeki Nakamura, Katsunori Akiyama and Kazue Abe
(Department of Legal Medicine, Tokyo Women's Medical College, Tokyo 162, Japan)

The eighth component of human complement C8 has a three chain structure composed of two subunits, α - γ and β subunits which are bound together by noncovalent bonds (Kolb and Müller-Eberhard 1976; Stechel et al.1980).

Genetic polymorphism of the C8 was first described by Raum et al.(1979), by using polyacrylamide gel isoelectric focusing (PAGIEF) of serum samples with a hemolytic assay containing a homozygous C8-deficient human serum (Petersen et al.1976) for development of patterns. Alper et al.(1983) demonstrated another C8 polymorphism which was defined by PAGIEF and development of specific patterns of hemolysis in an overlay gel containing C8 β subunit deficient serum and locus for C8 β subunit has been designated C82 with the alleles C82*A, C82*B and C82*A1. Recently, Tedesco et al. (1983) indicated that the C8-deficient serum which used for the detection of C8 polymorphism (Raum et al.1979) was characterized by the deficiency of the C8 α - γ subunit. Therefore, the locus for C8 α - γ subunit has been redesignated C81.

In the present investigation, the distribution of phenotypes and gene frequencies of C81 in the Japanese population are reported using PAGIEF followed by an electroblotting and enzymeimmunoassay with mono-specific anti C81 serum.

Materials and methods

Blood samples obtained from 448 unrelated healthy Japanese donors and 45 matings with 54 offspring were drawn onto EDTA (1.5mg/ml) as anticoagulants, and centrifuged at 2,500 rpm for 10 min to prepare the plasma.

Half millimeter thin layer polyacrylamide gel (T=5%, C= 3%) were prepared containing 3.1 M urea and 2.8 % Ampholine pH 3.5-9.5. 10 microliters of EDTA plasma were applied to the gel surface with Whatman 3 MM filter paper (5X5 mm) at a distance of 1.5 cm from the anodal end of the gel. 1.0 M H_3PO_4 (anode) and 1.0 M NaOH (cathode) were used for the electrode solution. IEF was carried out at a constant power of 10 W, maximally voltage of 1,000 V for 3 hours including the prefocusing without samples for 40 min. All experiment were conducted at 4°C.

Subsequent to the separation by IEF, transfer of protein onto a nitrocellulose membrane (0.45 μm), as previously described by Nakamura et al.(1984), was carried out for 50 min in the electrode buffer consisting 25 mM Tris-192 mM glycine and 20 % methanol, pH 8.3 at 400 mA with a cooling temperature of 4°C. For the identification of C81 pheno-

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TABLE 3: C3 gene frequencies in different parts of Italy

	n	S	F	Rare	ref
Tuscany	650	.803	.195	.002	-
Abruzzo	252	.817	.179	.004	4
Lazio	325	.791	.203	.006	4

TABLE 4: Bf gene frequencies in different parts of Italy

	n	S	F	S0.7	F1	ref
North-Western	191	.746	.237	.010	.007	3
North-Eastern	128	.785	.180	.016	.019	3
Central	123	.679	.280	.033	.008	3
Tuscany	1000	.713	.250	.024	.013	-
Southern	165	.715	.258	.018	.009	3
Sicily	79	.728	.228	.019	.025	3
Sardinia	139	.446	.295	.043	.216	3
Sardinia	217	.578	.219	.005	.198	1
Italy	62	.718	.250	.024	.008	5

TABLE 1: Bf distribution in Tuscany

phenotypes	observed		expected	
	n	%	n	%
S	518	51.8	508.37	50.84
F-S	340	34.0	355.79	35.58
F	68	6.8	62.25	6.23
S-S0.7	37	3.7	34.94	3.49
S-F1	13	1.3	18.54	1.85
F-S0.7	12	1.2	12.22	1.22
F-F1	11	1.1	6.49	0.65
Others	1	0.1	1.40	0.14
	1000	100.0	1000.00	100.00

Bf*S .7130 Bf*F .2495 Bf*S0.7 .0245 Bf*F1 .0130

Chi square 6.443 for 4 df (P .20-.10)

TABLE 2: C3 distribution in Tuscany

phenotypes	observed		expected	
	n	%	n	%
S	418	64.31	419.23	64.50
F-S	206	31.69	204.00	31.38
F	24	3.69	24.82	3.82
Others	2	0.31	1.95	0.30
	650	100.00	650.00	100.00

C3*S .8031 C3*F .1954 C3*Rare .0015

Chi square 0.0514 for 1 df (P .90-.80)

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DISTRIBUTION OF BF AND C3 TYPES IN TUSCANY (ITALY)

Ranieri Domenici, Alessandro Giari and Marino Bargagna (Istituto di Medicina Legale e delle Assicurazioni dell' Università di Pisa, Via Roma 55, 56100 Pisa, ITALY)

Bf and C3 serum polymorphisms have not been extensively investigated until now in Italy and they are not routinely used in paternity testing in our Country.

This paper is a contribution to a better knowledge of distribution of these system in continental Italy.

Material and methods

Serum samples were obtained, respectively, from 1000 and 650 unrelated healthy blood donors from Pisa Hospital Blood Bank (Tuscany, Italy). The specimens were subjected to C3 and Bf type determination within 2-3 days.

The phenotypes were determined by means of high voltage electrophoresis, following the methods described by Teisberg (1970) for C3 and by Dykes and Polesky (1976) for Bf, with minor modifications.

Results and discussion

Tables 1 and 2 show the distribution of Bf and C3 phenotypes, respectively, among 1000 and 650 individuals from Tuscany. The gene frequencies are the following: C3*S .8031, C3*F .1954, C3*Rare .0015; Bf*S .7130, Bf*F .2495, Bf*SO.7 .0245, Bf.F1 .0130. There is a good agreement between the observed and the expected frequencies of phenotypes, assuming Hardy- Weinberg equilibrium.

No significant difference between Tuscan and other Italian populations (Scacchi et al., 1979) were found, with regard to C3 system (table 3). On the contrary, a highly significant difference exists between continental Italy (included Tuscany) and Sardinia (table 4), as previously reported by Malavasi et al. (1981) and by Davrinche et al. (1984).

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FIGURE 1. F13A phenotypes observed by AGIF and NIB. The cathode is on top. From left to right, F13A 1, 1, 4-1, 3-1, 2, 2-1, 1, 1.

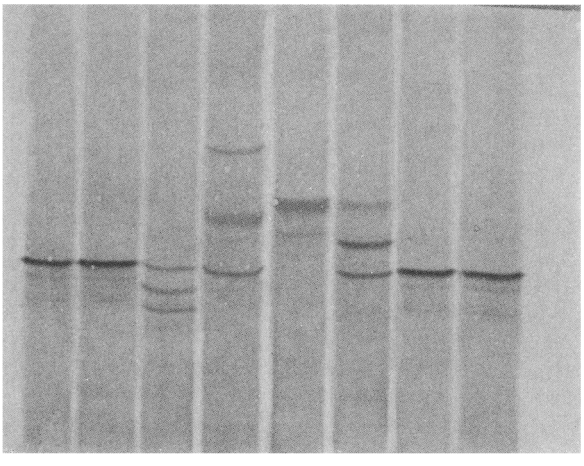

FIGURE 2. F13B phenotypes seen by AGIF followed by immunoblotting. From left to right F13B 3-2, 2-1, 3-1, 2, 3, 3-1, 1. The cathode is on top.

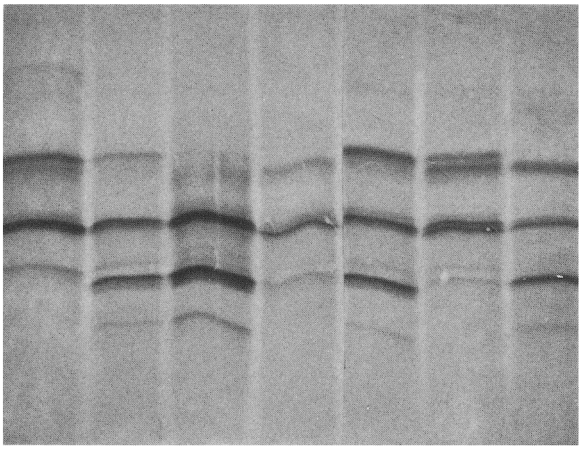

TABLE 4 PHENOTYPE AND GENE FREQUENCY DISTRIBUTION FOR F13B in FOUR US POPULATIONS

		PHENOTYPES						GENE FREQUENCIES		
Population	No.	1	2-1	3-1	2	3-2	3	F13B*1	F13B*2	F13B*3
White	328	195	47	72	3	5	6	0.776	0.088	0.136
Black	229	23	85	12	82	27	-	0.312	0.603	0.085
Mexican Amer.	47	10	6	20	1	3	7	0.489	0.117	0.394
Amer. Indian	129	32	2	66	-	6	23	0.512	0.031	0.457

White	$\Sigma\chi^2$	= 1.36	0.80 <p< 0.50	(df3)
Black	$\Sigma\chi^2$	= 2.23	0.80 <p< 0.50	(df3)
Mexican Amer.	$\Sigma\chi^2$	= 1.02	0.80 <p< 0.50	(df3)
Amer. Indians	$\Sigma\chi^2$	= 3.89	0.50 <p< 0.20	(df3)

TABLE 3

PHENOTYPE AND GENE FREQUENCY DISTRIBUTION
FOR F13A IN FOUR U.S. POPULATIONS

		PHENOTYPES				GENE FREQUENCIES		
Population	No.	1	2-1	4-1	2	F13A*	F13A*2	F13A*4
White	873	519	309	2	43	0.773	0.226	0.011
Black	148	92	52	-	4	0.797	0.203	-
Mexican Amer.	85	60	20	-	5	0.823	0.176	-
Amerindians	100	80	17	-	3	0.885	0.115	-

White	$\Sigma\chi^2$	= 0.717	0.95	<p< 0.80	(df3)
Black	$\Sigma\chi^2$	= 1.12	0.80	<p< 0.50	(df3)
Mexican Amer.	$\Sigma\chi^2$	= 3.08	0.50	<p< 0.20	(df3)
Amerindians	$\Sigma\chi^2$	= 2.72	0.50	<p< 0.20	(df3)

TABLE 2 IMMUNOBLOTTING TECHNIQUE

1. Passively blot to nitrocellulose (NC), 15 x 20 cm, presoaked in TBS (20mM Tris-HCl, pH 7.5, 0.5M NaCl). Place NC on gel, cover with piece of filter paper presoaked in TBS and 1cm of paper towels and blot for 30 minutes.

2. Air dry overnight or vacuum dry.

3. Block membrane 1 hour in TBS, 3% Hi Pure Gelatin, 0.05% tween -20.

4. To membrane add 10-15ml of 1:500 dilution antibody 1 (rabbit anti-human F13A or F13B) in wash buffer (TBS, 1% Hi Pure Gelatin, 0.05% tween -20). Gently agitate for one hour at room temperature.

5. Wash membrane 3 X 10 minutes in wash buffer.

6. To membrane add 10-15 ml of 1:1000-3000 dilution antibody 2 (HRP conjugated goat anti-rabbit IgG) in wash buffer. Gently agitate for 1 hour at room temperature.

7. Wash membrane 3 X 5 minutes in TBS.

8. Place membrane in dish with substrate mixture of 100 ml TBS, 10gm of 3,3' diaminobenzidine (tetrahydrochloride) and 200ul of 3% H202. Place dish in dark and use gentle agitation for 10-20 minutes.

9. Wash membrane briefly in tap water, and blot dry.

TABLE 1 IEF TECHNIQUE FOR F13A and F13B USING 0.5mm AGAROSE CELLS

	F13A	F13B
Agarose IEF (Pharmacia)	0.225 gm	0.225 gm
Sucrose	2.7 gm	2.7 gm
LKB Ampholyte 5-7	1.0 ml	0.8 ml
LKB Ampholyte 4-6.5	0.6 ml	0.8 ml
Electrolyte strips		
Cathode	0.2N NaOH	0.5 M glycine
Anode	1M H_3PO_4	0.025 M aspartic acid 0.025 M glutamic acid
Sample Application with Whatman # 1 filter paper	4 X 4mm wick soaked in plasma and blotted	5 X 5mm wick soaked in serum pretreated with Neuramindase (20µl sample + 10µl) of Clostridum perfringes, 10U/ml, in 2M Acetate buffer, pH 5.5), incubated overnight at 4°C.
Focusing		
Volts	2000	2000
ma	unlimited	unlimited
W	5 (10 minutes) 7 (10 minutes) 9 (15 minutes) 12 (35 minutes)	5 (15 minutes) 8 (15 minutes) 12 (50 minutes)

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GENE FREQUENCY DISTRIBUTION OF F13A and F13B in U.S. WHITES, BLACKS, AMERINDIANS AND MEXICAN-AMERICANS. Dale D. Dykes, Shirley Miller and Herbert Polesky. (Memorial Blood Center of Minneapolis, Minneapolis, Minnesota 55404, U.S.A.).

Coagulation factor XIII (fibrin stabilizing factor) is the precursor of fibrinoligase and calalyses the cross-linking of fibrin mononers into fibrin polymers. FXIII in plasma consists of two subunits, A and B, with the enzymatic activity residing in the A subunit. The B subunit is thought to act as a carrier and regulator of the A subunit. Genetic polymorphisms of the A and B subunits have been described. Reports on the distribution of these polymorphisms have primarily been restricted to Caucasian and Asian populations.(1-13). For this study we phenotyped local White, Black, Mexican American and Amerindian populations using a technique of isoelectric focusing (IEF) and immunoblotting.

METHODS

Blood samples from blood donors and paternity cases were collected at our institution and stored at -20°C until tested. F13A was tested using plasma from ACD anticoagulant tubes. F13B was tested using serum. The IEF and immunoblotting techniques are described in tables 1 and 2.

RESULTS

The IEF techniques clearly separated the F13A and B phenotypes, and the immunoblotting method makes it easy to define the bands of interest. (Figure 1 and 2.) The blotting technique was also capable of resolving the F13A type 3 homomer, which has previously been difficult or impossible to resolve by other methods. The gene frequency distribution of F13A seen in table 3, were not remarkable except for the high frequency of F13A*1 seen in the local Amerindian population. Phenotyping for F13B, however, shows significant differences between the four population groups. When analyzed for the frequencies of F13B*1,2 and 3, all were in Hardy-Weinberg equilibrium, Table 4.

The results of this study indicate that IEF combined with immunoblotting are effective methods for phenotyping F13A and B and further demonstrate the value of F13B as a marker for investigating population variations. In cases of disputed parentage the exclusion probabilities of F13B in Whites, Blacks, Amerindians and Mexican Americans is 19%, 26%, 23% and 30% respectively.

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Table II. Distribution of 455 mother/child combinations in Sweden

Mother		Child									
		1	2-1	2	3-1	3-2	3	1-rare	2-rare	3-rare	rare
1	obs	203	22	-	37	-	-	0	-	-	-
	exp	187.16	26.35	-	37.50	-	-	0.70	-	-	-
2-1	obs	22	41	1	0	4	-	0	0	-	-
	exp	26.35	30.06	3.71	5.28	5.28	-	0.10	0.10	-	-
2	obs	-	5	0	-	1	-	-	0	-	-
	exp	-	3.71	0.52	-	0.74	-	-	0.01	-	-
3-1	obs	33	4	-	38	8	7	0	-	0	-
	exp	37.50	5.28	-	45.01	5.28	7.51	0.14	-	0.14	-
3-2	obs	-	6	0	4	1	4	-	0	0	-
	exp	-	5.28	0.74	5.28	1.80	1.06	-	0.02	0.02	-
3	obs	-	-	-	9	1	2	-	-	0	-
	exp	-	-	-	7.51	1.06	1.50	-	-	0.03	-
1-rare	obs	1	0	-	0	-	-	1	0	0	0
	exp	0.68	0.10	-	0.14	-	-	0.68	0.10	0.14	0.00
2-rare	obs	-	0	0	-	0	-	0	0	0	0
	exp	-	0.10	0.01	-	0.02	-	0.10	0.01	0.02	0.00
3-rare	obs	-	-	-	0	0	0	0	0	0	0
	exp	-	-	-	0.14	0.02	0.03	0.14	0.02	0.03	0.00
rare	obs	-	-	-	-	-	-	0	0	0	0
	exp	-	-	-	-	-	-	0.00	0.00	0.00	0.00

$\chi^2 = 8.4$ $0.90 < p < 0.95$ 15.d.f.

Except the three common alleles with the six corresponding phenotypes eight rare phenotypes with two rare alleles involved were developed (see table I and figure 1). One of them is probably the type 4 variant which migrates between the type 2 and type 1 allele products. This type was reported by Kreckel et al (1983). The other rare allele has an electrophoretically migration in a position anodal to the type 1 allele product. This allele has been observed in a Swedish mother/child combination (see table II) as well as in another unrelated Swedish woman. To my knowledge this variant has not been described so far and the allele was therefore tentatively designated F13B*9.

F13B typing of 455 mother/child combinations is shown in table II. Unexpected combinations were not found. The data are in full accordance with the postulated genetic model of an autosomal locus with several codominant alleles. (χ^2=8.4, 0.90<p<0.95 15 d.f.)

The gene frequencies of F13B in Sweden are similar to those found in other European countries (Kreckel and Kühnl 1982; Mauff et al 1983; Olaisen et al 1983). The F13B-system is informative. The method of determination is sensitive and gives excellent band visualization. The classification results are highly reproducible. The system appears to be useful for both population studies and cases of disputed paternity.

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Results and discussion

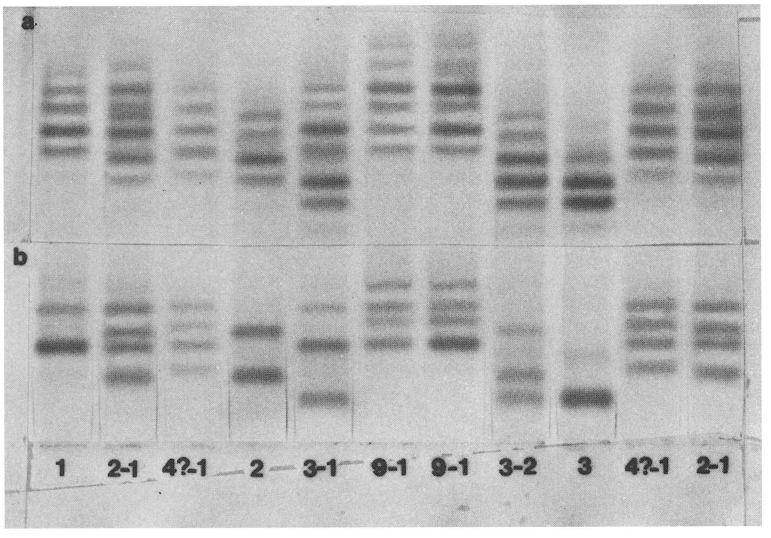

Fig. 1. Coagulation factor 13B patterns in serum revealed by high voltage electrophoresis and immunoblotting procedures. a and b show untreated and neuraminidase treated samples, respectively.

Table I. Distribution of F13B phenotypes in Sweden

Phenotype	Observed n	%	Expected n	%	Gene frequency
1	829	55.27	829.63	55.30	$F13B^1$ =0.7437
2-1	229	15.27	233.60	15.57	
2	20	1.33	16.44	1.10	$F13B^2$ =0.1047
3-1	336	22.40	332.43	22.16	
3-2	45	3.00	46.80	3.12	$F13B^3$ =0.1490
3	33	2.20	33.30	2.22	
1-rare	8*	0.53	6.02	0.40	$F13B^{rare}$=0.0027
2-rare	0	0	0.85	0.06	
3-rare	0	0	1.21	0.08	
rare	0	0	0.01	0.00	
Total	1500	100.00	1500.29	100.01	

χ^2 = 0.973 $0.80 < p < 0.90$ 3 d.f.

*Totally eight rare variants of which six probably are of the type 4-1. The other two are tentatively classified as 9-1.

The distribution of F13B phenotypes and gene frequencies in the population of Sweden are presented in table I and figure 1. The population was in Hardy-Weinberg equilibrium with a chi-square = 0.973, $0.80<p<0.90$ at 3 d.f.

Coagulation Factor 13B Polymorphism in Sweden.

Kerstin Hjalmarsson, State Institute for Blood Group Serology, SRL, Regionsjukhuset, S-581 85 Linköping, Sweden

Introduction

Human blood coagulation factor 13, the fibrin stabilizing factor, is a zymogen which after activation by thrombin and calcium ions catalyses the formation of γ-glutamyl-ε-lysine cross links between fibrin molecules (Losowsky and Miloszewski, 1977). Factor 13 consists of two A subunits and two B subunits (A_2B_2) (Schwartz et al 1973). Both A and B subunits are found in plasma but only B subunits in serum. The B subunit has no enzymatic activity and may serve as a carrier molecule in plasma (Schwartz et al 1973).

Electrophoretic polymorphisms of both A and B subunits have been described by Board (1979, 1980). Recently Olaisen et al (1983) have presented an enzyme-linked immunoblotting technique which improves the method for phenotyping products of the factor 13B locus after high voltage agarose electrophoresis.

The purpose of this investigation is to present the distribution of factor 13B phenotypes and gene frequencies from unrelated adult Swedes. Two rare alleles have been observed. A Swedish mother/child material is also presented.

Material and methods

Serum samples from 1500 unrelated Swedish adults and a mother/child material consisting of 455 pairs were investigated. All persons were involved in paternity cases.

After arrival at the laboratory the serum was separated from the blood cells by centrifugation and stored at +4°C. Factor 13B was typed in desialized serum samples (neuraminidase treatment) and in some cases in untreated serum samples. The phenotypes were determined twice or more.

The determination of factor 13B phenotypes was carried out by high voltage agarose electrophoresis followed by enzyme-linked immunoblotting technique mainly according to Olaisen et al (1983). 1.6 mm thick gels 0.5 % in agarose (Seakem ME), were cast onto glass plates 12 x 26 cm. The electrode buffer consisted of 186.6 mM Tris/374.3 mM glycine/ 31.8 mM Na-barbiturate/5.6 mM barbituric acid with pH 8.9. The gel buffer was diluted 1:4 with the electrode buffer. 10 ul serum or neuraminidase treated serum was applied on the cathodic side of the gel. The electrophoresis was performed for 2 h at maximum settings of 600V, 80 mA and 40W. The proteins were then transferred to nitrocellulose sheets (BIORAD) by passive blot for about 20 minutes.

Visualization of factor 13B bands was achieved by soaking the nitrocellulose sheets in rabbit anti factor 13B (Clotimmun-factor XIIIS, Behringwerke, Marburg) diluted 1:500 with 0.15 % Tween in PBS overnight washed 2 h with 0.15 % Tween in PBS, followed by a peroxidase conjugated goat anti rabbit antiserum (Behringwerke, Marburg) diluted 1:700 with 0.15 % Tween in PBS for 4 h and finally washed 1 h with 0.15 % Tween in PBS.

The development was performed in 50 ml of 0.1 M Tris-HCl, pH 7.5 with 35 mg 4 chloro-1-naphtol (BIORAD), 10 ml ethanol and 20 μl H_2O_2. The reaction was stopped with water.

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Population	N	Allele		
		Gc*1F	Gc*1S	Gc*2
Acquaviva Collecroce	77	0.2662	0.4805	0.2532
Montemitro	34	0.1618	0.5588	0.2794
S.Felice di Molise	63	0.1984	0.5238	0.2778
Heterogeneity analysis: $X^2 = 3.703$ (d.f. 4) $0.50 > p > 0.30$				

Table 2. Distribution of Gc allele frequencies in three Serbo-Croatian communities of Molise, Italy.

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Population	N	Allele		
		Gc*1F	Gc*1S	Gc*2
Montecilfone	74	0.1959	0.5743	0.2297
Ururi	84	0.1369	0.5774	0.2857
Portocannone	48	0.2187	0.6047	0.1771
Campomarino	154	0.1851	0.5162	0.2987
Heterogeneity analysis: $X^2 = 9.506$ (d.f. 6) $0.20 > p > 0.10$				

Table 1. Distribution of Gc allele frequencies in four Albanian communities of Molise, Italy.

equilibrium can be assumed for all seven samples under study.

The resulting frequencies of the three allotypes Gc*1F, Gc*1S and Gc*2 are shown in tables 1 and 2. Furthermore one Gc variant was found, which is not yet identified.

For heterogeneity analysis the X^2 test has been applied. Intragroup analysis of the Albanian and Serbo-Croatian samples revealed statistically significant homogeneities (tables 1 and 2). Analysis of both groups combined revealed as well significant homogeneity: $X^2 = 2.80$, $p = 0.2464$ (d.f. 2). When comparing these results with those so far published for the central-southern Italian population (Bargagna et al., 1983; Petrucci and Congedo, 1983; Kannapinn, not published), it reveals a highly significant heterogeneity: $X^2 = 52.38$, $p = 0.0001$ (d.f. 20).

Conspicuously there is an increased frequency of the Gc*1F allotype in the investigated communities, particularly in Acquaviva Collecroce, which could be of special interest, because the endogamy rate of just this village is the highest of all under study. An effect of drift can be assumed in this case.

The evaluation of the total study is still in progress and will cover altogether 18 polymorphic genetic systems. The results may hopefully lead to some more knowledge about isolated populations and their genetic structure.

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DISTRIBUTION OF Gc SUBTYPES IN FOUR ALBANIAN AND THREE SERBO - CROATIAN COMMUNITIES OF MOLISE, ITALY.

Peter Lindenberg and Gianfranco Biondi

(Universität Bremen, Abt.f. Humanbiologie/Anthropologie, D-2800 Bremen 33, F.R.G. and Università di Roma, Istituto di Antropologia, I-00185 Roma, Italy)

Introduction

During the 15th century many Albanian and Serbo-Croatian groups came to the Adriatic side of central-southern Italy as a consequence of the Turkish occupation of the Balkan area. At the present time, only in the considered villages the population has maintained the use of the Albanian and Serbo-Croatian language, respectively (Biondi et al., 1983; Biondi et al., 1984).

This investigation is part of a greater study about genetic consequences of isolation and its breakdown, resulting from the increase of social mobility, in order to understand the microevolution of complex societies.

Material and methods

This study was carried out on N = 360 schoolboys and -girls living in the four Albanian communities of Montecilfone (n = 74), Ururi (n = 84), Portocannone (n = 48) and Campomarino (n = 154) and N = 174 schoolboys and -girls living in the three Serbo-Croatian communities of Acquaviva Collecroce (n = 77), Montemitro (n = 34) and S. Felice di Molise (n = 63).

The determination of the Gc (group specific component) subtypes was done by isoelectric focusing (IEF) on polyacrylamide gels (PAG) according to Dannewitz (1985).

Results and discussion

The distribution of observed and expected phenotype frequencies are in good agreement, and the Hardy-Weinberg

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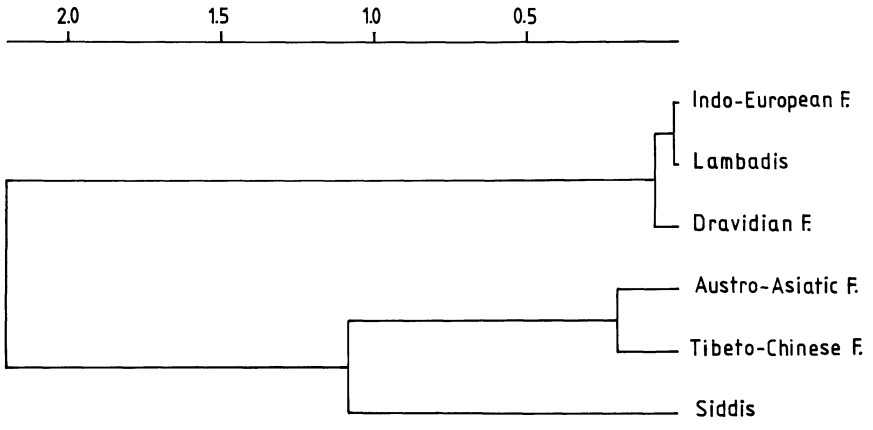

Fig. 3 Genetic distances among language families.

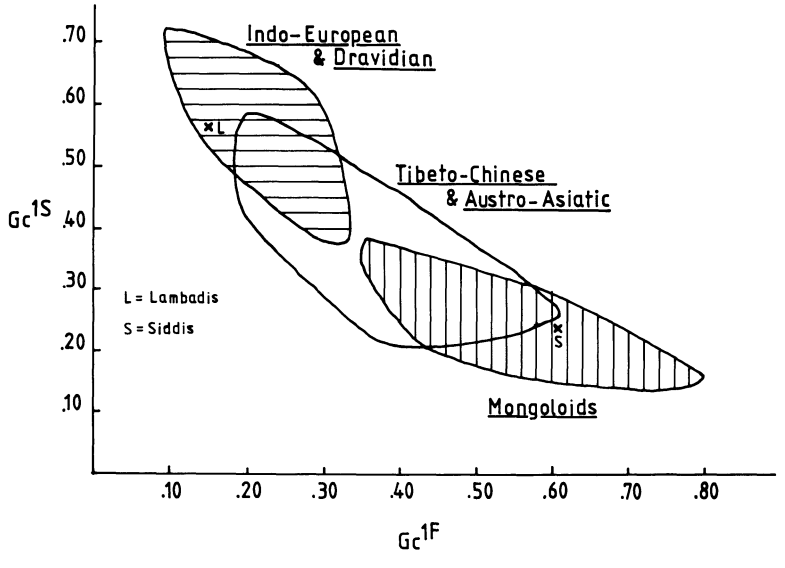

Fig. 4 Variability of Gc^{1F} and Gc^{1S} frequencies in Mongoloids as compared with language families.

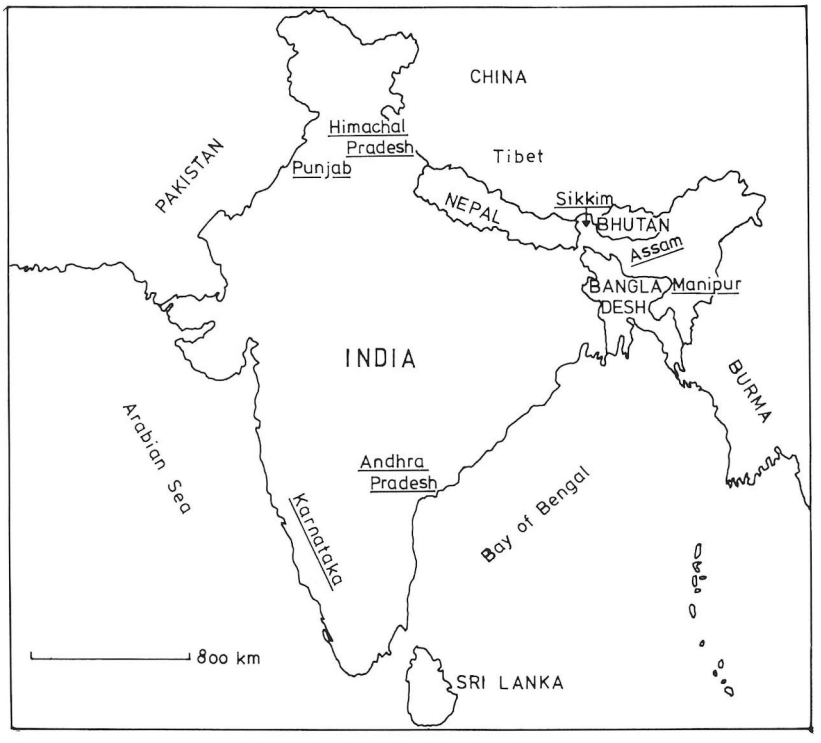

Fig. 1 Location of Indian populations, which have been Gc-subtyped.

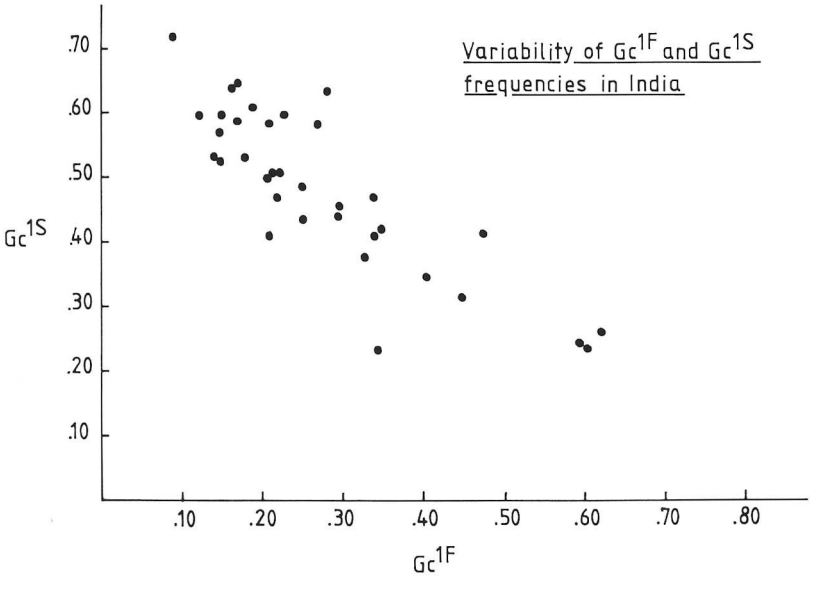

Fig. 2 Variability of Gc^{1F} and Gc^{1S} frequencies in India.

Walter, H., Dannewitz, A., Veerraju, P. and Goud, J.D.:
Gc subtyping in South Indian tribal and caste populations. -
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into the Mongoloid range of variability, which applies in particular to the Tibeto-Chinese family, whereas the Austro-Asiatic family is closer to the Indo-European and Dravidian group. This, of course, must be discussed in detail elsewhere. Concluding this one can point out that Indian populations with affinities to the Mongoloid racial group - as the Tibeto-Chinese speakers are - are striking by rather high Gc*1F and low Gc*1S frequencies, whereas those belonging to the Caucasoids (Indo-European and Dravidian speakers) and Australoids, the original inhabitants of India, show the contrary. Thus Gc subtypes prove to be of considerable importance for the analysis of genetic differentiation processes in India.

This is finally also corroborated by the distribution of rare Gc subtype variants in India, as e.g. the Meiteis from Manipur and the Karbis from Assam show Gc 1A8 variants (gene frequency in each of these two populations 0.014), which have also been reported for other Mongoloid populations (Japanese; Constans 1979). One can assume that this rare Gc subtype allele attained to the gene pool of Meiteis and Karbis by gene flow, which is reasonable considering the racial history of the Northeastern parts of India.

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ON THE VARIABILITY OF GC-SUBTYPES IN INDIA

Hubert Walter and Angela Dannewitz (Dept. of Human Biology/ Physical Anthropology, University of Bremen, D-2800 Bremen 33, F.R.G.)

In close co-operation with some Indian anthropologists (Indera P. Singh and M.K. Bhasin, Delhi; B.N. Mukherjee and K.C. Malhotra, Calcutta; B.M. Das, Gauhati; and P. Veerraju, Waltair) we are conducting several research projects in order to analyse the distribution of genetic markers in Indian populations. These studies consider a great number of blood group, serum protein and red cell enzyme polymorphisms as well as haemoglobin variants. The results of our Gc subtypings will be reported here briefly.

Up to now a total of 35 population groups from various parts of India have been Gc subtyped (Fig. 1). With the exception of the data from Himachal Pradesh (Papiha 1981, Papiha et al. 1983) and Punjab (Papiha et al. 1982) all the others have been reported by our group; some of them are published already (Walter et al. 1984). Disregarding here the Gc*2 frequencies (which vary among Indian populations between 0.089 in Brahmans from Sikkim and 0.409 in Karbis from Assam) and focusing on Gc*1F and Gc*1S alleles one can see a considerable variability of these two alleles (Fig. 2),which will be described in detail elsewhere. This variability, however, is not due to chance, but was found to be closely associated with language families (Fig. 3): Indo-European and Dravidian speaking populations are forming one cluster (together with the linguistically not classifyable Lambadis, a tribal population from South India), Austro-Asiatic and Tibeto-Chinese speaking another one (together with the Siddis, a South Indian population of Negroid origin). As the first cluster is characteristic by rather low Gc*1F and high Gc*1S frequencies, the other one by high Gc*1F and low Gc*1S frequencies we supposed that this genetic distance pattern might be associated with the racial affiliations of the population groups under study. We therefore have plotted in Fig. 4 the variability of Gc*1F and Gc*1S frequencies in Mongoloid populations from East and Southeast Asia and in comparison that of the language families. The result is clear: Indo-European and Dravidian speaking populations are obviously different from the Mongoloids, whereas Austro-Asiatic and Tibeto-Chinese speakers are falling

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This method is simple, fast, and more reliable than conventional immunoelectrophoresis.

The data on Pi polymorphism are received from isoelectrofocusing carried out for Tf subtyping. The presented Pi gene frequencies with a very frequent Pi^M and rare genes Pi^S, Pi^Z and Pi^F show a good agreement with many reports on population of different human populations.

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Table 3. Result of Pi-typing

	Obs.		Exp.		Gene
Type	No.	%	No.	%	frequencies
M	563	94.6	562.4	94.5	Pi^{M3} = 0.972
M S	23	3.9	24.3	4.1	Pi^{S} = 0.021
M Z	2	0.3	2.0	0.3	Pi^{Z} = 0.002
F M	6	1.0	5.8	1.0	
S	1	0.2	0.3	0.1	Pi^{F} =00.005
Total	595	100.0	594.8	100.0	

The distribution of Gc subtypes studied in a northern Italian population is shown in table 2. The observed and expected values assuming a HARDY-WEINBERG equilibrium were in good agreement and only one Gc variant (Gc2-1F-1S bands) found by means of immunofixation technique.

The table 3 demonstrates the results of Pi subtyping received by PAGIF. 94,6 % of the tested samples have the same phenotype PiMM.

Discussion

The distribution of Tf is nearly similar to the results observed in European populations: Tf^{C2} = 0.1691 in Berlin, GDR (Geserick and Patzelt), Tf^{C2} = 0.1689 in Hessen, FR^{G} (Kühnl). Moreover, in a population of GDR the gene frequency of Tf^{C3} was calculated to be 0.0632.

Testing the Gc system a polymorphism was recognized including the three common genes and only one variant (Gc2-1F-1S bands). This system is certainly useful for forensic medicine in cases of identification and disputed paternity. Using the immunofixation technique we were not able to identify uncommon variant of Gc.

Table 1. Results of Tf-typing

	Obs.		Exp.		Gene
Type	No.	%	No.	%	frequencies
C1	369	62.0	369.5	62.1	Tf^{C1} = 0.788
C1C2	159	26.7	158.5	26.6	Tf^{C2} = 0.169
C1C3	40	6.7	39.4	6.6	Tf^{C3} = 0.042
C1D	1	0.2	0.9	0.2	Tf^{D} = 0.001
C2	18	3.0	17.0	2.9	
C2C3	6	1.0	8.4	1.4	
C3	2	0.3	1.0	0.2	
Total	595	100.0	594.7	100.0	

Table 2. Result of Gc-typing

	Obs.		Exp.		Gene
Type	No.	%	No.	%	frequencies
1F	14	2.4	14.0	2.3	Gc^{1F} = 0.153
1F1S	104	17.4	104.3	17.5	Gc^{1S} = 0.571
1S	196	32.8	194.7	32.7	Gc^{2} = 0.275
2 1F	51	8.5	50.2	8.4	Gc^{V}(variant)
2 1S	186	31.2	187.5	31.4	= 0.001
2	45	7.5	45.2	7.6	
2 V	1	0.2	0.3	0.1	
Total	597	100.0	596.2	100.0	

focusing with a higher resolution capacity new genetic heterogeneities of proteins were discovered.

With the isoelectric focusing technique Frants and Eriksson were able to identify three subtypes of the commom phenotype PiM of the Pi-system. In the same way, Constans and Viau showed that the Gc^{1} allele divides into two allele: Gc^{1F} and Gc^{1S}. Kühnl and Spielmann have found two subtypes of the common Tf^{C} allele: Tf^{C1} and Tf^{C2}. In 1979 and 1980 new Tf^{C} subtypes were observed: Tf^{C3} (Kühnl and Spielmann), Tf^{C4} and Tf^{C5} (Constans et al.).

The data on the distribution of Tf-, Gc- and Pi-polymorphisms in a northern Italian population presented in this paper were evaluated to be important for population genetics and forensic medicine.

Material and Methods

Fresh blood samples were obtained from unrelated donors of a northern Italian district mainly from the Vicenza area. Serum was separated promptly and transported by airplane with dry ice.

The polyacrylamide gels isoelectric focusing (PAGIF) for Gc, Tf and Pi were agreement with Geserick et al.[1] and Nagai.[2]

Results

Table 1 summarizes the results of Tf-subtyping by PAGIF. The position of the main bands of TfD is similar to the electrophoretic mobility observed after gel electrophoresis. The gene allele frequencies of Tf^{C1}, Tf^{C2} and Tf^{C3} were calculated, but it was not possible to calculate the frequency of Tf^{C4} and Tf^{C5} because the sample of 595 sera were not containing Tf^{C4} and Tf^{C5} subtypes.

ISOELECTRIC FOCUSING OF Gc, Tf AND Pi SUBTYPES IN A NORTHERN ITALIAN POPULATION

T. Nagai[+], T. Ikeda[+], G. Rossi[+], M. Salviati[++] and O. Prokop[+++]

+: Department of Legal Medicine, Teikyo University School of Medicine, Tokyo-173, Japan

++: Laboratory of the Central Immunotransfusion and Human Genetics, Hospital of Vicenza, Vicenza-36100, Italy

+++: Department of Forensic Medicine, Humboldt University, Berlin-1040, GDR

Summary

The data on the distribution of Gc-, Tf- and Pi-polymorphisms in the northern Italian population presented in this paper are important for population genetics and forensic medicine. Fresh blood samples were obtained from unrelated northern Italian people mainly from the Vicenza area. The polyacrylamide gel isoelectric focusing technique for Gc, Tf and Pi was in agreement with Nagai and Geserick et al.

The following gene frequencies in the northern Italian population are calculated:

Gc^{1F}=0.153 Gc^{1S}=0.571 Gc^{2}=0.275 Gc^{V}=0.001 (Variant)

Tf^{C1}=0.788 Tf^{C2}=0.169 Tf^{C3}=0.042 Tf^{D}=0.001

Pi^{M} =0.972 Pi^{S} =0.021 Pi^{Z} =0.002 Pi^{F}=0.002

The observed and expected values assuming a HARDY-WEINBERG equilibrium were in good agreement and only one Gc variant (Gc2-1F-1S) found by means of immunofixation technique.

Introduction

The determination of protein polymorphisms for genetic and forensic investigations was at first based on electrophoretic procedures: gel electrophoresis, immunoelectropheresis and immunofixation electrophoresis. After introduction of the isoelectric

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Table I. PI and Gc in Cagliari and Nuoro (Sardinia)

CAGLIARI			NUORO		
PI	obs	exp	PI	obs	exp
M1	118	118.35		93	88.75
M2	20	17.74		9	8.78
M3	1	0.66		1	1.03
M1M2	87	91.66		51	55.84
M1M3	19	17.79		18	19.15
M1M4	20	19.33		5	5.93
M2M3	8	6.89		8	6.02
M2M4	6	7.48		3	1.87
M1S	23	21.65		8	9.71
M2S	8	8.38		3	3.05
M3S	1	1.62		1	1.05
M4S	1	1.76		1	0.32
M1Z	2	1.54		2	1.88
M2Z	1	0.60		1	0.59
S	1	0.99		1	0.26
n.	316			205	

Gc	obs	exp	obs	exp
1S	103	96.98	78	72.33
1F	14	11.05	9	5.65
1F1S	59	65.47	37	40.42
21S	86	90.68	50	58.45
21F	31	30.93	13	13.33
2	23	21.19	18	11.81
n.	316		205	

Gene frequencies

Cagliari	PI	M1	0.611	Gc	1S 0.554
		M2	0.236		1F 0.187
		M3	0.045		2 0.259
		M4	0.049		
		S	0.055		
		Z	0.004		
Nuoro	PI	M1	0.653	Gc	1S 0.594
		M2	0.217		1F 0.166
		M3	0.071		2 0.240
		M4	0.022		
		S	0.030		
		Z	0.007		

uencies from Nuoro are tightly clustered around mean values. These results confirmed on the whole our assumption that geographic individuality of Sardinia does not automatically involve a genetic uniqueness of its population structure.

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SERUM GENETIC MARKERS IN SARDINIA.II. Gc AND PI IN CAGLIARI AND NUORO

Romano Petrucci(*), Piero Congedo(*),Ernesto D'Aloja (°), and Vincenzo L.Pascali(°)
(*)Dept Genetics and Molecular Biology, University of Rome
(°)Istituto di Medicina Legale, Università Cattolica del S. Cuore, Rome, Italy

This study is part of a general survey aimed at investigating the genetic variability of blood markers in Sardinia(1). Sardinia island was long since an isolated region where malignant malarian endemia would impress a peculiar selective pressure(2). For this reason special attention has been as yet paid by Anthropologists and Geneticists to the structure of Sardinian populations. Thus detailed maps aeepresently available of the distribution of erythrocyte markers (3). Conversely data are still lacking on further classes of markers, such as serum isoproteins. The purpose of this study is to contribute at filling the lack of data concerning two major isoprotein systems (Gc and PI) in Cagliari and Nuoro. One more reason to acquire more data on serum markers is that they are needed for biostatistical analysis of paternity.

Materials and methods

Sera were collected from unrelated individuals in Cagliari (316) and Nuoro (205). The Sardinian origin of the blood donors was specially cared. Both sexes were almost equally sampled. Gc separation was performed as usually (4), thereafter the simple immersion of focused gels in 10% sulfosalycilic acid was chosen as detection system. PI typing was performed as elsewhere described (5) with special care to select suspected M4 types, whose diagnosis was confirmed by shallow IPGs (6).

Results and discussion

Table I gives distribution and gene frequencies of PI phenotypes in the two localities. When compared with those reported for the Italian mainland (7), PI gene frequencies showed no peculiar distribution of the M gene products, but in the sample from Cagliari PI M3 was slightly lower (.046) than PI M4 (.056). As for the rest of alleles, while no special remark is to be paid to PIS, the very low frequency of PIZ seemed us to be only close to Greek values among all the European countries (8).

Table I reports as well phenotypes and alleles of Gc. If compared with values we already grouped along the Italian peninsula (4), only Gc1S in Cagliari is slightly differing from the Continental Italy mean frequency; conversely allele freq

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focusing becomes one of the most useful markers in paternity testing.

The distribution of Tf subtypes are shown in Table 3, and it can be seen that there was a good agreement between the observed distribution of phenotypes and that expected according to the Hardy-Weinberg law.

The distribution found in the sample differs slightly from the results observed in other European populations, being the Tf^{C3} allele frequency one of the lowest within the population of European origin. The same fact is found in the Galicia population (6).

The chance of exclusion using the Tf system is 17.05% and the EM value 9.87.

PLG phenotypes and gene distribution can be seen in Table 4. A good Hardy-Weinberg equilibrium was observed.

The observed allele frequencies are lower than it is observed in North-European populations and similar to the observed in italians.

The chance of exclusion using the PLG system in this population is 14.04% and the EM value=9.90.

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Table 3. Frequencies of Tf phenotypes and Tf alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
C1-C1	517	516.55	0.44	0.00
C1-C2	219	223.22	-4.22	0.08
C1-C3	61	57.17	3.82	0.25
C1-B	5	5.48	-0.48	0.04
C2-C2	27	24.11	2.88	0.34
C2-C3	10	12.35	-2.35	0.44
C2-B	2	1.18	0.81	0.56
C3-C3	1	1.58	-0.58	0.21
C3-B	0	0.30	-0.30	0.30
B-B	0	0.01	-0.01	0.01
Total	842	841.99		2.26

Tf C^1 = 0.7832
Tf C^2 = 0.1692
Tf C^3 = 0.0433
Tf B = 0.0041

X^2 = 2.26526
df = 6
P > 0.75
CE = 17.05
EM value = 9.865737

Table 4. Frequencies of Plg phenotypes and Plg alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
1-1	465	462.89	2.10	0.00
1-2	250	254.20	-4.20	0.06
2-2	37	34.89	2.10	0.12
Total	752	752		0.20

Plg^1 = 0.7845
Plg^2 = 0.2154

X^2 = 0.2054947
df = 1
P > 0.50
CE = 14.04
EM value = 9.896515

Table 1. Frequencies of Pi phenotypes and Pi alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
M1-M1	338	338.52	-0.52	0.00
M1-M2	219	215.06	3.93	0.07
M1-M3	111	112.33	-1.33	0.01
M1-S	116	117.74	-1.74	0.02
M1-F	3	2.40	0.59	0.14
M1-Z	2	2.40	-0.40	0.06
M2-M2	36	34.15	1.84	0.09
M2-M3	32	35.68	-3.68	0.38
M2-S	34	37.40	-3.40	0.30
M2-F	0	0.76	-0.76	0.76
M2-Z	1	0.76	0.23	0.07
M3-M3	11	9.32	1.67	0.30
M3-S	21	19.53	1.46	0.10
M3-F	1	0.39	0.60	0.90
M3-Z	0	0.39	-0.39	0.39
S-S	12	10.23	1.76	0.30
S-F	0	0.41	-0.41	0.41
S-Z	1	0.41	0.58	0.81
F-F	0	0.00	0.00	0.00
F-Z	0	0.00	0.00	0.00
Z-Z	0	0.00	0.00	0.00
Total	938	937.99		5.22

Pi M^1 = 0.6007
Pi M^2 = 0.1908
Pi M^3 = 0.0996
Pi M^S = 0.1044
Pi M^F = 0.0021
Pi M^Z = 0.0021

X^2 = 5.222971
df = 15
P > 0.99
CE = 34.64
EM value = 9.759441

Table 2. Frequencies of Gc phenotypes and Gc alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
1S-1S	252	252.20	-0.20	0.00
1S-1F	125	121.16	3.83	0.12
1F-1F	15	14.55	0.44	0.01
1S-2	291	294.42	-3.42	0.03
1F-2	66	70.72	-4.72	0.31
2-2	90	85.92	4.07	0.19
Total	839	839		0.68

Gc^{1S} = 0.5482
Gc^{1F} = 0.1317
Gc^2 = 0.3200

X^2 = 0.68363 df = 3
P > 0.75
CE = 30.12 EM value = 9.830011

Samples were stored at -30°C prior to analysis and used without previous treatment for Pi, Gc and PLG typing. For Tf subtype determination the serum samples were diluted 1:5 with 0.5M ferrous ammonium sulphate and incubated for 18h at 4°C.

PAGIF was carried out in 0.3 mm polyacrylamide gels at a gel concentration of T=5.5% and C=3%. Ampholyte concentration was 5%. Polymerization was carried out with riboflavine and ultraviolet light.

A mixture of Ampholine and Pharmalyte (pH 2.5-5, 3.5-5, 4-6 and 4.2-4.9) were used for Pi typing. For Gc typing was used a mixture of Ampholine and Pharmalyte pH 4-6, 4-6.5 and 4.5-5.4. Ampholine pH 5-7 and 5-8 were used for Tf and PLG typing respectively.

Staining of the gels was carried out with Coomassie Blue R 250 for Pi, Tf and PLG. Gc bands were read after simple precipitation with sulphosalicylic acid.

Results and Discussion

The distribution of Pi subtypes and their allele frequencies are shown in Table 1. 14 different phenotypes were found. A fair agreement was found between observed and expected values, assuming a Hardy-Weinberg equilibrium.

The Pi^S frequency found in our population is one of the highest found within European populations in agreement with the progressive decrease of the Pi^S frequency from southwestern toward the northern European countries (the highest frequencies are found in the Galicia population). The distribution of the other alleles in European countries seems to be more uniform.

Pi subtyping offers in our population a theoretical exclusion rate of 34.64% and an EM value of 9.77, being the most useful electrophoretic marker for paternity testing in our population.

Table 2 summarizes the results of the Gc subtyping. Good agreement was noted for the Hardy-Weinberg distribution.

Gc alleles frequencies in Barcelona populations are in the range expected for caucasians.

With a theoretical chance of exclusions of non-fathers=30.12% and an EM value=9.83, the Gc polymorphism subtyped by isoelectric

FREQUENCY AND DISTRIBUTION OF Pi, Gc, Tf AND PLG SUBTYPES BY ISOELECTRIC FOCUSING IN BARCELONA

Manel Gené, Emili Huguet, Angel Carracedo, Guadalupe Ercilla, Jacint Corbella (Departaments de Medicina Legal de les Facultats de Medicina de Barcelona i Santiago). *

Introduction

The application of isoelectric focusing to the analysis of some polymorphisms has revealed considerably more genetic heterogeneity than was apparent by conventional methods.

The separation of the Pi components by isoelectric focusing has led to reliable characterization of four Pi^M alleles at the Pi gene locus (1,2).

Subtyping of TfC by isoelectric focusing has now demonstrated eight separate variants (3). The alleles commonly encountered in white people are Tf^{C1}, Tf^{C2} and Tf^{C3}.

Constans and Viau(4) in 1977 reported further subclassification of Gc by isoelectric focusing in polyacrylamide gels. This method permitted distinction of six common subtypes called 1S, 1S-1F, 1F, 2-1S, 2-1F and 2.

Finally Hobart (5) using isoelectric focusing first identified PLG variants in 1979 and named the common alleles PLG^1 and PLG^2.

Despite the vast array of population studies until now concerned with Pi, Tf and Gc, subtyping of these markers in Spanish populations has been published once in Galicians (6).

From our knownledge studies on PLG polymorphism in Spanish populations has not been reported until now.

In this paper we report the results of a survey of these serum proteins in 800 donors from the metropolitan area of Barcelona.

Material and Methods

Serum from freshly collected blood samples from around 800 healthy donors was used.

Donors were classified according to their ancestral origin as catalans or mixed populations.

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* Av. Joan XXIII s/n, E-Barcelona 08028, Spain

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Results

The results of testing 316 blood donors from southern Germany for Malic Enzyme are listed in table 1:

Table 1: Malic Enzyme

Phenotype	n observed	n expected	χ^2
1	124	125.42	0.02
2-1	151	147.32	0.09
2	41	43.26	0.12
	316	316.00	0.23

$MEM^1 = 0.63$ $0.5 < p < 0.7$

The results of testing 367 blood donors from southern Germany for Alpha-L-Fucosidase are listed in table 2:

Table 2: Alpha-L-Fucosidase

Phenotype	n observed	n expected	χ^2
1	136	134.60	0.015
2-1	108	109.95	0.033
2	23	22.45	0.011
	267	267.00	0.059

$FUCA^1 = 0.71$ $0.8 < p < 0.9$

Observed and expected values are in good agreement; so are the respective gene frequencies compared with earlier findings (Kühnl and Spielmann, 1977; Kondler and Wiebecke, 1981; Kömpf and Ritter, 1983).

From the gene frequencies given here for the two leucocyte systems their potential value in paternity testing and in forensic medicine is obvious. Since the formal genetics are well established and population data are at hand, Malic Enzyme and Alpha-L-Fucosidase appear as reliable and helpful tools.

Polymorphisms in Leucocytes: Population Data on Malic Enzyme and Alpha-L-Fucosidase

G. A. Siebert

Institut für Immunologie und Serologie der Universität
Im Neuenheimer Feld 305
6900 Heidelberg
Direktor: Prof. Dr. K. Rother

Introduction

The genetically determined polymorphisms of mitochondrial Malic Enzyme (E.C. 1.1.1.40) and Alpha-L-Fucosidase (E.C. 3.2.1.51) have been first described in 1972 (Cohen et al.) and in 1974 (Turner et al.), respectevely. Data on population genetics are still scarce, however. The two enzyme systems are readily detectable in human white blood cells and can be demonstrated by routine methods such as starch gel electrophoresis (Malic Enzyme) or Isoelectric Focusing (Fucosidase). In this paper population data obtained from blood donors in southern Germany are presented.

Material and methods

Malic Enzyme: Sampling, treatment and electrophoresis are carried out as published elsewhere (Siebert et al., 1979).

Alpha-L-Fucosidase: White cell samples must be desialinised prior to electrophoresis. 50µ of sample are diluted with an equal amount of neuraminidase (1 Unit/ml aqua dest.) and stored overnight at 4°C. 10µl of neuraminidase treated leucocyte sample were run for 180 min. on commercially available polyacrylamid gels pH 3.5-9.5 under normal conditions. Staining and visualisation of gene products were performed according to Turner et al. (1972).

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The observed frequencies for common PGM_1 types are not significant different from the results of previous studies in other Spanish populations (5) being the frequency of the PGM_1^- allele slighltly higher.

With a theoretical chance of exclusion of 33.78% and an EM value of 9.77, the PGM polymorphism subtyped by IEF becomes the most useful polymorphic enzyme for paternity testing in our population.

Similar results can be observed in the most of caucasian populations studied until now.

The distribution of the AcP phenotypes is shown in Table 2, and it can be seen that there was a good agreement between the observed and expected Hardy-Weinberg numbers.

A comparison of our results to previously published ones shows no great difference with South-European populations.

The chance of exclusion using this marker in Catalonia is 24.38% and the EM value=9.85, becoming an useful marker for paternity testing.

Finally, the distribution of GLO phenotypes is shown in Table 3. A good agreement was found between observed and expected values.

The gene frequencies observed for GLO were within the range expected for European populations and are very similar to other spanish populations. The chance of exclusion with this marker is 18.6% and the EM value=9.88.

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Table 3. Frequencies of Glo phenotypes and Glo alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
1-1	127	124.09	2.90	0.06
1-2	303	308.80	-5.80	0.10
2-2	195	192.09	2.90	0.04
Total	625	625		0.22

$Glo^1 = 0.4456$
$Glo^2 = 0.5544$

$X^2 = 0.2205453$
df = 1
P > 0.50
CE = 18.6
EM value = 9.887267

Table 1. Frequencies of PGM phenotypes and PGM alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
1+ 1+	270	264.98	5.01	0.09
1+ 1-	112	111.97	0.02	0.00
1+ 2+	208	212.22	-4.22	0.08
1+ 2-	44	49.83	-5.83	0.68
1- 1-	13	11.82	1.17	0.11
1- 2+	42	44.83	-2.83	0.17
1- 2-	11	10.52	0.47	0.02
2+ 2+	45	42.49	2.50	0.14
2+ 2-	22	19.95	2.04	0.20
2- 2-	4	2.34	1.65	1.17
Total	771	770.99		2.70

PGM^{1+} = 0.5862
PGM^{1-} = 0.1238
PGM^{2+} = 0.2347
PGM^{2-} = 0.0551

X^2 = 2.70817
df = 6
P > 0.75
CE = 33.78
EM value = 9.772357

Table 2. Frequencies of AcP phenotypes and AcP alleles in the Barcelona population

Phenotypes	Obs.	Exp.	Dif.	X^2
A-A	78	72.43	5.56	0.42
A-B	332	339.06	-7.06	0.14
A-C	35	39.05	-4.00	0.42
B-B	399	396.76	2.23	0.01
B-C	94	91.41	2.58	0.07
C-C	6	5.26	0.73	0.10
Total	944	943.99		1.18

AcP^{A} = 0.2770
ACP^{B} = 0.6483
ACP^{C} = 0.0746

X^2 = 1.18433
df = 3
P > 0.75
CE = 24.38
EM value = 9.847938

STUDY OF THE POLYMORPHIC VARIANTS OF AcP, PGM_1 AND GLO IN THE POPULATION OF BARCELONA (SPAIN).

Emili Huguet, Manel Gené, Guadalupe Ercilla, Angel Carracedo, Ricardo Castillo, Jacint Corbella (Departaments de Medicina Legal de les Facultats de Medicina de Barcelona i Santiago. Servei d´Hemoterapia i Hemostasia de l´Hospital Clínic de Barcelona)

Introduction

In the last years, the distribution of enzyme polymorphisms has been analyzed in large population groups in many areas of the world. However, in Catalonia, reasonably large population studies on enzyme polymorphisms have not been reported.

In this paper we report the results of a survey of red cell PGM_1, AcP and GLO in more than 600 samples from people living in the metropolitan area of Barcelona.

Material and Methods

The study was carried out with blood obtained from blood donors from the Hospital Clínic Blood Bank.

Red cells were washed three times in a physiological saline solution and subsequently frozen at -30°C until typing.

PGM_1 subtypes and AcP phenotypes were determined by PAGIF.

PAGIF was carried out in 0.3 mm polyacrylamide gels at a gel concentration of T=5.5% and cross-linking of C=3%. Ampholine (pH 5-8 for AcP and pH 5-7 for PGM) concentration was 5%.

Polymerization was carried out with riboflavin and UV light.

Isoenzyme visualizations were done according to Burdett and Whitehead (1) for AcP and according to Sutton and Burgess (2) for PGM.

GLO phenotypes were determined in agarose-starch gel electrophoresis according to Wetterling (3) with a staining technique according to Parr et al. (4).

Results and Discussion

The distribution of PGM subtypes and their allele frequencies are shown in Table 1. As can be seen, good agreement was found for the Hardy-Weinberg distribution.

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RED CELL ENZYMES (N = 154 for every marker)

EAP	AA	AB	BB	AC	BC	CC
Obs.	6	51	96	0	1	0
Exp.	6.4403	48.8975	96.6474		1.0148	

Gene freq. A=0.2045 B=0.7922 C=0.0033 X^2=0.0590 for 3 d.f. P > 0.99

PGM_1	1-1-	1+1+	1-1+	1-2-	1-2+	1+2-	1+2+	2-2-	2+2+	2-2+
Obs.	15	56	60	2	6	5	10	0	0	0
Exp.	15.5927	56.7598	59.4991	2.2324	5.0863	4.2633	9.7046	0.0799	0.4147	0.3654

Gene freq. 1+=0.6071; 1-=0.3182; 2+=0.0519; 2-=0.0288

X^2=I.2218 for 6 d.f. 0.98 P 0.99

6-PGD	AA	AB	BB	B-V	A-V	V-V
Obs.	105	42	5	0	2	0
Exp.	104.7400	42.8765	4.3879		1.9956	

Gene freq. A=0.8247 B=0.1688 V=0.0650 X^2=0.1030 for 3 d.f. P > 0.99

EsD	1-1	2-1	2-2
Obs.	144	10	0
Exp.	144.1527	9.6847	0.1626

Gene freq. 1=0.9675 2=0.0325 X^2=0.1730 for 1d.f. 0.50 < P < 0.70

ADA	1-1	2-1	2-2
Obs.	145	9	0
Exp.	144.1527	9.6847	0.1368

Gene freq. 1=0.9700 2=0.030 X^2=1.388 for 1 d.f. 0.50 < P < 0.70

Ak	1-1	2-1	2-2
Obs.	154	0	0

RESULTS: Serum protein (N = 160 for every marker)

Gc	1S-1S	1F-1F	1S-1F	2-1S	2-1F	2
Obs.	29	18	49	31	25	8
Exp.	29.7493	18.9118	47.4389	31.0464	24.7536	8.1000

Gene freq. 1S=0.4312 1F= 0.3438 2=0.2550

X^2=0.1172 for 3d.f. $0.98 < P < 0.99$

Pi	M_1M_1	M_1M_2	M_2M_2
Obs.	10	59	91
Exp.	9.7516	59.4969	90.7515

Gene freq. M_1=0.2469 M_2=0.7531 X^2=0.011 for 1 d.f. $0.90 < P < 0.95$

PLG	1-1	2-1	2-2
Obs.	122	36	2
Exp.	122.5000	35.0000	2.5000

Gene freq. PLG1=0.8750 PLG2=0.1250 X^2=0.1306 for 1d.f. $0.70 < P < 0.80$

Tf	C_1C_1	C_2C_1	C_2C_2
Obs.	141	18	1
Exp.	140.6250	18.7499	0.6251

Gene freq. C_1=0.9375 C_2=0.0625 X^2=0.2557 for 1 d.f. $0.50 < P < 0.70$

Hp	1-1	2-1	2-2
Obs.	106	49	5
Exp.	106.4391	48.1219	5.4390

Gene freq. Hp1= 0.8156 Hp2=0.1844 for 1 d.f. $0.80 < P < 0.90$

MATERIAL and METHODS

160 blood samples were taken from donors living around Agats, and stored at 4°C , as soon as possible, until their arrival in Italy. The samples were recived in our Institute in a satisfactory condition for testing. We typed 160 serum samples and 154 cloth samples. Six cloth samples were discarded for their degradation. The samples (serum and cloth) were stored at -40°C.

The method used for each genetic marker is reported above:

Tf, Pi, PLG and Gc	I.E.F.
Hp	Vertical Electrophoresis
ADA, Ak, EsD and 6-PGD	Electrophoresis on Cellogel sheets
PGM_1 and EAP	I.E.F.

POPULATION GENETIC STUDIES ON ASMAT (IRIAN-JAYA INDONESIA)

P.Cortivo[1], M.Tommaseo[2], L.Caenazzo[1], C.Crestani[1], C.Scorretti[1] and P.Benciolini[1].

1 Istituto di Medicina Legale e delle Assicurazioni dell'Università di Padova. Via Falloppio 50 35100 PADOVA (ITALY)

2 Istituto di ANTROPOLOGIA - Università di Padova.Centro Studi e Ricerche Ligabue, Venezia ITALY.

The samples were obtained from individuals living in the districts around the village of AGATS in the territory of ASMAT.

New Guinea is divided politycally into two parts; one of which is the Irian Jaya area. This area covers 260000 sq Km with about 1000000 inhabitants.

The Asmat region, located on the Southern coast, has an area of about 30000 sq Km with 35000 inhabitants.

The district of Agats belongs to the Bisman Asmat area(about 100 sq Km and 1300 inhabitants) known as the Flamingo Bay area. The people of Asmat have been in continuous contact with outside agents only since 1953, but the region around Agats has not yet been affected by social changes in the family structure and marriage system. The Bisman-Asmat populations share a common dialect and a common ecological zone,thus they can be regarded as an homogenous group under a physical, linguistical, social and cultural point of view.

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Fig.1- A cluster based on gene distance coefficients resulting from the comparision between arab populations. The traits are ABO,MNSs,CcDDe,K,P,and Fy.

Fig2-A cluster based on gene distance coefficients resulting from a comparis'on involving the systems :6-PGD ACP,PGM,AK,and Hp.

Tab.2- A comparision with previous studies using x^2-test.‡

System	ABO	MN	P_1	K	Hp *
Population -Jordanians					
.Bedouin N (1)	1.5393	21.7629	0.5000	0.1439	
.Bedouin NN (1)	3.7630	<.001 4.7699	0.8781	0.4990	
.Transjord. (1)	2.3798	.10-.05 0.7948	1.6514	0.4258	
.Jordanians-81 (2)	4.2872				3.8018
-Palestinians					
.in Jordan (1)	5.0923	0.8953	6.3714	0.6833	
.in Palestine (3)	5.8909	0.0099	<.05 5.3733	1.1337	
.in Kuwait (4)	2.9592		<.05		
.in Palestine (5)					1.4298

‡ - x^2-values were estimated. Segnificant P-values are under corresponding x^2-values .

* Hp-0 phenotypes were considered.

1) Taleb& Ruffié1968 ; 2)Banerjee et al.1981 ;3) Mourant et al.1976 ; 4) Onsi &El-Alfi 1968 ;5) Ramot et al. 1962 .

Tab.3- Predicted changes in some of the gene frequencies of the jordanian population according to data on population growth (Samha,1980),under the assumption of an existing panmixy.

Allele	P_a	P_a^2	$P_a^2-P'_a$	X	t	$\bar{P}$	P(obt.data)
ABO							
p	0.2979	.2977	-.0006	-.0030	.2947	.2330	
q	0.1122	.1124	+.0002	+.0030	.1154	.1125	
r	0.5899	.5890	-.0009	-.0135	.5755	.6445	
M	0.5899	.5990	-.0008	-.0120	.5870	.5833	
P_1	0.5502	.5532	+.0030	+.0450	.5982	.4677	
K	0.0405	.0393	-.0012	-.0180	.0213	.0339	

P'_a= Gene frequency after the immigration of 344000 Palestinian

P_a^2= Gene frequency due to a 6.5% population growth per year.

$\bar{P}$ = Expected gene frequency to-day.

t = Time = 15 years .

Tab.1- The obtained results and estimated frequencies .(N=120)

System		n	x^2	Gene Freq.
ABO	A	41	2.645	p=0.2330
	B	20		q=0.1225
	AB	11		
	O	48		r=0.6445
MNSs	MMSS	12	134.3113	MS=0.1650
	MMSs	10		Ms=0.4183
	MMss	21		NS=0.0515
	MNSS	13		Ns=0.3652
	MNSs	20		
	MNss	21		M=0.5833
	NNSS	4		S=0.2165
	NNSs	3		
	NNss	16		
Rehsus	CCDEe	5		CDE=0.0442
	CCDee	27		CDe=0.4540
	CcDEE	1		Cde=0.0172
	CcDEe	45		CdE=0.0237
	Ccddee	1		cDE=0.1565
	ccDEE	5		cDe=0.0469
	ccDEe	6		cdE=0.0172
	ccDee	3		cde=0.2404
	ccddEe	1		
	ccddee	7		
P	P_1+	86		P_1=0.4677
Kell	K+	8		K=0.0339
JK	JK^a+	115		JK^a=0.7959
FY	FY^a+	86		FY^a=0.4677
ACP	ACP-A	5	19.6658	ACP^A=0.3333
	ACP-AB	70		ACP^B=0.6333
	ACP-B	37		
	ACP-CB	8		ACP^C=0.0333
6-PGD	PGD-A	115	67.6804	PGD^A=0.9667
	PGD-AC	2		
	PGD-C	3		PGD^C=0.0333
PGM_1	PGM_1 1	59	0.2844	PGM_1^1=0.7083
	PGM_1 2-1	52		
	PGM_1 2	9		PGM_T^2=0.2917
ADA	ADA 1	88	0.1081	ADA^1=0.8542
	ADA 2-1	29		
	ADA 2	3		ADA^2=0.1458
AK	AK 1	114	0.0525	AK^1=0.9790
	AK 2-1	5		
	AK 0	1		AK^2=0.0210
Hp	Hp 1	13	0.7558	Hp^1=0.3613
	Hp 2-1	59		
	Hp 2	47		Hp^2=0.6387
	Hp 0	1		
Gc	Gc 1	83	0.1032	Gc^1=0.8292
	Gc 2-1	33		
	Gc 2	4		Gc^2=0.1708

1. Selection: e.g. differences in the O-frequency could be related to infections (Vogel & Helmboldt, 1972).
2. endogamy: e.g. consanguineous marriages were common; geographical isolation of some bedouine tribes (e.g. in Sinai; Bonné et al. 1971).
3. gene flow (Migration): e.g. slavery; break down of isolates by civilisation. The gene-flow/Migration-hypothesis is supported by the observation that the negroid element, e.g. cDe-haplotype frequency, in Arabia increases from north to south and the European elements from south to north.

The jordanian population is made up of bedouins and mostly Palestinians and seems to be a mixture of these two main clusters.

The populations of Hijas and Najd seems to be a binding ring between these populations of the north and the south, genetically as well as historically (Fig.1. and 2.; Al-Madani, 1965).

Conclusion: hence, our results are interpretable in the background of historical and ethnical events, which have played an important role in changing the genepool of the jordanian population.

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BLOOD GROUPS, HAPTOGLOBINS, Gc AND RED CELL ISOENZYMES - A POPULATION GENETICS STUDY OF THE JORDANIAN ARABS

Abdalla Nabulsi and Alexander L. Rodewald (Institute of Anthropology of the University, D-2000 Hamburg, W.Germany)

Introduction: Jordan is an islamic arabic kingdom, which shares its history and culture with the other arabic states. The borders and population of to days Jordan are the mere results of the events following the first world war and the arab-jewish conflict. The arabic origin of the jordanian population is historically established.

The aim of this study is to investigate the distribution of bloodgroups in Jordan and compare with other arabic populations. The results are interpreted in the light of historical and ethnic evidences.

Material and methods: 120 venal blood-samples were collected in Amman, Jordan. The following bloodgroups were serologically determined: ABO, CcDEe, MNSs, Fy, Jk, K and P1; the erythrocyte enzymes ACP, ADA, AK, 6-PGD and PGM1 and the serum proteins Hp and Gc were electrophoretic examined. X^2-tests and genetic distance analysis were used to compare the data with those of other studies of Jordan and other arabic countries.

Data and Discussion: The data in table 1. show that to days jordanian population with its high A-,CDe-,Ms,Ns-, ADA2-, lower 6-PGDB- and cde-, and middle O-,B-,cDe,K-, P1-,Jka-,Fya-,AK2-,PGM1,Hp^1-, and Gc^1-frequencies is nearer to the mediterranian arabic populations than to the nuclear Arabia. In case of MNSs,ACP-, and 6-PGD-systems, the comparison of observed and expected frequencies show significant differences. However, it is difficult to interpret these differences.

The comparison with previous studies on Jordan (Table 2.) shows that the gene frequencies of the tested sample lies nearer to those of the Transjordanians than to any other subgroup of the jordanian population. There is evidence of a limited gene flow (panmixy) between the transjordanian and the palestinian Jordanians (Table 3.).

According to the distance analysis (Edwards & Cavalli-Sforza, 1972), graphically represented with clustering (Table 4; Fig.1,2), the arabic population can be divided in two main clusters:

1.- The "Arabian"-cluster (all bedouins, the inhabitants of the arabic peninsula and the Socatrans) with distinctly higher O-,M-,S-,cDe-,K-,Jk^a- and Fy-, relat tively higher Hp^1-,$6\text{-}PGD^B$- and P-, and lower Gc^1- frequencies tahn those observed in Europe. High Hp^0- and ACP^R-frequencies are genetic marker of this cluster.

2.- The "Mediterranian & Iraq"-cluster with higher A-,ACP^R- and Gc^1-, and lower O-,M-,S-,cDe-,Fy^-- and Hp^1-frequencies than those of the "Arabian"-cluster.

The differences in the gene frequencies between the two clusters could be explained as a result of the following factors:

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Table 2. The degree of HLA-D compatibility obtained by mixed lymphocyte culture test (MLC) and HLA-D typing using homozygous typing cells (HTC)

HLA-D compatibility in tested couples	MLC	HTC
HLA-D identical	1	1
HLA-D haploidentical	23	20
HLA-D different	38*	41

* disagreement in 3 couples (4.8%)

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These results were compared to the results obtained by HLA-D typing using HTC. The HLA-D frequencies are shown on the Table 1. The most frequent HLA-D antigens are HLA-Dw5 (28.23%) and HLA-Dw1 (26.61%) and there was no significant difference in HLA-D antigen frequencies between Croatian population and other Caucasoid populations in Europe.

Table 1. The HLA-D antigen frequencies in 124 nonrelated individuals from the population of Croatia

Antigen HLA-D	women N=62	men N=62	total N=124
HLA-Dw1	25.81	27.42	26.61
-Dw2	20.97	19.35	20.16
-Dw3	17.74	16.13	16.94
-Dw4	14.52	12.90	13.71
-Dw5	29.03	27.42	28.23
-Dw6	19.35	17.74	18.55
-Dw7	14.52	16.13	15.32
-Dw8	8.06	6.45	7.26

The evaluation of HLA-D compatibility between two individuals using lymphocyte culture test seems to be useful as well as the HLA-D typing with HTC, Table 2.

The disagreement in the determination of HLA-D compatibility using these two methods was found only in 4.8% of tested couples.

It is evident that the HLA-D typing may be useful in some cases of paternity evaluation, but further analysis of HLA-D and DR determinants would bring a final decision upon this matter.

THE FREQUENCIES OF HLA-D ANTIGENS IN THE POPULATION OF SR CROATIA

Marijan Gerenčer, Marija Tomašković, Slobodan Pfeifer, Vesna Mezulić and Andrija Kaštelan (Tissue Typing Centre and Blood Bank, KBC Rebro, 41 000 Zagreb, Yugoslavia).

Despite of the significant correlation between the majority of serologicaly defined HLA-DR antigens and HLA-D antigens defined by homozygous typing cells (HTC), the correlation between HLA-Dw6/DRw6 and some newly defined HLA-D/DR antigens is rather low. Assuming these facts and the findings of crossing over between HLA-D and DR loci (1,2,3), one may draw conclusion that HLA-D and DR are two distinct but very closely linked loci. However, the relationship between HLA-D and DR determinants is not clearly understood.

The HLA-D antigens are not commonly used in paternity testing, but they may be useful in some cases as well as in the population and in HLA and disease studies (4).

We have typed 62 couples randomly taken from the forensic medicine cases of paternity testing. The HLA-D typing was performed using 4 HTC for each HLA-D specificity, except 3 for determining HLA-Dw7 and 2 for HLA-Dw8 specificity. The 11 HTC were of local origin, and the others were submited by Prof.J. Van Rood (Leyden) and Prof. H. Grosse-Wilde (Essen). The relative responses to each HTC were double normalized (DNRR) according to the method of Mendell et al. (5). DNRR below 40% represented a typing response. An individual was considered to be positive for certain HLA-D specificity when 3 out of 4 typing responses to relevant HTC were obtained. The mixed lymphocyte culture test (MLC) was also performed in all couples to determine the degree of HLA-D compatibility between the woman and man in each couple. DNRR below 35% in both directions identified HLA-D compatibility, and DNRR below 70% HLA-D haploidentity.

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$$Q_{i,j}^{m} = \frac{N_1 \times Q_{i,j}^{1} + N_2 \times Q_{i,j}^{2} + \ldots\ldots + N_n \times Q_{i,j}^{n}}{N_1 + N_2 + \ldots\ldots + N_n}$$

in which $Q_{i,j}^{1}$, $Q_{i,j}^{2}$, etc. represent the Q values that are deduced from various haplotype frequency tables for a given HLA-A,B haplotype, and N_1, N_2 etc. are the sample sizes belonging to the various tables.

These mean Q values are used, together with the estimated gene frequencies of a sample from a local or regional population of limited size to compute the two-locus haplotype frequencies of that population:

$$p(Ai,Bj) = p(Ai).p(Bj).Q_{i,j}^{m} \quad \text{or} \quad p(Bj,Ck) = p(Bj).p(Ck).Q_{j,k}^{m}$$

This procedure may be assumed to yield relatively reliable two-locus haplotype frequency estimates, especially if the total size of the samples on which the various applied haplotype frequency tables were based, was considerably large.

Our second preliminary study concerned three-locus haplotype frequencies. It resulted into the conclusion that in most cases (for most of the B-alleles) three-locus haplotype frequency estimates can be calculated reasonably reliably by the formula

$$p(Ai,Bj,Ck) = \frac{p(Ai,Bj).p(Bj,Ck)}{p(Bj)}$$

For a few B-alleles the formula appeared unreliable; the most relevant of them concerns three-locus haplotypes with the B44 allele. Most probably this is due to the fact that B44 represents a so called 'broad specificity' that can be expected in the next future to be splitted up into a number of 'short specificities', each of them with its own linkage relations with certain HLA-A and C alleles.

Combination of the two aspects described above leads to the formula:

$$p(Ai,Bj,Ck) = p(Ai).p(Bj).p(Ck).Q_{i,j}^{m}.Q_{j,k}^{m}$$

Especially with regional population samples of limited size, and with two-or three-locus haplotypes of rather low frequency, the results with the calculation methods that are described above may be assumed to be much more reliable than the estimates that would be deduced from the distribution of the phenotypes in the sample.

Estimation of HLA haplotype frequencies in national or regional populations.

L.E.Nijenhuis, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands

With about 20 HLA-A alleles, 40 B alleles and 10 C alleles, the total number of different HLA-A,B,C haplotypes amounts to 20 x 40 x 10= 8000. This implies that the greater part of the three-locus haplotypes will have low or even very low frequencies and that, even in rather large population samples, the estimates of these frequencies will have relatively considerable standard errors.
Even if those estimates could be obtained from direct countings of haplotypes, the sampling errors of most of them would be considerable. This is the more so where haplotype frequencies must be calculated from observed phenotypes, due to the multiple genotypical interpretations that are possible for each phenotype.
Each two-locus HLA-phenotype has at least two different genotypical possibilities; with three-locus phenotypes that number amounts to minimally four. These minimal numbers of genotypical possibilities are valid for full-house phenotypes, i.e. if visible heterozygosity exists for each of the two or three (A and B, or B and C, or A and B and C) genes involved in it. However, with part of the phenotypes, one or more of the genes may be represented by only one visible allele. Since one can never be sure whether such a situation should be explained as homozygosity for that allele, or rather as heterozygosity with a 'blanc' allele, the number of genotypical interpretations of certain phenotypes may be increased from 2 up to 5 for two-locus phenotypes, or from 4 up to 14 for three-locus phenotypes. And this situation greatly increases the standard errors of the estimated haplotype frequencies.
In comparison to the errors of the haplotype frequency estimates, those of the calculated gene frequencies of the separate HLA-A,B or C genes are relatively small.
Consequently, also the reliability of estimates of the haplotype frequencies could be assumed to be markedly improved if it were possible to calculate haplotype frequencies directly from gene frequencies.
It is, however, generally believed that such procedures are impossible, due to the wellknown linkage disequilibria between the separate HLA genes. Two preliminary studies have shown that that impossibility may appear less absolute than is generally believed.
The first study concerns two-locus haplotypes.
The results of the study suggest that, although gene-frequencies may vary between related populations, linkage disequilibrium relations are much more stable.
These equilibrium relations can be expressed as the quotient (Q) of the 'observed' haplotype frequency and its expectation (i.e. the product of the frequencies of the composing alleles):

$$Q_{i,j} = \frac{p(Ai,Bj)}{p(Ai).p(Bj)} \quad \text{or} \quad Q_{j,k} = \frac{p(Bj,Ck)}{p(Bj).p(Ck)}$$

Concerning a group of related populations (e.g. Caucasians), if a number of two-locus haplotypefrequency tables are available from the literature, mean Q values can be deduced from them:

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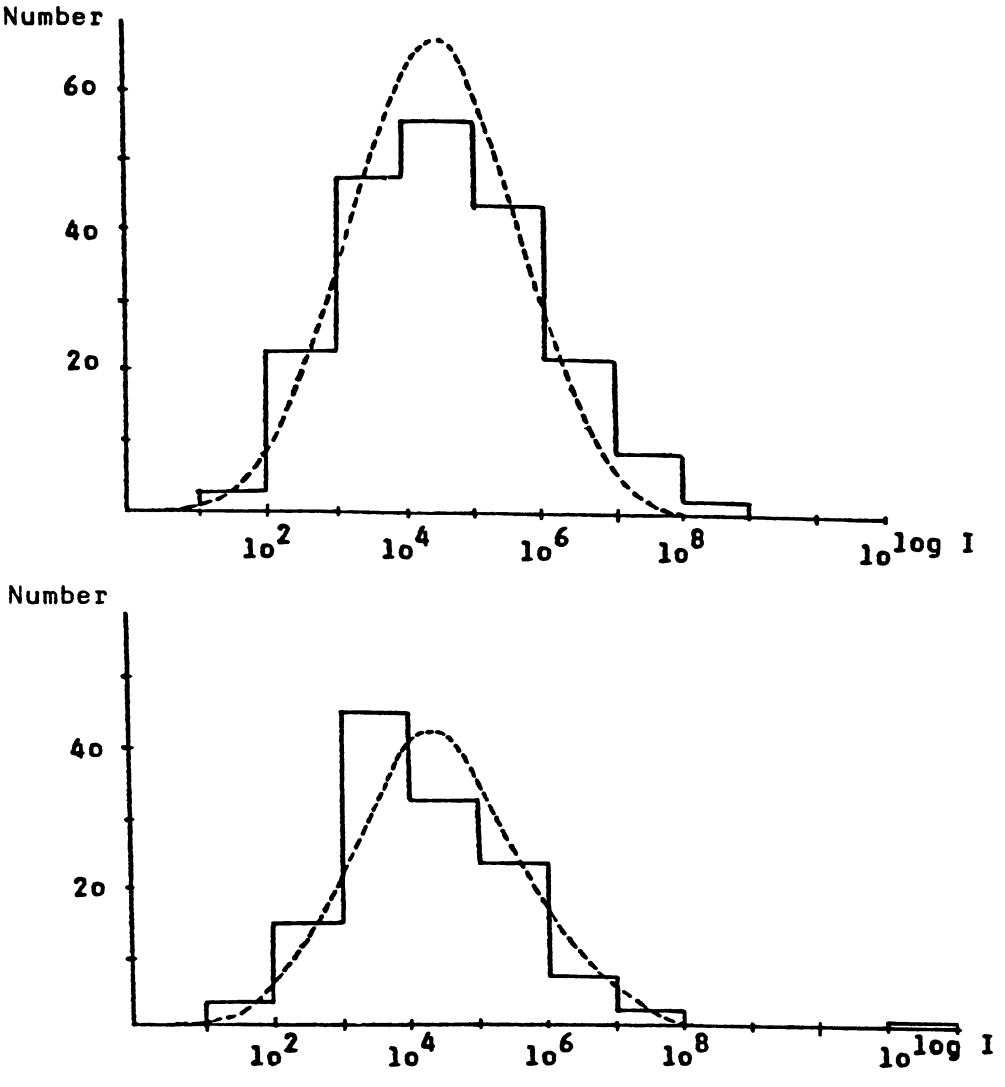
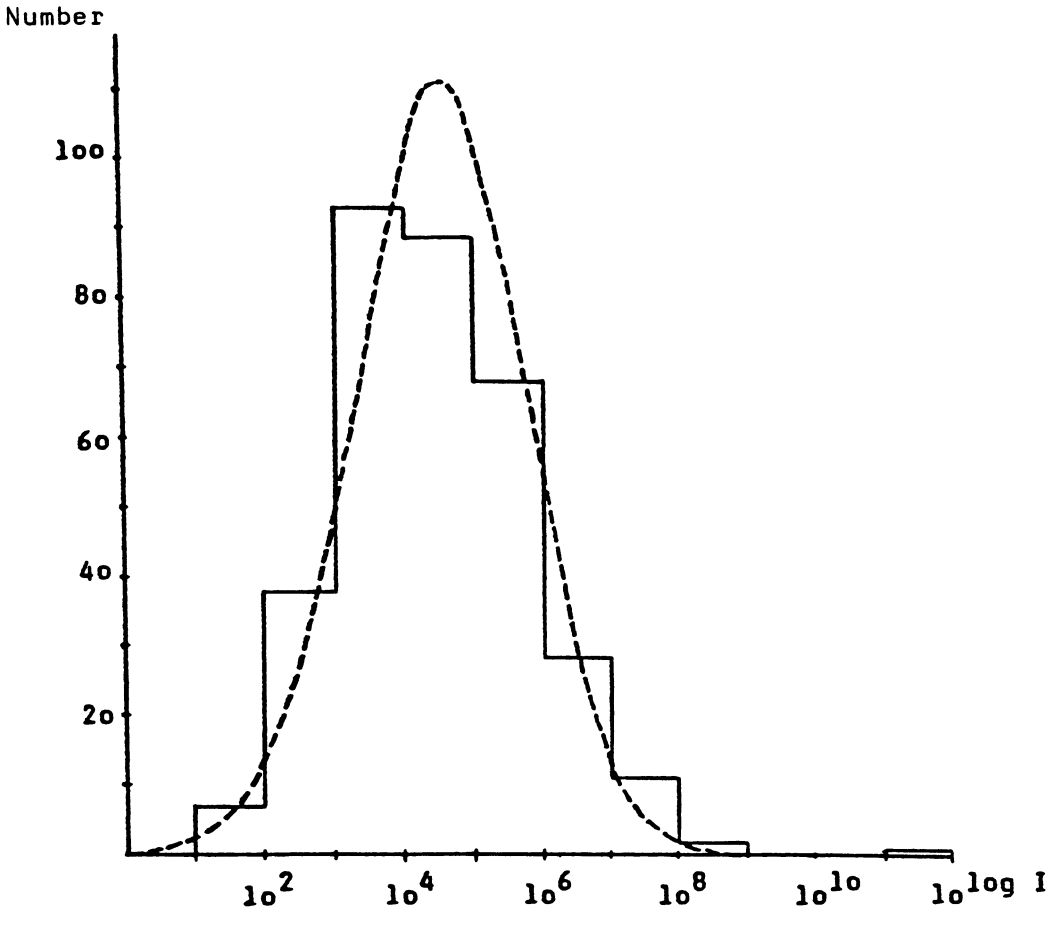
IV. Population Genetics



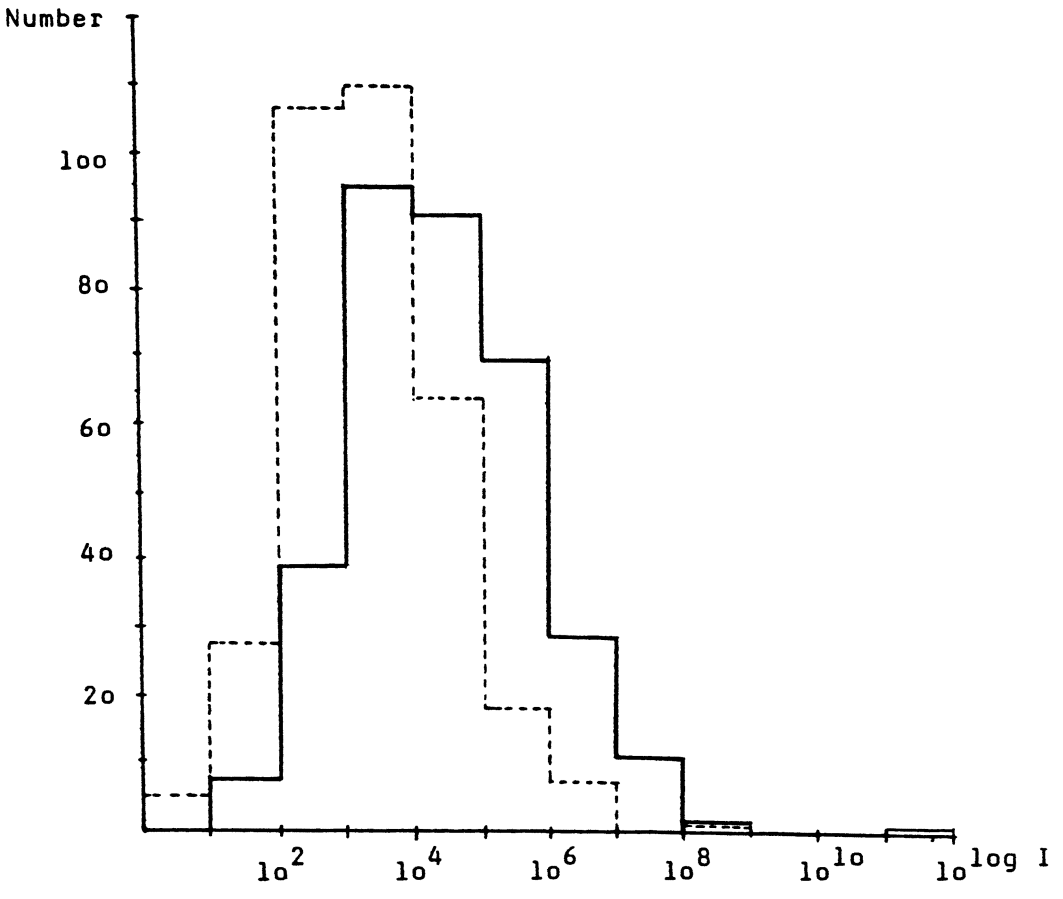
OBSERVED AND EXPECTED DISTRIBUTION OF THE PATERNITY INDEX AMONG 208 ALLEGED FATHERS FROM ONE MAN CASES (ABOVE) AND 130 ALLEGED FATHERS FROM CASES INVOLVING MORE THAN ONE MAN (BELOW).

FIGUR 3.



OBSERVED AND EXPECTED DISTRIBUTION OF THE PATERNITY INDICES AMONG 338 ALLEGED FATHERS.

FIGUR 2.



OBSERVED DISTRIBUTION OF THE PATERNITY INDICES AMONG 338 ALLEGED FATHERS BEFORE ''''''''' AND AFTER CHROMOSOME EXAMINATION ————,

FIGUR 1.

NUMBER AF EXCLUSIONS AMONG THE 178 NON-FATHERS.

CHROMOSOME	OBSERVED	EXPECTED	χ^2
1	2	6,22	2,97
9	7	12,18	2,36
3	41	43,41	o,17
4	4	2,45	o,99
13	36	52,4o	7,26 *
14	14	21,o3	2,66
15	24	23,92	o,oo
21	15	18,51	o,74
22	15	25,73	5,23 *
Y	11	7,42	1,8o

TABLE 3.

370 DANISH PATERNITY CASES WITH 515 MEN.

CHROMOSOME VARIANTS	HLA EXCLUDED	HLA NOT EXCLUDED	TOTAL
EXCLUDED	112	13	125
NOT EXCLUDED	47	343 *)	390
TOTAL	159	356	515

HLA RATE OF EXCLUSION: 0,89

EXP. NUMBER OF NON-FATHERS: 159/0,89 = 178,65

EXP. NUMBER OF CHROMOSOME EXCLUSIONS: = 178,65 x 0,7333

= 131

*) 5 EXCLUSIONS ACCORDING TO OTHER TYPE SYSTEMS.

TABLE 2.

THEORETICAL RATE OF EXCLUSIONS.

CHROMOSOME	RATE OF EXCLUSIONS	TOTAL
1	0,0348	0,0348
9	0,0682	0,1006
3	0,2430	0,3191
4	0,0137	0,3284
13	0,2933	0,5254
14	0,1177	0,5813
15	0,1339	0,6373
21	0,1036	0,6749
22	0,1440	0,7217
Y	0,0831	0,7448

TABLE 1.

paternity cases in which the ordinary blood- serum, and enzym typing has left more than one possible father. Such cases represents a selection of mother/child type constellations with a low exclusion-possibility and therefore also with a low value of the paternity index for the biological father. This is in agreement with the observed distrbution of the combined paternity indices for the 13o men.

The first exclusion of paternity according to chromosome variants (the Y chromosome) was published by de la Chapelle et al. in 1967. Examination for chromosome variants has proved usefull within different fields of human genetics. It should be emphasized that the hetermorphism described above is strongly simplified. Olson et al. have recommended the use of several staining methods i order to improve the efficiency of the examination for chromosome variants in paternity cases. However, even in the simplified version discribed above chromosome examination has proved usefull in paternity testing.

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732 persons has shown rates of discordance between o.3% and 1.7% for the different heteromorphic chromosomes and has clearly demonstrated that in order to avoid mother/child exclusions and false exclusions of paternity only clearcut differences should be considered conclusive (Niebuhr and Gürtler, 1981).

Among the 515 men in 37o paternity cases exclusion of paternity according to the HLA-system and / or chromosome variants was obtained for 172 men and futher 5 men were excluded according to other type systems. 33 cases were left without a possible father, 336 cases were left with only one possible father, and one case was left with two possible fathers, one with a paternity index of 563 and the other with a paternity index of 15.

Any close linkage between serological type systems and chromosome variants has so far not been observed. Paternity indices obtained from serological typeresults and from chromosome variants may therefore be multiplied in order to obtain a combined paternity index which include all the obtained information concerning the paternity of the alleged father.

The distribution of the combined paternity indices obtained for the remaining 338 men is shown in figur 1. A combined paternity index exceeding lo ooo was obtained for 59% of the men, a combined paternity index exceeding 1 ooo for 86% of the men, and a conbined paternity index exceeding loo for 98% of the men.

The distribution of the paternity indices obtained from the serological typeresults including the HLA type results but without the chromosome variants is drown with a dotted line in figur 1. It occurs that the chromosome variants have increased the mean of the obtained paternity indices with a factor lo.

The observed distribution of the combined paternity indices deviates from the expected normal distribution as shown in figur 2. However, as shown in figur 3 the distribution obtained for the 2o8 men from one man cases is in accordance with the expected Normal distribution. The remaining 13o men belongs to

children are known to maintain their structure. Thus the paternity of non-fathers may be excluded according to one or both of the following two rules:

1) A chromosome variant shall not occur in a child unless it is present in at least one of the parents.

2) A child must have at least one variant of each of the nine heteromorphic autosomes in common with each of the two parents.

The obtained estimate of the frequencies of the different chromosome variants in the Danish population may be used for calculating the expected rate of exclusions of paternity among non-fathers (Gürtler and Niebuhr, 1981) as shown in table 1. The expected rate of exclusions obtained by use of all the heteromorphic chromosomes is o.72 for paternity cases concerning girls and o.74 for cases concerning boys.

Table 2 concerns 37o unsolved Danish paternity cases with 515 men in which examination for chromosome variants have been used alongside with HLA typing. Exclusions of paternity according to the HLA system were obtained for 159 men. The expected rate of exclusions of paternity among non-fathers according to the HLA system during the periode within which the material was collected has been calculated as o.89. Thus the expected number of non-fathers among the 515 men shall be 159/o.89 = 178.65 and the expected number of exclusions of paternity according to chromosome variants shall be 178.65 x o.7333 = 131. The observed number was 125.

In table 3 the observed number of exclusions of paternity obtained by use of variants of the different heteromorphic chromosomes has been compared with the calculated expected number of exclusions. There is a general agreement between the observed and the expected numbers. However for chromosome 13 and chromosome 22 the observed number of exclusions is significantly lower than the expected values.

This deficit refers to cases in which the difference between the child and the mother and/or the alleged father is too small to support an exclusion. A double blindtest including

The size of the satellite as well as the size of the brilliant p 11 band is compared with that of the q 31 band on chromosome 1 and chromosome 7 and with the short arm of chromosome 16 in order to classify the chromosome variants as NN, NS, NM, SN, SM, MN, MS, MM, LN, or vLM. The symbol for the satellite is placed in front of the symbol for the p 11 band. For example SM denotes a chromosome with an intensely stained or brilliant satellite which is smaller than the q 31 band on chromosome 1 and a brilliant p 11 band which is bigger than the q 31 band on chromosome 1 but smaller than the q 31 band on chromosome 7.

The polymorphism of chromosome 1 and 9 concerns the size of the juxtacentromeric band qh which by staining with quinacrine mustard remains without any fluorescense and therefore occurs as a secondary constriction. The variants are classified as SM, L, and vL. SM denotes a chromosome on which the secondary constriction is shorter than the short arm of chromosome 16. L denotes a chromosome on which it is longer than the short arm of chromosome 16 but shorter than the short arm of chromosome 9, and vL denotes a chromosome on which it is longer than the short arm of chromosome 9.

The polymorphism of the Y choromosome concerns the size of the fluorescent distal part of the long arm and is classified as O, S, ML, or vL. O denotes an Y chromosome without a fluorescent band on the long arm. S denotes an Y chromosome which is smaller than chormosome 21 (only small fluorescent spots), ML denotes an Y chromosome which is bigger than chromosome 21 but smaller than chromosome 16, and vL denotes an Y chromosome which is bigger than chromosome 16 (three fluorescent bands).

The frequency of the different chromosome variants in the Danish population was obtained from a material of 1119 unrelated Danish males and females (Niebuhr and Gürtler, 1981). The observed distribution of the different chromosome variants in the kariotypes of the invenstigated 1119 persons was in perfect agreement with the expected Hardy-Weinberg proportions.

Chromosomes which have passed on from parents to their

CHROMOSOME POLYMORPHISMS IN LEGAL PATERNITY CASES.

H. Gürtler and E. Niebuhr.

University Institute of Forensic Hemogenetics and
University Institute of Medical Genetics, Copenhagen.

Examination for chromosome variants has been used alongside with HLA typing in unsolved paternity cases in Denmark since 1977. Preliminary results have been presented in 1977, 1979 and 1981.

Cultures of lymfocytes obtained from blood samples are used for the preparation of metaphase chromosomes from the parties. The preparations are stained with quinacrine mustard. By use of an UV photomicroscope (Zeiss III) and Agfachrom positive color film (CT 18) at least five color slides of the preparations are produced for each person. The use of color slides makes it possible to recognize the brilliancy of intensely stained chromosomal markers.

The polymorphism of chromosome 3 and 4 concerns the brilliant juxtacentromeric band q 11 which may occur on the long arm of these chromosomes. They are classified as N, S, M, L, vL, and INV.

N denotes a chromosome without a brilliant q 11 band. S denotes a chromosome for which the size of the brilliant q 11 band is smaller than that of the band q 31 on the long arm of chromosome 1. M denotes a chromosome for which the size of the brilliant q 11 band is bigger than that of the q 31 band on chromosome 1 but smaller than that of the band q 31 on the long arm of chromosome 7. L denotes a chromosome for which the size of the brilliant q 11 band is bigger than that of the q 31 band on chromosome 7 but smaller than the short arm of chromosome 16 and vL denotes a chromosome for which the size of the brilliant q 11 band is bigger than the short arm of chromosome 16. INV stands for partial inversion.

The polymorphism of the chromosomes 13, 14, 15, 21, and 22 concerns the intensely stained or brilliant satellite and also the brilliant band p 11 which may occur on the short arm of these chromosomes.

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allele alligned with one of the alleged father's alleles, he was included as a possible biological father. If on the other hand the child's paternal allele and the alleged father's alleles did not comigrate, he was excluded as an alleged biological father.

The following results were obtained. There were 29/102 (28.4%) alleged fathers excluded by DNA and also 29/102 excluded by HLA 25 of which were excluded by both HLA and DNA. The average paternity index of included alleged fathers based on the racial frequency of the observed alleles was 91.6% for DNA. This compares well with HLA A and B which has an average paternity index of about 90%. Although the estimation of paternity is subject to interpretation by the courts, a number of 90.0% is often accepted as evidence along with other proof that an alleged father is the true biological father.

There were 8 cases where there was disagreement between the DNA and HLA results. In 4 cases, the alleged father was excluded by DNA but included by HLA. In 4 cases, an alleged father was excluded by HLA but not DNA. These discrepancies point out the difficulty in relying on only a few genetic systems to establish biological paternity. In combination DNA and HLA would have excluded 33/102 (32.4%).

Of the 29 alleged fathers excluded by DNA RFLP analysis, 26/29 (89.7%) were excluded by Eco R1 and 11/29 (37.9%) were excluded by Taq 1 digestions. Thus as might be expected by the larger number of alleles, Eco R1 digested DNA hybridized with the pAW101 probe was much more informative in assignment of biological paternity. As a mater of fact, in only 2/29 (6.9%) cases were alleged fathers excluded by Taq 1 and not Eco R1 digestions. Finally, in 9/29 (31.0%) cases were alleged fathers excluded by both Eco R1 and Taq 1 digestions.

Conclusion

The assignment of paternity in legal situations is aided by biological testing of alleged fathers. The use of DNA restriction fragment length polymorphisms can help in these determinations. A comparison of the results obtained by HLA and DNA RFLP analysis indicated that DNA can help in the determination of biological paternity. Among 102 paternity trios examined by the two methods, similar numbers of alleged fathers were excluded by both methods and the paternity index calculated for both methods was comparable. These results indicate that DNA restriction length polymorphisms can add greatly to the assignment of biological paternity.

pLM0.8. The probe pAW101 (kindly supplied by R. White) consists of a 6.5 kilobase (kb) Eco R1 insert in the plasmid pBR322, derived from the D14S1 locus on chromosome 14, and is polymorphic with Eco R1 (4). The probe pLM0.8 is a 879 base pair Cla 1-Sph 1 insert in pBR322 derived from the 3' flanking region of the Harvey Ras oncogene. This locus is localized in chromosome 11 (5) and is polymorphic with Taq 1. Each probe was used as an insert, purified from the pBR322 vector.

Results and Discussion

The two probes used in this study detect RFLPs corresponding to unique sequences in the human genome that are inherited in a Mendelian fashion (4,6). With each probe, one (homozygote) or two (heterozygote) bands are seen in each individual.

Allele frequency data was collected for Caucasians, Hispanics, and Blacks from random, non-related blood donors from the New York City area. The number of alleles observed for the pLM0.8 probe hybridized with Taq 1 digested DNA was 18 with sizes ranging from 1.8 to 4.5 kb. The size of the fragments were measured to the nearest 10 base pairs. Under the standard conditions used to fractionate the DNA, the resolution of two adjacent alleles was approximately 40 base pairs in the 2.60 kb size region and 60 base pairs in the 4.0 kb region. However, an increased separation of the DNA fragments, by the use of longer gels, failed to reveal alleles varying in size by 20 or 30 base pairs. In all three racial groups, the 2.59 Kb allele predominated. Although most of the alleles were observed among the three races, the allele frequencies were different among the three groups. For the pAW101 probe hybridized with Eco R1 digested DNA we could resolve bands varying in size by 200-300 base pairs. Therefore, the D14S1 alleles could be grouped in over 30 size groups, ranging from 14.3 to 32.5 kb. In this size range the fragments were measured to the nearest 100 base pairs. For all three racial groups, the majority of fragments were between 14.3 and 17 kb, but again the frequency of the various DNA fragments varied among the groups.

The allele frequencies obtained for these two polymorphisms were used to calculate the likelihood that the allele present in both the child and the alleged father was the same.

The results of 102 paternity trios analysed for DNA RFLPs were compared with the HLA and protein determinations. DNA determinations were done in duplicate and the samples were displayed on gels with the child between the mother and alleged father flanked by size standards. With this type of layout it was easy to visualize which of the alleles of the child had been inherited from the mother and which one might correspond to the alleged father's alleles. If the child's paternal

7.6; 5 mM MgCl; 1.0% Triton X-100) followed by centrifugation at 2,000 x g for 10 minutes at 4°C. The nuclei were resuspended in 1 - 2 cc of DNA lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 10 mM MgCl) containing 100 ug/ml Proteinase K and 1.0% SDS. After 4 to 16 hours incubation at 37°C with gentle rocking, sodium perchlorate was added to 1.0 M. Samples could be stored at 4° C for months or processed into DNA by extracting twice with phenol-chloroform (1:1) followed by two extractions with chloroform, then dialysis against 3 changes of a 1000 fold excess of TE (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). DNA concentrations were determined by absorbence at 260 nm.

Restriction Endonuclease Digestion, Electrophoresis, and Hybridization

Five micrograms of each DNA was digested to completion with a five fold excess of Eco R1 or Taq 1 (Bethesda Research Laboratories) restriction endonuclease using conditions recommended by the manufacturer. Samples were routinely concentrated by the addition of ammonium acetate to 0.3M and 2.5 volumes of cold 95% ethanol, stored at -20°C overnight, and the DNA pelletted by a 10 minute centrifugation in a microcentrifuge (10,000 x g). After briefly drying the pellet, the DNA was redigested with a five fold enzyme excess in a final volume of 20 microliters.

Digested DNAs were size fractionated at room temperature by agarose gel electrophoresis in recirculated TAN buffer (10 mM Tris-HCl, pH 7.9; 5mM sodium acetate; 1 mM EDTA). Eco R1 digested DNA was electrophoresed in 0.4% agarose at 0.6 volts/cm for 65 hours while Taq 1 digested DNA was electrophoresed in 1.2% agarose at 1.8 volts/cm for 20 hours. The length of the agarose gels varied from 15 to 22 cm. Appropriate DNA size markers were included and DNA was visualized by ethidium bromide staining with ultraviolet illumination.

After soaking the gel in denaturation solution (0.3 M NaOH, 0.5 M NaCl) followed by neutralization solution (0.5 M Tris-HCl, pH 7.0; 0.4 M NaCl), the DNA was transferred to a nylon membrane (Zetabind, AMF Cuno). Specific cloned DNA sequences were labelled with ^{32}P by nick translation and hybridized to the the nylon filters using standard procedures (3). After washing away unhybridized probe, the filter was exposed to x-ray film (Kodak, X-omat) at -70° C in a cassette with Lightning Plus screens (DuPont) for 20 to 72 hours. Fragment sizes were determined by relative mobility to standards using a digitizing pad interfaced with a DEC PDP-11 computer.

Recombinant DNA Probes

The two DNA probes used in this study were pAW101 and

APPLICATION OF DNA POLYMORPHISMS TO THE DETERMINATION OF PATERNITY

I. Balazs, K. Wexler, L. Nicholas, L. Miyazaki, A. Giusti, M. Baird, *P. Rubinstein, *F. Allen, *L. Sussman, J. Glassberg (Lifecodes Corporation, Elmsford, N.Y. 10523; *New York Blood Center, New York, New York 10029, U.S.A.)

Abstract

The Mendelian inheritance of polymorphic DNA sequences makes them useful for paternity determinations. We examined the DNA polymorphisms associated with the genetic loci D14S1 and HRAS1 in more than 100 paternity cases. The objective of these tests was to first determine the phenotypes (ie. the size of polymorphic fragments) of the mother, child and alleged father and second to use this information in assignment of paternity. In addition, the HLA haplotype of these individuals was established and used to determine paternity. A comparison of these two methods for the analysis of paternity cases indicated: 1) the two DNA polymorphisms used in this study excluded the same number of alleged fathers from paternity cases as HLA, 2) both methods resulted in similar paternity indices.

Introduction

Biological testing to help determine paternity includes ABO, HLA, and protein polymorphism analysis. In combination, these tests can exclude or include an alleged father with a high degree of certainty (1). Current technology allows the direct examination of DNA from specific heritable changes through the use of restriction endonucleases and specific probes (2). This report examines the application of DNA restriction fragment length polymorphism analysis to the assignment of paternity.

Materials and Methods

Sample Origin

Blood samples were collected at the New York Blood Center in New York City as either non-related volunteer blood donors or as paternity trios. Information concerning race was provided. All samples were collected using potassium EDTA as anticoagulant.

DNA Purification

DNA was isolated from the white cells. First the red cells were lysed by the addition of 4 volumes of 4°C blood lysis buffer (0.32 M sucrose; 10 mM Tris-HCl, pH

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Recent Research on Human DNA-polymorphism

Lars Bolund, Institute of Human Genetics
University of Aarhus, Denmark

The recent developments in recombinant DNA technology have opened up entirely new possibilities in the study of genetic variation and inheritance. Thousands of fragments from the human genome have been amplified by bacterial cloning and used as probes to detect polymorphisms in the restriction enzyme cleaving pattern of the homologous sequences in the genome. Many laboratories, including our own, have mapped and characterized a number of such restriction fragment length polymorphisms (RFLPs). Approximately 1000 such chromosomally localized RFLP markers have now been described and more are coming.

The RFLP analysis is presently done by digestion of extracted DNA from a cell sample from different individuals with suitable restriction enzymes followed by electrophoretic separation of the generated fragments according to size and visualization of the relevant fragments by hybridization with radioactively labelled probes and autoradiography.

Since the analysis can be performed on relatively small quantities of DNA and even on DNA from blood and semen stains, this opens up the theoretical possibility of identification and paternity testing with next to unlimited precision. However, the analysis involves the use of a great number of different probes and will thus be time consuming and expensive even if the methodology is being simplified by automatization and non-radioactive labelling.

Of particular interest in this connection are some RFLP markers that consist of tandemly repeated sequences. Especially the so called α-repeats that are clustered in the centromere regions of the chromosomes and are organized as a number of basic units (approximately 170 base pairs long) that form a block that in turn is reiterated manyfold. Recently probes have been developed that selectively hybridizes to chromosome specific variants of the basic repeat. If the DNA is cleaved with a restriction enzyme that does not cleave in the basic repeat and then analyzed with such a probe most of the homologous DNA is in very long fragments but due to individual variation in rare basic repeats a ladder of small fragments is also generated. It has been claimed that every X chromosomal centromere can be recognized by its unique pattern of fragments with this type of analysis. Such highly informative polymorphisms of course obviates the need for multi marker analyses. Though mendelian inheritance of these markers have been demonstrated, it is not yet completely clear whether they are stable enough to permit practical use even in paternity testing.

Another possibility is to use a probe that is homologous to repetitive, highly polymorphic sequences spread throughout the genome. With this technique one can obtain a complex pattern of fragments that is more or less unique to the individual. It is presently unclear if this analysis is reproducible enough to be clearly interpreted and practically managable in forensic medicine.

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III. DNA and Chromosomes

18K by SDS electrophoresis, were less evident or almost lack in parotid secretions, and did not show high affinity to hydroxyapatite. Some monkeys possessed several other protein bands, each having different mobility, suggesting that genetic variation of the "Fast" proteins occur in macaques.

By starch-iodine method individual saliva showed one or two main amylase isozymes with accomapnying minor ones. Various phenotypes were observed and 5 different main isozymes (presumable alleles) have been discriminated in the samples. In contrast to humans, salivary amylase appears to be highly polymorphic in macaques.

Salivary Protein Variation in Macaque Monkeys

Goichi Ishimoto (Department of Legal Medicine, Mie University School of Medicine, Tsu, Japan)

Several human salivary proteins are known to exhibit genetic polymorphism, but little information is sofar available in non-human primates as to such phenotypic variability. We tried to collect a considerable number of saliva samples from Japanese monkey(Macaca fuscata) and also other species of macaques to examine their salivary proteins.

Whole saliva was obtained by drooling from pilocarpine-stimulated animals of 42 M.fuscata, 6 M.mulatta and other 18 monkeys from 6 species. Parotid saliva was obtained by ductal cannulation in some selected animals. Analysis was mainly performed by polyacrylamide gel electrophoresis in alkaline buffer system.

Compared with humans macaque salivas showed unique patterns in anionic gels. When stained with dimetoxybenzidine, individual salivas disclosed one broad zone. This component constituted of major protein in monkey saliva, and probably corresponds to so-called MPRP(macaque proline-rich), since proline was highest in amino acid composition in the purified proteins. Same pattern was observed in all of 66 samples, indicating that macaque PRP is monomorphic.

Intra-species variation was detected in far-anodal region of the protein-stained gels, in which 2 bands and 3 types were distinct in M.fuscata. Differing from MPRP, the proteins, whose molecular weight were calculated to be about

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Results and Discussion

Out of 8686 investigated mother-child combinations ten incompatible pairs were found, which gives a frequency of 0.0012 (table I).

Eight of these exclusions show contrary homozygosity. No extra bands have been observed by seven of them, pointing towards some rare allele, consequently the probable explanation is the occurence of a silent allele. Neither family studies nor quantitative enzymatic investigations have been performed. The eighth mother-child exclusion a1/a3 show a discrepancy against the AGE typings where both mother and child were typed as 2-1. A closer investigation revealed a very weak blur in the a2 position on the IEF-gel. This problem is earlier discussed (Svensson, Wetterling, 1982) and if the explanation is some rare allele, unsensitivity in the method or both I will leave unsaid.

Furthermore, two pairs show contrary heterozygosity (a4a1/a3a2 and a3a2/a1). A larger list of a Swedish PGM_1^a material exhibits an excess of the type a3a2 and a corresponding deficit of a2a1 (table II). This would imply that certain a2a1 were mistyped as a3a2. But there is no such risk due to similarities between the phenotype patterns. Experimentally by repeated freezing and thawing of the blood samples the PGM_1^{a1} allele shows a clear trend to go over to the PGM_1^{a3} allele after about three weeks. This indicates that the PGM_1^{a1} allele in some way is labile. However, no discrepancies of the type a3a2 against a2a1 have been observed since the subgrouping of PGM started and the child a3a2 with mother a4a1 have been clearly typed on fresh samples by three occasions. Heterozygote mother-child exclusions have also been reported by Martin (1981) a3a2/a4a1 and by Vivian Johnsson, Helsinki, a3a2/a1 (personal communication).

Conclusion

Due to problems with the relatively high frequency of silent and rare alleles and even supposed incorrect typings within PGM_1^a, it is recommended not to base any exclusions in paternity cases on this system solely.

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Table I. Mother-Child exclusions within PGM_1 during 1980-1984 in Sweden (8686 combinations tested).

	Child					
Mother	a1	a2	a3	a4	a3-a2	Total
a1		1	1	1		3
a2	2					2
a3	3					3
a3-a2	1					1
a4-a1					1	1
Total	6	1	1	1	1	10

Table II. Distribution of PGM_1^a phenotypes in a sample of 6800 unrelated adult Swedish persons.

Phenotypes PGM_1^a	Observed n	Expected n	X^2
a1	2804	2751.9	0.9864
a2a1	1186	1241.9	2.5162
a3a1	1313	1356.6	1.3630
a4a1	546	547.6	0.0047
a2	146	140.1	0.2485
a3a2	349	306.1	6.0124
a4a2	127	123.6	0.0935
a3	169	167.2	0.0194
a4a3	132	135.0	0.0667
a4	28	27.2	0.0235
Total	6800		11.3343

For d.f. = 3 $p < 0.05$
The rare phenotypes are not included.

INCOMPATIBLE MOTHER-CHILD PAIRS FOUND IN THE PGM_1 SYSTEM.

Gunilla Wetterling (State Institute for Blood Group Serology, SRL, Regionsjukhuset, S-581 85 Linköping, Sweden)

Introduction

Since Spencer et al. (1964) demonstrated polymorphism within human red cell phosphoglucomutase incompatible mother-child pairs have been reported. When Bark et al. (1976) succeeded in subtyping PGM_1 this deviation from the rules of inheritance also appeared within the subgroups revealing incompatible homozygosity (Prokop et al. 1981 and Polesky et al. 1983) as well as incompatible heterozygosity (Martin, 1981).

The present paper calls in question the use of PGM_1^a as a single excluding marker in paternity cases.

Material and Methods

Hemolysates

Venous blood samples from 8686 mother-child pairs involved in cases of disputed paternity in Sweden during the years 1980-1984 were collected without additive. The blood cells were washed three times with isotonic saline and lysed by the addition of 0.2 % mercaptoethanol followed by freezing and thawing to achieve complete hemolysis. The lysates were tested within a week after venepuncture. All samples were examined twice and the mother-child exclusions were confirmed by repeated sampling.

Isoelectric focusing (IEF)

Apparatus: LKB 2117 Multiphor; LKB 2103 Power Supply. Gel: LKB ampholine PAGplates pH 5-6,5 and 0.5 mm gels (T = 5.5 %; C = 3.3 %) containing 4 % LKB ampholine pH 5-7. Electrode solutions: 0.01 M sodium hydroxide (cathode) and 1 % acetic acid (anode). Temperature: + 5°C. Application: The hemolysates were applied directly onto the gel surface with 25 ul microcaps 1.5 cm from the anode. Focusing procedure: Prefocusing was performed during 30 min at 2000 V, 20 mA and 25 W followed by 2.5 h of focusing at the same adjustment. Identification: The phenotypes were visualized by an agar overlay method (Sutton and Burgess, 1978).

Agarose gel electrophoresis (AGE)

All samples have also been investigated by thin layer agarose (0.8 % Litex HSC) gel electrophoresis. This was performed with a 0.025 M tris-maleic buffer as bridge buffer and 1/5 dilution as gel buffer during 2 h and at 12 V/cm.

Nomenclature

The nomenclature proposed by Kühnl et al. (1977) was used.

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Address: Institute of Forensic Medicine, C-Skłodowskiej 3 a, 80-210 Gdańsk, Poland.

There are several advantages of PGP polymorphism with respect to its application in forensic serology:

1 - the isozyme types can be detected by simple method in starch gel electrophoresis;

2 - good distribution PGP alleles in the population studies and its established formal genetics;

3 - high relative chance of exclusion non-father of this system;

4 - possibility detectable isozyme types in bloodstains material.

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sults and the values reported by other authors (1,2,3,4,5,6,7). Tabele 2 presents the distribution of PGP phenotypes in 174 mother-child pairs.

Tabele 2. Determination of the PGP types in mother-child pairs.

Mother	Child						Total
	1	2-1	2	3-1	3-2	3	
1	102	18	-	2	-	-	122
2-1	15	12	2	3	2	-	34
2	-	4	-	-	-	-	4
3-1	5	2	-	4	-	-	11
3-2	-	3	-	-	-	-	3
3	-	-	-	-	-	-	-
Total	122	39	2	9	2	-	174

Investigation of mother-child pairs didn't reveal any deviation from Mendelian proportions. A mother-child exclusion was not observed. Up to now no "silent" allele and rare variants has been found in our material. The possibility to exclude non-father from paternity by means of the PGP-system is 12.6% in the Polish population.

In the material interpreted experimental stains the correct determination of PGP phenotypes was possible in all the cases of absorbed bloodstains up to 5 weeks old. In 3 cases it was also possible to determine PGP phenotypes in older blood stains - up to 7 weeks old, Tabele 3.

Tabele 3. Summary of experimental bloodstains of known PGP phenotypes.

No. of stains	Stain substrate	Age of stains		Correctly typed
		35 days	49 days	
20	cloth	17	3	20

factors was observed in 174 mother-child pairs.

Determination of PGP phenotypes from blood stains has been obtained. 20 samples of blood with known PGP phenotypes were poured on strips of white cloth and dried at room temperature. Strips of the stained cloth of size of about 4 square mm were put directly on the starch gel with a drop of the electrophoresis buffer. Electrophoresis conditions and staining were the same as the method used for fresh blood.

Result and discussion.

PGP isoenzyme bands are found between 6 and 8 cm from the origin on the anodal side of gel. Six isoenzyme patterns 1,2, 2-1,3-1,3-2 and 3 were recognized. Basing on phenotype frequencies, frequency of PGP genes in the Polish population were calculated. The results of the present investagation are given in Tabele 1.

Tabele 1. Phenotypes and gene frequencies of PGP in a population sample of North Poland.

Phenotyp	Phenotypes frequencies				Gene frequencies
	Observed		Expected		
	n	%	n	%	
1	462	73.22	456	72.27	
2-1	118	18.70	123	19.49	PGP^1=0.8502
2	10	1.58	8	1.27	
3-1	31	4.91	34	5.39	PGP^2=0.1165
3-2	9	1.43	4	0.63	
3	1	0.16	6	0.95	PGP^3=0.0333
Total	631	100.oo	631	100.oo	

Observed and expected values are in good agreement under Hardy-Weinberg conditions. The estimate for the gene frequency of PGP a comparison of different caucasian gene frequency data shows that there are no significant differences between our re-

Szczerkowska Zofia
Institute od Forensic Medicine, Gdańsk, Poland.

APLICATION OF THE POLYMORPHISM OF PHOSPHOGLYCOLATE PHOSPHATASE IN FORENSIC MEDICINE

Phosphoglycolate phosphatase (PGP, EC. 3.1.3.18) found in red blood cells as well as in other tissues is considered to have an influence on oxygen transport by inactivating phosphoglycolate and increasing the level of 2,3-diphosphoglycerate(8).

The polymorphism of PGP was first described by Barker and Hopkinson(2). The six common phenotypes PGP 1,2,2-1,3,3-1 and 3-2 have been interpreted by these authors as a products of the different combinations of three alleles PGP^1, PGP^2 and PGP^3 at an autosomal locus.

Numerous population and family study (2,3,5,6) as well as possibility detectable PGP isozyme in blood stains material (4) proved that this genetic marker may be useful in the forensic serology.

This paper reports on phenotype and gene frequencies in a population sample from North Poland and detemination of PGP phenotypes from bloodstains.

Material and method.

Blood samples of 631 healthy and unrelated adults were investigated according to the method of Barker and Hopkinson (2) with following modyfications: the buffer system used was a 0,2 M TEMM pH 7,2. The gel buffer was prepared by diluting the stock buffer 1 in 15. The starch gel were made with 11% hydrolysed starch and 1 mM 2-mercaptoethanol. Electrophoresis was carried out at 110V 40mA for 22 h at $4^{\circ}C$. The staining procedure was the same as the original method. The mode of inheritance of PGP

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always "subtyped" in contrast to isofocusing where in practice only ESD 2-1 and 2 are retyped to detect ESD 5. ESD variants are very rare in our material. ESD 4-1 is situated very cathodically. Staining of this part of the gel is imperative to detect these variants. The most frequent variant encountered was ESD 7-1.

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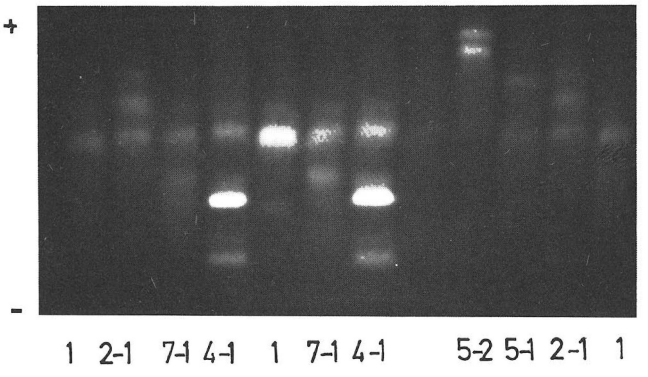

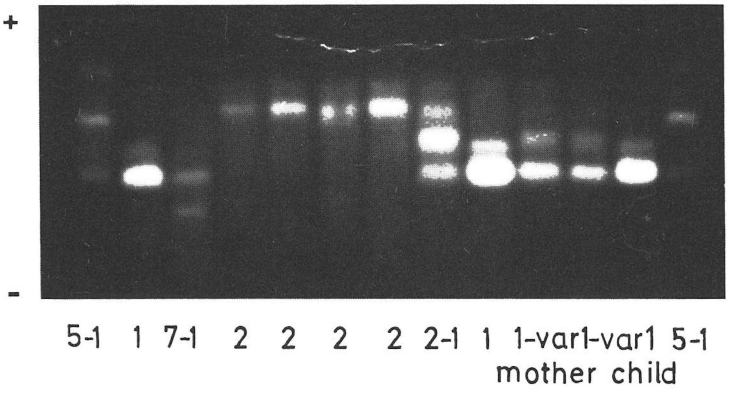

ELECTROPHORETIC "SUBTYPING" OF RARE ESD VARIANTS

W. Bär and V. Biedermann, University of Zürich, Switzerland, Institute of Forensic Medicine

1. Introduction

The common allozymes 1,2 and 5 of red cell esterase D can be determined in a clear-cut manner by agarose gel electrophoresis using a continuous buffer system at pH 5.35.-5.45 composed of malic acid (1). At acidic pH values the allozyme 5 migrates definitely more anodically than the allozyme 2; at neutral pH this distinction is ambiguous. With this new electrophoretic method, isoelectric focusing of ESD 2-1 and 2 is not necessary anymore to detect the gene product.of ESD 5. To show the usefulness of the electrophoretic method to detect rare variants was the aim of this investigation.

2. Methods

2.1. Sample Preparation

Blood was collected by venepuncture and hemolysates were made from washed and packed red cells by dilution 1:1 with redistilled water followed by freezing. Destromatisation of the hemolysates was effected by CCl4.

2.2. Electrophoresis

Gel buffer: 12 mM malic acid
Bridge buffer: 100 mM malic acid
pH-value: 5.35 - 5.45 (adjust pH definitely after 24 h)
Agarose: Seakem LE50002 FMC Corp.
Sample Application: 6 cm from the cathodical edge with a sample applicator(Code No. 19-29411-11 Pharma.)
Electrophoresis: 24 v/cm, 3,5 - 4 hrs, 5 C
Paper wicks: 4 layers of Whatmann No. 1
Sample Staining: 1 mg umbelliferyl acetate disolved in acetone and buffered by a a phosphate buffer og pH 6.9. Filter paper overlay placed both anodic- and cathodically

3. Results and Discussion

Different red cell ESD variants can be detected by electrophoresis at acidic pH values. The split of ESD 1 in ESD 7-1 described by Nishigaki (2) for isofocusing is also detectable with our method (3). Furthermore, when using the electrophoretic method, ESD 1 is

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In a case of paternity testing we observed an incompatibility between a mother and her child. The mother seemed to be homozygous for ESD*2 and the child homozygous for ESD*1.
Measurement of enzyme activity showed the following results:
ESD1-1: 128.48 U ± 21.18; ESD2-1: 115.20 U ± 16.04; ESD2-2: 98.30 U ± 14.78; ESD5-1: 82.15 U ± 16.98 .
The ESD 2-QO type had an activity of 50.3 U, which is 51.2 % of normal ESD2-2 and the ESD 1-QO type had 58.9 U, which is 45.8 % of normal ESD1-1.

Quantitative and IEF results point to a mutational event that deleted or completely inactivated one allele and results in a silent allele ESD*QO with the phenotypes ESD 1-QO and ESD 2-QO.

Acknowledgement

We like to thank Dr. W. Bär, Institute of Forensic Medicine, University of Zurich for providing us reference samples of ESD 7-1 and ESD 5.

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phenotype	observed		expected	
	n	%	n	%
ESD 1-1	518	76.85	515.54	76.49
ESD 2-1	118	17.51	124.15	18.42
ESD 2-2	11	1.63	7.48	1.11
ESD 5-1	23	3.41	21.48	3.24
ESD 5-2	2	0.30	2.63	0.39
ESD 5-5	0	0.00	0.20	0.03
ESD 1-VAR	2	0.30	1.75	0.26
ESD 2-VAR	0	0.00	0.20	0.03
	674	100.00	673.43	99.97

df = 2, X^2 = 0.865

Table 1: Observed and expected phenotypes

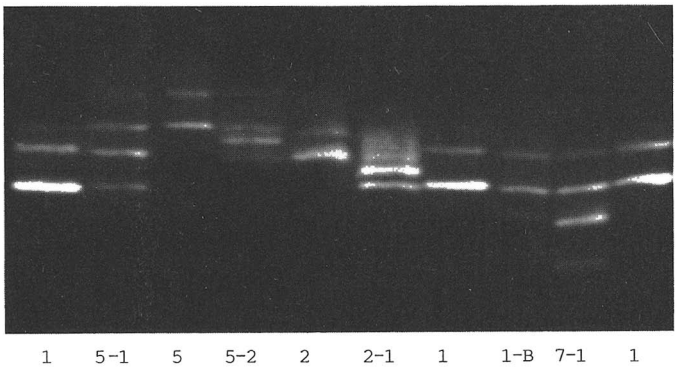

Figure 1: ESD phenotypes 1, 5-1, 5, 5-2, 2, 2-1, 1, 1-B, 7-1 and 1 as obtained by IEF on agarose gel (pH 4.5 - 5.5).

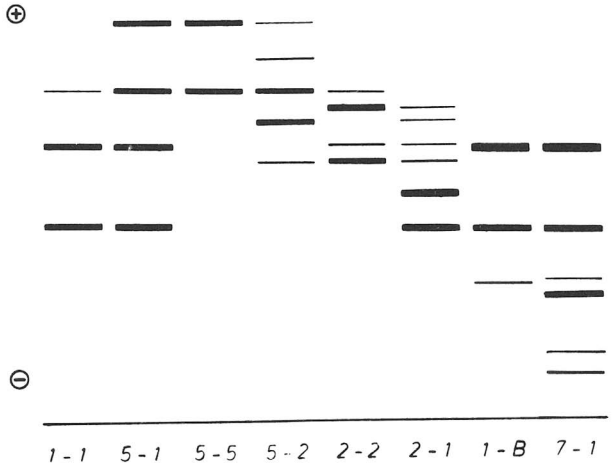

Figure 2: Schematic presentation of ESD phenotypes

thode with an application strip (SERVA).

Isoelectrofocusing: Prefocusing was carried out 25 minutes at constant power. Settings were: 1.7 W, 1500 V, 15 mA, 5°C.

Focusing was carried out 15 minutes at constant voltage (settings: 3.4 W 400 V, 15 mA, 5°C) and 60 minutes at constant power (settings: 3.4 W, 1500 V, 15 mA, 5°C).

Isoenzyme visualisation: Cellulose acetate membranes (micro solid CAF ATX -57-BBN, Biotec Fischer) were soaked in 0.1 M sodiumacetate-buffer pH 7.5 containing 2 mg 4-Methylumbelliferylacetate/5 ml and laid on the gel surface. After incubation for 10 minutes at 37°C the gel was viewed under ultraviolet light (365 nm).

Quantitative assay: Measurement of enzyme activity was carried out on a Perkin-Elmer MPF-44 A Fluorescence Spektrophotometer. The increase of fluorescence of 4-Methylumbelliferon after hydrolysis of 0.1 mM 4-Methylumbelliferylacetate was monitored with excitation at 320 nm and emission at 540 nm in 0.1 M sodium acetate pH 5.5 at 20°C. 1 Unit of enzyme was defined as the hydrolysis of 10^{-6} mol 4-Methylumbelliferylacetate per hour per gram hemoglobin.

Results and Discussion

ESD-phenotyping was easily possible by ultrathinlayer agarose gel-IEF. Addition of TAURINE resulted in a higher resolution of isoenzymes (12). In a sample of 674 unrelated persons from Southern Germany the following phenotypes have been observed: ESD1, ESD2-1, ESD2, ESD5-1, ESD5-2 and ESD7-1 (Fig. 1). In a case of disputed paternity we observed a "new" ESD variant in a father and his child. The phenotype looks like an ESD1 with an additional faint cathodal band, which was not detectable by starch gel electrophoresis. The IEF-results point to the existence of a genetically determined "new" ESD-variant, which we provisionally called ESD-Berlin (ESD B, Fig.1). In vitro storage effects, especially modification by oxidised gluthathione could be avoided by reduction with DTT. After reduction even several years old hemolysates did not differ from fresh samples.

The following allele frequencies were observed:
ESD*1 = 0.8746, ESD*2 = 0.1053, ESD*5 = 0.0185, ESD*VAR = 0.0015 (Tab. 1, Chi-square values were calculated with three classes only).

GENETIC STUDY OF RED CELL ESTERASE D POLYMORPHISM BY ISOELECTRIC FOCUSING

G. Gradl, S. Weidinger, H. Cleve and F. Schwarzfischer
(Institute of Anthropologie and Human Genetics, University of Munich, Richard Wagner-Str. 10/I, D-8000 München 2, FRG)

Introduction

The polymorphism of human esterase D (ESD) was first detected 1973 (1) with three common phenotypes, determined by two autosomal codominant alleles, ESD*1 and ESD*2. Other investigators described some rare alleles ESD*3 (2), ESD*4 (3), ESD*6 (4), ESD*7 (5), ESD*Cph (6), ESD*D (7), and the common allele ESD*5 (8). There is evidence for the existence of a silent allele ESD*0 (9,10) and a variation of activity between phenotypes(11).

In the present study the polymorphism of ESD was studied by ultrathinlayer agarose gel isoelectric focusing (IEF). The enzyme activity was measured by fluorometry according to Sparkes et al (10) with a slight modification.

Materials and Methods

Sample preparation: Red cells of venous blood were washed twice and hemolysed by sonification. For IEF the hemolysates were diluted with an equal volume of 50 mM Dithiothreitol (DTT) and incubated overnight at 4°C. For the quantitative assay stromafree hemolysates were prepared by dilution 1:4 with 25 mM DTT and ultracentrifugation at 100000 g for 30 minutes. Hemoglobin concentration was estimated as cyanmethemoglobin.
Gel casting: Gels were casted on gelbond film (LKB) in a prewarmed cassette of two glass plates 125 x 260 mm with an U-shaped spacer of four layers of tesafilm or two layers of parafilm (Ø 0.25 mm).
Gel solution contained 80 mg Agarose IEF from Pharmacia 0.8 % w/v), 1 g Sorbitol (10 % w/v), 80 mg 4-aminoethansulfonic acid (TAURINE, 0.8 % w/v) 125 µl Servalyt pH 4.5-5.0 and 375 µl Servalyt pH 5.0-5.5 (2 % v/v) in 9.5 ml destilled water. Gels were precooled overnight at 4°C. Anode was soaked with 1.5 ml 0.25 M acetic acid and cathode with 1 ml of 0.25 M NaOH.
Sample application: 2 µl of hemolysate were applied 1.5 cm from the ca-

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Die Tab. 2 zeigt, daß A2HS mit einer AVACH von 17.81 einen guten Mittelplatz einnimmt. Der höchste Informationswert kommt dabei nach wie vor dem PGM1-Subtypen-System mit 31.91 % zu, gefolgt vom GC- und PI-System mit 29.74 % bzw. 29.29 %. Der Informationswert des A2HS liegt somit zwischen dem der 6. Komplementkomponente (C6) und dem der Alpha-Fucosidase mit 15.10 %. Wie eingangs erwähnt, war bislang noch keine chromosomale Zuordnung des A2HS-Genortes möglich, somit ergab sich keine Einschränkung des Informationswertes durch Kopplung an andere genetische Marker wie dies z.B. beim C2-, BF- und HLA-System der Fall ist.

Zusammenfassend läßt sich sagen, daß das A2HS-System ein weiterer, informativer Marker in der forensischen Blutgruppenkunde ist. Durch die Möglichkeit einer Simultantypisierung mit zwei weiteren IEF-Systemen, dem PI- und GC-System, dürfte eine baldige Einführung des A2HS in die Routinediagnostik der Paternitätsserologie sowie für humangenetische Fragestellungen durch die hier vorgestellte Technik wesentlich erleichtert werden.

Anschließend wurde das Gel mit CBB R 250 gefärbt und entfärbt, so daß eine Bestimmung der Alpha-1-Antitrypsin-Phänotypen in den Ablesezonen 4,6 und 8 möglich war. Die Hintergrundsfärbung durch den verdünnten A2HS-Antikörper war minimal, so daß auch die Identifizierung der Produkte der Defektgene PI*S und PI*Z zweifelsfrei möglich war.

Dies gelang auch auf selbst hergestellten Gelen des pH-Bereichs 4.2 bis 4.9 (mit Zusatz von pH 4-6 Ampholyten). Unsere Trenngele wurden in einer Standardgelkonzentration von T= 5 % sowie einem Vernetzungsgrad C (cross-linkage) von 3 % hergestellt, wobei sich die Ampholytkonzentration zwischen 2.08 und 2.20 % bewegte. Bei Geldimensionen von 245x110x0.4 mm ergab sich gegenüber den 1 mm dicken Fertiggelen für die selbst hergestellten Gele eine beträchtliche Materialeinsparung.

Anschließend sei auf den Informationswert des A2HS-Systems im Zusammenhang mit den übrigen, mittels IEF in der Abstammungsbegutachtung untersuchten genetischen Marker hingewiesen.

	%	%	
		31.91	PGM1
		29.74	GC
		29.29	PI
		26.90	GDH
		22.58	PLG
		22.29	F13B
		19.43	TF
		19.27	C8
		18.77	C6
A2HS	17.81		
		15.10	FUCA
		9.77	ESD
		8.33	GAA
		4.52	AMY2
		2.83	C2

Tab.2. AVACH-Werte einiger Isofokussierungssysteme. Das A2HS-System bietet eine isolierte Ausschlußchance von 17,81%

Da die Position der A2HS-Glycoprotein-Banden nach IEF eine partielle Überschneidung mit dem polymorphen Alpha-1-Antitrypsin erkennen läßt, bot sich eine Simultantypisierung auf Fertiggelen des pH-Bereichs 4 bis 5 an, wobei sich die für die Ablesung entscheidenden Zonen 4, 6 und 8 zwischen pI 4.4 und 4.7 befinden. Kathodenwärts der Auftragsstelle liegt die GC-Ablesezone.

Zur IPAGIF verwendeten wir A2HS-Antiseren der Firma Behringwerke sowie von Atlantic Antibodies. Mit dem Antikörper wurde in einer 1:1 Verdünnung eine Celluloseacetatfolie getränkt und für 5 Minuten luftblasenfrei auf die korrespondierende Geloberfläche aufgelegt. Nach Entfernen der Folie wurde diese über Nacht in 0.9 % NaCl-Lösung ausgewaschen, das Trenngel fixiert und zunächst bezüglich der kathodenwärts zur Startlinie liegenden GC-Phänotypen ausgewertet.

Die Abb. 2 zeigt die GC-Phänotypen nach einfacher Fixation des Trenngels. Hervorzuheben ist hier, daß unterhalb der Auftragszone keine störende Überlagerung des A2HS-Antikörpers eintritt, die GC-Präzipitate also gut gegen einen dunklen Hintergrund abgelesen werden können.

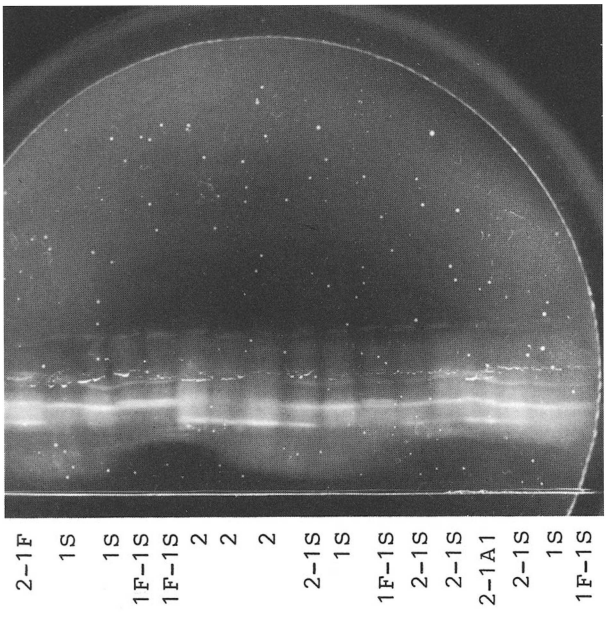

Abb.2. GC-Phänotypen auf einem pH 4-5 Gel nach vorheriger 5 min A2HS-Immunfixation.Die Anodenseite ist oben.

Familienuntersuchungen umfassen 26 Familien mit 29 Kindern. Dabei ergab die Aufspaltung der Kinderphänotypen keine Abweichung von dem angenommenen Modell zweier autosomal-kodominanter Allele. Vier der sechs in einem 2-Allelensystem möglichen Eltern-Phänotypen-Konstellationen wurden bisher beobachtet. Zusätzlich zu den kompletten Familien werteten wir Daten von 37 Mutter/Kind-Kombinationen aus. Auch hier ergab sich keine mit den Erbregeln unvereinbare Mutter/Kind-Kombination der A2HS-Phänotypen.

Die nach dem Gen-Abzählverfahren errechneten Allelfrequenzen im A2HS-System ergaben für A2HS*1 einen Wert von 0.655, A2HS*2 einen Wert von 0.340 und für die beiden beobachteten "Varianten" einen Wert von 0.005. Eine Identität des unter "Varianten" zusammengefaßten Allels mit A2HS*3 erscheint dabei möglich.

Bei Europiden wurden bislang nur spärliche populationsgenetische Daten erhoben. Neben der eingangs bereits erwähnten Studie von COX und ANDREWS (Kanada, 1983), die sich auf die Analyse von 68 Individuen stützt, berichteten OLAISEN et al. (1983) über eine gleichfalls kleine Stichprobe aus Norwegen. Von WEIDINGER et al. (1984) stammen Daten von 166 Blutspendern aus Süddeutschland. Die in der vorliegenden Arbeit ermittelten Allelfrequenzen differieren dabei nur unbeträchtlich von denen der süddeutschen Stichprobe. Gravierende technische Probleme bei der Phänotypendifferenzierung traten bei uns nicht auf. Wir beobachteten jedoch regelmäßig in gealterten Proben eine Verschiebung des A2HS-Bandenmusters in Richtung Anode; dies konnte bei länger gelagerten 2-Typen zur Vortäuschung eines Phänotyps 2-1 führen, womit dann nicht immer zweifelsfrei die Abgrenzung zum heterozygoten Typ A2HS 2-1 möglich war.

Ein geschlossenes Bild vermitteln auch die von UMETSU et al. (1984) und YUASA et al. (1985) bei Japanern erhobenen Daten, die insgesamt bereits über 3000 Personen umfassen. Die auf den japanischen Hauptinseln ermittelten Frequenzen bewegen sich dabei zwischen 0.24 und 0.27, lediglich die Population aus Okinawa unterschreitet diesen Wert mit 0.2065 für A2HS*2 signifikant.

Wir verwendeten zur Darstellung des A2HS-Polymorphismus die pH-Bereiche 4 bis 5, wobei wir handelsübliche Gele vom Typ PAGplate benutzten sowie eigene Gele vom pH-Bereich 4.2 bis 4.9. Hervorzuheben ist noch eine Gesamtdauer von 5 Stunden Trennzeit und die kurzen Immunoprint-Zeiten, die sich bei Verwendung einer A2HS-Antikörper-getränkten CAF ergeben. Auf die Möglichkeit, nach Immun-Abklatsch das Gel konventionell zu fixieren und die GC-Isoproteine, sowie nach anschließender Färbung mit CBB R 250 (0.1%) auch die PI-Banden abzulesen, wird später eingegangen.

Die folgende Tabelle zeigt die Verteilung der A2HS-Phänotypen in einer Stichprobe von 197 hessischen Blutspendern. Neben den drei häufigen Phänotypen 1, 2-1 und 2 beobachteten wir in zwei Fällen abweichende Bandenmuster, die jeweils neben dem A2HS*1-Genprodukt auftraten. Ein Vergleich von Beobachtungs- und Erwartungswerten läßt eine recht gute Übereinstimmung erkennen. Die Summe der χ^2-Werte liegt bei 0.361; unter Zugrundelegung eines Freiheitsgrades ergibt sich somit ein p-Wert von 0.5 bis 0.7. Dies bedeutet, daß die Unterschiede zwischen Beobachtungs- und Erwartungswerten nicht signifikant differieren.

Tab.1. A2HS-Phänotypen und -Allelfrequenzen in Hessen

Phänotypen	n(beob.)	n(erw.)	Allelfrequenzen
1	84	84,47	A2HS*1 = 0,655
2-1	88	87,74	A2HS*2 = 0,340
2	23	22,79	A2HS*V = 0,005
1-Var	2	1,32	
	197	196,32	1,000

$\Sigma\chi^2 = 0,361$; $0,5 < p < 0,7$; FG = 1

häufiger Allele in der weißen Bevölkerung, die die Bezeichnung A2HS*1 und *2 erhielten. Daneben konnte die Arbeitsgruppe Hinweise auf die Existenz eines dritten, die Kriterien eines Polymorphismus (>0.01) knapp überschreitenden Allels (A2HS*3) gewinnen. Von WEIDINGER et al. (1984), UMETSU et al. (1984) und YUASA et al. (1985) in Japan stammen Hinweise auf zwei weitere Allele, A2HS*4 und *5.

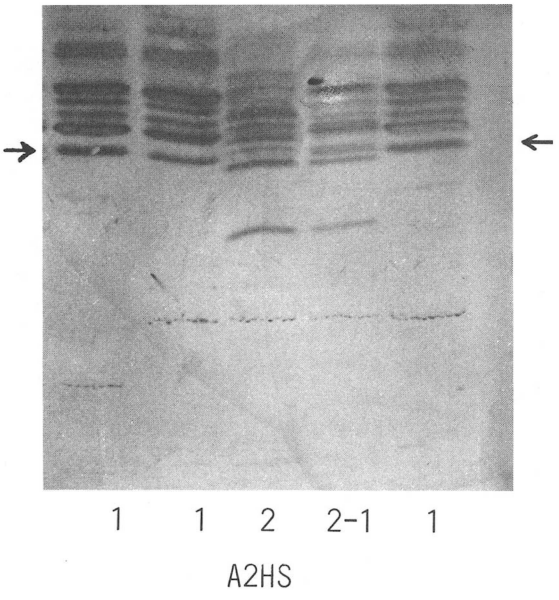

Abb.1. Die drei häufigen A2HS-Phänotypen 1,2-1 und 2 nach Immunoprint-PAGIF. Die Anodenseite ist oben

Die Abb. 1 zeigt die drei häufigen Phänotypen A2HS 1, 2-1 und 2 nach Auftrennung in einem Polyacrylamidgel des pH-Bereichs 4 bis 5. Dabei läßt der Phänotyp 2-1 ein aus den korrespondierenden Banden der entsprechenden Homozygoten zusammengesetztes Muster erkennen. Ein Dosiseffekt ist dabei eindeutig vorhanden. Beim homozygoten Phänotyp A2HS 2 ist die kräftige, kathodennahe Bande gut zu erkennen. Sie fehlt beim Phänotyp A2HS 1 vollständig. Als kritische Ablesezone wird dabei die mit Pfeil markierte Region angesehen. Oberhalb dieser eigentlichen Ablesezone findet sich eine Reihe weiterer Banden in unbehandelten (d.h. ohne Neuraminidase-Vorbehandlung eingesetzten) Serum-Proben. Ihre Position und Intensität ist bei der Typendifferenzierung gleichfalls von Bedeutung.

ALPHA-2-HS GLYCOPROTEIN POLYMORPHISMUS

Tarja Tarkkala Mendner und Peter Kühnl

Institut für Immunhämatologie, Universität Frankfurt,
Sandhofstraße 1, D-6000 Frankfurt am Main 71

Seit der Einführung der Technik der isoelektrischen Fokussierung (IEF) auf Polyacrylamidgelen in die forensische Blutgruppenkunde im Jahre 1976 wurde eine erhebliche Zahl informativer, genetischer Marker entdeckt und bereits bei Routinefragestellungen analysiert. Die IEF erwies sich dabei bezüglich der Trennschärfe und der Möglichkeit einer Kombination mit immunologischen Verfahren den bis dahin eingesetzten Standard-Elektrophoresetechniken auf verschiedenen Trägermaterialien wie Stärke, Agarose, Celluloseacetatfolie oder Polyacrylamidgel als überlegen.

Untersuchungen von HEREMANS im Jahre 1960 bzw. der Arbeitsgruppe von SCHMID und SCHULTZE in den darauffolgenden Jahren führten zur Isolierung und Reinigung des A2HS-Glycoproteins (A2HS-GP). Der Syntheseort ist die Leber, die durchschnittliche Plasmakonzentration des Proteins liegt zwischen 40 und 80 mg/dl. Das Molekulargewicht liegt bei ~49 KD, der pI-Wert zwischen 4.5 und 4.9. Über die biologische Funktion ist wenig bekannt, doch diskutiert man eine Opsonierung der weißen Blutkörperchen. Eine Chromosomenzuordnung des A2HS-Genortes gelang bisher nicht. 1977 wiesen ANDERSON und ANDERSON durch eine Kombination von Polyacrylamidgel-Isofokussierung (PAGIF) und einer 2-dimensionalen Polyacrylamidgel-Elektrophorese (2D-PAGE) erstmals einen genetischen Polymorphismus des A2HS nach. Hinzuweisen ist auf die Synonyma AHS, HSGA oder Alpha-2-HS, die von anderen Arbeitsgruppen verwendet wurden und von der auf der Human Gene Mapping Conference 7 (Los Angeles, 1983) empfohlenen Abkürzung A2HS abweichen.

Untersuchungen von COX und ANDREWS aus dem Jahre 1983, die mit einer ein-dimensionalen IEF-Technik und anschließender Silberfärbung arbeiteten, bestätigten die Existenz zweier

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Distribution of ORM-phenotypes in 215 Danes determined by Isoelectric Focusing

	F1	F2	S	F1F2	F1S	F2S
obs	77	0	33	5	91	9
exp	72.7	0.2	32.0	8.1	96.5	5.4

chi^2= 4.4315 d.f.2 0.1<p<0.2

Gene frequencies:	ORM*F1	ORM*F2	ORM*S
This study :	0.581	0.033	0.386
Eiberg et al. n=1679 (unpublished) :	0.611		0.389

Segregation of ORM-phenotypes in 33 families with 82 children.

Matings	n	Children (n=82) F1	S	F1F2	F1S	F2S
F1 x F1	5	11	-	-	-	-
F1 x S	4	-	-	-	10	-
F1 x F1F2	1	0	-	1	-	-
F1 x F1S	9	12	-	-	13	-
F1F2 x F1S	2	2	-	0	2	3
F1F2 x S	1	-	-	-	1	1
F1S x F1S	5	3	3	-	6	-
F1S x F2S	1	-	2	0	1	0
F1S x S	2	-	2	-	4	-
F2S x S	1	-	1	-	-	1
S x S	2	-	3	-	-	-
Total	33	28	11	1	37	5

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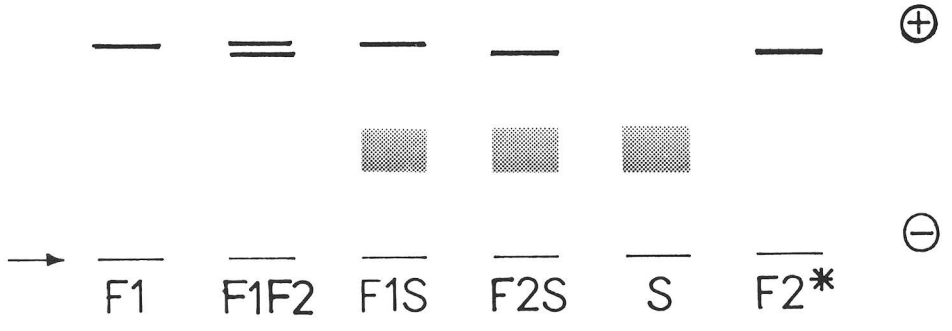
ORM-phenotypes as demonstrated either by immunoprinting (cellulose acetate) or by immunoblotting (nitrocellulose)



* hypotetic pattern
origin

and within the main groups F, FS, and S all the results agreed.
The distribution of the phenotypes observed in 215 Danes, and the frequencies of the three genes, calculated by gene counting, is given in table 1. The material was found to be in Hardy Weinberg equillibrium, and the total frequency of F1 plus F2, and the frequency of S, is in accordance with the results obtaind by conventional electrophoresis (Johnson et al. 1965, Eiberg unpublished).
The results of the examination of 33 families with 82 children are given in table 2, and 11 of the expected 21 mating classes were observed. In each combination group of parental types, the segregation of phenotypes in the children is in accordence with the assumtion of autosomal, codominant inheritance.
The method described using immunoprinting for identification of the ORM-subtypes is rather expensive, as the specific antibody has to be used undiluted, in order to obtain sharp and well-defined F-bands. Therefore, experiments with development of the ORM-types by immunoblotting was initiated. Both the primary antibody (anti-orosomucoid) and the phosphatase conjugated secondary antibody was used in high dilutions (1/250 - 1/1000), and the "anti-antibody" was visualised by a very sensitive histochemical staining method. 5-bromo-4-chloro-indoxyl phosphate was used as substrate and staining was performed with nitroblue tetrazolium (Blake et al 1984), but compared to the immunoprinting technique the pattern of the ORM-subtypes were more diffuse, and further experiments with the immunoblotting method is planned.

In summary, determination of Orosomucoid by isoelectric focusing extends the number of common phenotypes from three six, and the theoretical chance of exclusion of non-fathers from 18% to 21%. However, before application to paternity testing can be recommended analysis of a larger material, including mother/child pairs, should be performed.

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Ampholine pH 4-6 (LKB). Isoelectric focusing was performed at 6°C for 30 min. with 1500 V as maximum and using a constant power mode adjusted after 0, 10 and 20 min. to 10, 20 and 25 W, respectively. Samples were applied on Whatman paper no 1 (4x5mm) at the cathodic side of the gel. Electrode solutions were 0.5M NaOH for the anode, 0.5M CH_3COOH for the cathode.

Immunofixation

After conventional electrophoresis the ORM phenotypes were identified by immunofixation. Unfractionated, undiluted rabbit anti-serum against orosomucoid were applied directly on to the surface of the gel. The specific antibody was raised in rabbits ad modum Johnson et al. 1969.
After isoelectric focusing immunoprinting (10min) was carried out on Cellulose Acetate (Satorius 25G 11200) using undiluted anti-orosomucoid (Dakopatt a/s) and staining at 70°C for 15 min with 0.12% Kenacid Blue R (BDH) dissolved in ethanol:acetic acid:water (25:8:67).

Immunoblotting

The technique used was mainly according to Bjerrum et al. 1983. NC-membrane: Nitrocellulose BA 85-SB (Schleicher & Schuell). Protein tranfer: capillary pressure for 10 min. The NC membrane, presoaked in water,was smoothed on the gel surface followed by a piece of wet filter paper, a stack of paper towels and a glass plate (470 g). After air drying (30-60 min) the additional binding sites were blocked by agitating the membrane 15min in 15 ml 2% Tween 20 (polyoxyethylene sorbitan monolaurate) in washing-buffer (0.5% Tween 20, 50 mM Tris, 150 mM NaCl, pH 10.5), followed by 1x5min washing in the same buffer. Reaction with the primary antibody was performed overnight by agitating the membrane at 20°C with anti-orosomucoid (Dakopatt a/s) diluted 1/250 or 1/500 with washing buffer. The following morning the membrane was agitated 3x5 min in washing-buffer. The reaction with 15 ml of the secondary antibody (swine anti-rabbit immunoglobuline conjugated with alkaline phosphatase (Dakopatt a/s)) diluted 1/250, 1/500 or 1/1000 was performed for 1 hour, 20°C. Finally the membrane was washed three times (2x5 min in washing-buffer, 1x5min in 0.1M ethanolamine buffer pH 9.6) before development of the phenotypes with 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium (Blake et al. 1984).The reaction was stopped by washing with destilled water.

Results and Discussions

By isoelectric focusing it was found, that the electrophoretic F-band was subdivided as two bands with different pI values. The most acidic band was named F1, the other F2, and five phenotypes (F1, F1F2, F1S, F2S, and S) were observed (figure 1). Only the most infrequent of the expected six phenotypes , the homozygote F2, still has to be demonstrated. 182 neuraminidase treated samples were examined both by conventional electrophoresis and by isoelectric focusing,

Orosomucoid polymorphism: Determination by Separator Isoelectric Focusing and Demonstration of ORM*F subtypes.

Mariann Thymann & H.Eiberg

Institute of Forensic Genetics and Institute of Medical Genetics, University of Copenhagen

Genetic polymorphism of Orosomucoid (ORM=alpha$_1$-acid glycoprotein) was demonstrated already twenty years ago, and appeared to be based on two allelic genes (Schmid et al.1965). The only population study published comprised 220 Caucasians living in U.S.A. and the frequencies of the two alleles, S and F, were 0.36 and o.64, respectively (Johnson et al. 1969). Linkage on chromosome 9 of ORM to ABO , AK$_1$(Adenylate kinase), and ALADH (delta-Amino-levulinate dehydrase) was recently demonstrated (Eiberg et al. 1982). Based on the above frequencies the discriminative power of Orosomucoid is 60% and the theoretical chance of exclusion of non-fathers in paternity testing 18%. As application to Forensic Hemo genetics seems obvious, it was decided to reinvestigate the Orosomucoid polymorphism using modern techniques.

Materials

172 serum samples from unrelated Danes and sera from 33 families with 82 children were examined. Half of the family material originated from staff members with relatives, the other half was families from North Zealand. A great deal of the sera has been stored at - 20^{0}C up to four years.

Methods

Treatment of serum

6 drops of serum were dialysed 30 min. against 50 ml 0.1M CH_3COONa pH 5.5 using Millipore Filters (VSWP 02500). 25 µl dialysate was then incubated 16-18 hours at 37^{0}C with 15 µl neuraminidase (Clostridium perfringens, Boehringer, Mannheim, 1U/mg. 1mg of the enzyme was dissolved in 2.5 ml 0.1M CH_3COONa).

Electrophoresis

Conventionel agarose electrophoresis was performed ad modum Alper & Propp 1968 using a discontinuous buffer system (Ashton & Braden 1961). Wessel-buffer pH 8.6: LiOH 1.2g/l, H_3Bo_3 11.8g/l. Gel-buffer pH 8.4: 1 volume wessel-buffer, 9 volumes of a buffer containing Tris 6.2g/l, citric acid 1.6g/l.

Isoelectric focusing

The gel (0.5x110x205mm) contained 0.62% agarose (Isogel,LKB), 10.5% sucrose, 0.4% MES (2(N-morpholino)-ethane sulfonic acid,Sigma) 1.1% ACES (N-(2-acetamido)-2-aminoethane sulfonic acid,Sigma) 1.9 v/v % Ampholine pH 3.5-5 (LKB), and 5.5 v/v%

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Table 3

LOCATION OF THE EPITOPES, DETECTABLE WITH THE McAbs 5A1 AND 1C2 BY HAI

McAb	5A1	1C2	
dilution	10^{-6}	10^{-4}	10^{-4}
	Eza	Eza	Ef
specificity	G1m(z)	G1m(a)	IgG
no sample	++	++	++
myel.prot.G1m(za)	-	-	-
Fab fragment	-	++	++
Fc fragment	++	-	-
pFc' fragment	++	-	-
	CH1	CH3	CH3

++ = strong agglutination
+ = inhibition

Table 4

REACTIVITY OF McAb 5A1 AND 1C2 IN DIRECT ELISA

McAb	5A1	1C2
dilution	10^{-5}	10^{-3}
coat		
G1m(za)	+	+
G1m(zax)	+	+
G1m(f)	-	+
IgG2	-	+
IgG3	+	+
IgG4	-	+
IgA	-	-
IgM	-	-
specificity	G1m(za)+IgG3	IgG

+ = positive
- = negative

Table 5

SPECIFICITY OF McAbs 5A1 AND 1C2 IN ELISA-INHIBITIONTEST WITH G1m(za)-COATED PLATES

McAb	5A1	1C2
dilution	10^{-5}	10^{-3}
no sample	+	+
serum G1m(z+a+)	-	-
serum G1m(z+a-)	-	+
serum G1m(z-a+)	+	-
serum G1m(z-a-)	+	+
specificity	G1m(z)	G1m(a)

Table 1

McAbs 5A1 AND 1C2 IN HAEMAGGLUTINATION TEST

McAb[1]	Erythrocytes coated with: G1m(za)	G1m(zax)	G1m(f)	IgG2	IgG3	IgG4	IgA	IgM
5A1	11[2]	11	2	1	2	0	0	0
1C2	⩾12	⩾12	10	8	10	10	0	0

1) series of 12 ten-fold dilutions, starting 1:100
2) last tube in which agglutination was seen

Table 2

McAbs 5A1 AND 1C2 IN THE HAI-TEST

McAb	5A1		1C2	
dil.	10^{-6}	10^{-2}	10^{-4}	10^{-4}
	Eza	Ef	Eza	Ef
no sample	++	+	++	++
serum Gm(zaxg)	-	+	-	-
serum Gm(fnb)	++	+	++	-
serum Gm(zag)	-	+	-	-
serum Gm(afnb)	++	+	-	-
serum Gm(zfnb)	-	+	++	-
myel.prot.G1m(za)	-	+	-	-
other IgG myel.prot.	++	+	++	-
IgA	++	+	++	++
IgM	++	+	++	++
specificity	G1m(z)	aspecific	G1m(a)	IgG
strength	very strong	weak	strong	strong

++= strong agglutination
+= agglutination
-= inhibition

typed sera (diluted 1:20 and 1:60) and myeloma proteins (in several dilutions, starting 0.1 mg/ml) were used as inhibitors.
Only G1m(z) positive sera and proteins inhibited the agglutination by McAb 5A1 of G1m(za) coated cells. However the weak agglutination by McAb 5A1 of G1m(f) positive cells could not be inhibited. The agglutination by McAb 1C2 of G1m(za) positive cells was inhibited by G1m(a) positive samples only. This was in contrast to the agglutination of G1m (f) positive cells which was inhibited by all sera and IgG proteins. Therefore these results make the impression that McAb 1C2 has a dual specificity, depending on the circumstances that it can react as anti-G1m(a) and as anti-IgG antibody. Additional cloning experiments did not alter the specificity of this McAb. The usefulness of the McAbs 5A1 and 1C2 in the HAI test as typing reagents for G1m(z) and G1m(a) resp. could be confirmed by typing more than 1000 sera of different races. No differences were shown between the results with these McAbs and the results obtained with the conventional antisera of the same specificity.
The location of the epitopes, detectable with the McAbs 5A1 and 1C2 were investigated with Fab, Fc and pFc' fragment of a G1m(za) positive myeloma in the HAI assay (table 3). The G1m(z) specific reaction of McAb 5A1 was inhibited by the Fab fragments and not by Fc or pFc'. The G1m(a) specific reaction with McAb 1C2 was inhibited by Fc and by pFc' but not by the Fab fragment. These results are in complete agreement with the results which we have obtained in inhibition experiments with the conventional anti-G1m(z) and anti-G1m(a) antisera. This is also in agreement with the aminoacid analysis of $\gamma 1$ chains with different allotypes. It has been shown that the G1m(z) allotype depends on a difference in aminoacid 214 and the G1m(a) allotype on differences in amino acids 356-358.
The reactivity of the McAbs 5A1 and 1C2 was also tested in the direct ELISA (table 4) as well as in the ELISA inhibition test (table 5). In the direct tests McAb 5A1 reacts not only with the G1m(za) and (zax) positive myeloma proteins but also with IgG3 myeloma proteins, G3m(b) as well as G3m(g). Although the reaction with IgG3 is remarkable, it is not necessarily in contrast with the results of the HA test, because in our hands the ELISA test with IgG3 coated plates is more sensitive than with IgG of the other subclasses and thus this reactivity with IgG3 can be a reflection of the already observed weak anti-IgG reactivity of the antibody. Anyhow in the direct ELISA McAb 5A1 is not specific for G1m (z). McAb 1C2 reacts with IgG of all subclasses and allotypes in this direct test. However in the ELISA-inhibition test with G1m(za) coated plates McAbs 5A1 and 1C2 can be used as specific typing reagents for G1m(z) and G1m(a) respectively.
These results again demonstrate that the reactivity of McAbs with their epitope is strongly influenced by the presentation of the epitope on the molecule.

Km allotypes could be made with this panel of sera. For comparison with conventional antisera more than 1000 non-selected human sera were used. IgG, IgA and IgM myeloma proteins of the different subclasses and allotypes were used as coating antigens and as (non)inhibitors. These myeloma proteins were isolated by $(NH4)_2SO4$ precipitation followed by ionexchange chromatography (DEAE-50, Pharmacia), gelfiltration (G200, Pharmacia or AcA34, LKB) and/or affinity chromatography.
Fragments of a G1m(za) positive IgG1λ myeloma protein, were produced by digestion with papain or pepsin. Fab, Fc and pFc' fragments were isolated by gelfiltration (AcA44) and ionexchange chromatography by FPLC (Mono Q, Pharmacia).

Haemagglutination-inhibition assay

Human erythrocytes (OR2R2) were coated with incomplete Rh antibodies, mainly IgG1 and/or IgG3, or isolated IgG, IgA and IgM myeloma proteins using the chromic chloride method. The Ig coated cells were used in a 0.1% suspension in phosphate buffered saline (PBS). McAbs containing ascites was diluted in PBS with 5% foetal calf serum.

All HAI tests were performed in V-shape bottom microtitre plates by addition of 25 µl of successively (non)inhibitor, anti-allotype antibody and Ig coated cells. The plates were incubated overnight at 4°C. The results were read macroscopically over a light source after tilting the plates for about 10 minutes at an angle of about 60°.
The sera, proteins and peptides, used as (non)inhibitors were diluted in PBS.

Direct ELISA and ELISA-inhibition assay

Flat bottom polysterene plates were coated with myeloma protein (100 µl, 2 µg/ml in PBS) overnight at 4°C. After washing with PBS + 0,005% Tween 20 in the direct test 100 µl McAb containing supernatant or ascites fluid, diluted in PBS + 0.02% Tween 20 + 0.2% gelatine, were added and incubated 1 hour at 37°C.
After washing 100 µl horseradish peroxidase conjugated goat anti-mouse Ig (GM17, CLB) in a dilution of 1:5000 were added, followed by incubation at 37°C during 1 hour. As substrate 100 µl of a 0.1 mg/ml solution of tetramethyl benzidine in 0.11 M sodium acetate buffer pH 5,5 + 0.003% H2O2 was used. The reaction was stopped with 30 µl 2M $H2SO_4$ after a few minutes. The plates were read with an automated ELISA reader (Titertek, R Multiscan) at wavelength 450 nm.
In the ELISA-inhibition (or indirect ELISA) test the McAb was first incubated with the (non)inhibiting sample during one hour before the mixture was added to the Ig coated plate.

Results and discussion

Ascites fluid of two McAbs, 5A1 and 1C2, were tested in the haemagglutination (HA) test in a series of 12 ten-fold dilutions against a panel of erythrocytes coated with IgG of the different subclasses and allotypes and with IgA or IgM (Table 1). McAb 5A1 reacts strongly with the G1m(za) positive cells and weakly with some of the other IgG coated cells. McAb 1C2 reacts not only strongly with G1m(za) positive cells but also with all other IgG coated cells.
Haemagglutination-inhibition tests were performed with both McAbs with G1m(za) and with G1m(f) coated cells (Table 2). Our panels of Ig allo-

MONOCLONAL ANTIBODIES TO IMMUNOGLOBULIN ALLOTYPES: SPECIFICITY AND REACTIVITY IN HAEMAGGLUTINATION AND ELISA TECHNIQUES.

Gerda G. de Lange, Fred M. van Leeuwen, Peter H. van Eede, Patrick J. Lincoln and C. Paul Engelfriet (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands and London Hospital Medical College, University of London, U.K.).

Summary

Monoclonal antibodies (McAbs) were prepared to further study the allotypes of immunoglobulins. By screening supernatants in the haemagglutination-inhibition (HAI) as well as in the ELISA techniques anti-G1m(z) (=5A1) and anti-G1m(a) (=1C2) antibodies could be obtained.
These two antibodies are specific for the corresponding allotypes in inhibition assays performed with the HAI and the ELISA, but only under special circumstances. When McAb 5A1 is used in the direct haemagglutination test (HA) there is some cross-reactivity with other IgG coated cells.
This reaction is not inhibitable. However in the HAI with G1m(z) coated cells and in the indirect ELISA with G1m(z) coated plates only G1m(z) positive samples inhibit. McAb 1C2 shows another picture, because it reacts in the direct HA as well as in the direct ELISA with IgG of all subclasses and allotypes. In inhibition tests with G1m(a) coated cells only G1m(a) positive samples inhibit; with other IgG coated cells IgG of all subclasses and allotypes inhibit. It could be shown that the epitope, detected by 5A1 is located in the CH1 domain and the epitope(s) detected by 1C2 is/are located in the CH3 domain.
In conclusion, McAbs 5A1 and 1C2 are both useful anti-Gm reagents. However McAb 1C2 seems to have a dual specificity, namely anti-Gm(a) and anti-IgG. The question is, is the G1m(a) specificity a pseudospecificity, in other words are we dealing here with an anti-IgG antibody with a prevalence for G1m(a) positive molecules.

Introduction

The immunoglobulin (Ig) allotypes belong to a polymorphic genetic system, which is very useful for paternity testing and bloodstain analysis. As the immunoglobulins are stable molecules, the allotypes can also be determined in old serum and plasma samples and bloodstains.
However it is rather difficult to obtain sufficiently strong and specific antisera. For this reason and for further study of the allotypes we undertook the production of monoclonal anti-allotype antibodies.

Material and Methods

Monoclonal antibodies

Two monoclonal antibodies (McAbs), 5A1 and 1C2, were produced by immunization of Balb/c mice with human G1m(za) positive IgG1 myeloma proteins, followed by fusion of spleen cells with cells of the non-secretor mouse myeloma cell line SP2, according to the method of Köhler and Milstein (Nature: 256, 495, 1975).Hybridomas were selected by screening of supernatants in the haemagglutination-inhibition assay (HAI) and in the ELISA. After limiting dilutions the antibody producing cells were injected into mice for the production of ascites fluid.

Human sera, myeloma proteins and fragments

Thirty Ig allotyped human sera from individuals of different races were used in the inhibition tests. Distinction between all known Gm, A2m and

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Gm activities after chemical modification or protein A binding. Myeloma IgG3 (Kam) and protein A (Sigma) were mixed at a molar ratio 1:2, and then the mixture was passed through a Protein A-Sepharose CL-4B (Pharmacia) column in order to remove the non-bound IgG3. No effect of a IgG3-protein A interaction resulted in G3m(s) and G3m(t) activities. Amidation and trinitrophenylation had not influence on the both Gm activities. After carboxymethylation, the activities were completely missing. These results were obtained on controlled and modified proteins. Iodination of tyrosine allowed to lose the Gm activities, while photoxidation of histidine influenced on G3m(s) activity at x12 dilution level.

DISCUSSION

Myeloma protein Kam was purified by cold precipitation in the same way as myeloma protein Jir (9). However, the property of cryoglobulin faded with purification; the purified protein had not precipitated at 4°C in concentration 30 mg/ml. The peptide mapping of Kam was identical to that of Jir on FPLC chromatograms. This protein had methionine at position 379 and histidine at 435 by partial sequence analysis of tryptic peptides PT8 and PT12. This results are consistent with that of the myeloma protein Jir. The IgG3 Kam carrying Gm(s,t) reacted with protein A.

After chemical modification or protein A binding, G3m(s) and G3m(t) activities were examined to inspect the chemical enviroments around their epitopes. G3m(s) and (t) activities were not influenced by IgG3-protein A interactions. Only G3m(s) activity was detected on the pFc' fragment. These results suggested that epitope of G3m(s) marker localized on only CH3 domain, and that the formation of G3m(t) epitope was necessary for a CH2-CH3 domain interaction. The epitopes of G1m(f) and G1m(z) markers were found to be located on the Fd fragment and required the presence of both L and H chains (15). The chemical modification of aspartic acid, glutamic acid, lysine, histidine residues of protein Kam had no effect on both G3m(s,t) activities, while iodination of tyrosine resulted in complete inhibition of those activities. In the case of carboxymethylation, the protein must lose the activities because of the breakage of secondary structure in the acidic solution. From these results, we speculate that the epitopes of G3m(s) allotype is present on the last C-terminal beta-strand of CH3 domain, which is formed by antiparallel beta-pleated sheet (16), and that epitope of G3m(t) is present on the portion including the 3rd, 4th beta-strand and turn structure between them, which have tyrosine at position 391. And the CH2 domain was closely associated with this G3m (t) epitope.

Prealbumin from a patient with heredofamilial amyloid polyneuropathy had a substitution of methionine for valine at position 30 (16). This substitution resulted in the amyloid fibril formation. As a domain structure of immunoglobulin, prealbumin is composed of eight beta-strands with forming the two antiparallel beta-sheet (18). Cryoprecipitation and amyloid fibril formation, which are aggregation of protein molecules, are thought to be attributable to some abnormalities on the surface of protein molecule. The substitution, which is methionine instead of valine, may be responsible for these phenomena. IgG3 protein Jir carrying G3m(t) was characteristic of strong cryoprecipitation. Nishimura (19) suggested that one of the aggregation sites reside on the Fc portion of Protein Jir. However, purified protein Kam and protein Goe (20) carrying the same markers have no properties of cryoprecipitation. It is a matter of interest to clarify the mechanisms and relationship between the amino acid substitution and the molecular abnormalities.

The digest was applied to a Shimpack DIOL-150 column (7.9x50 cm, particle size 5 um) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.2 M sodium sulfate.

After peptic digestion of this protein in acidic solution, the pFc' fragment (CH3 domain) was purified by gel permeation chromatography on high-performance liquid chromatography (HPLC-GPC). The tryptic peptides of completely reduced and carboxymethylated pFc' fragment (Kam) were separated by reversed-phase chromatography on fast protein liquid chromatography (FPLC-RPC). The chromatogram and the amino acid composition of peaks were were identical to those of pFc' (Jir). Peptide PT8 and 12, which had a substitution specific to G3m(t) or G3m(s), were partially sequenced by manual Edman degradation and carboxypeptidase treatment.

Chemical modification. 0.5 mg of myeloma protein (Kam) was employed for chemical modification. Each modified sample was dialized against buffered saline for the serial titration of Gm typing. Amidation of aspartic acid or glutamic acid on the surface of myeloma protein (Kam) was carried out in 0.5 ml of 1 M glycineamide-HCl buffer (pH 4.75) with 9.6 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for 1 hr at room temperature (10). Methylthio group of methionine was reacted to monoiodoacetic acid under an acidic condition (11). The protein was dissolved in 0.5 ml of diluted HCl (pH 2.5) and added to 3 mg of monoiodoacetic acid. The mixture was stood for 4 hrs at 37°C. For iodination of hydroxy group of tyrosine, the protein in 0.5 ml of 0.1 M carbonate buffer (pH 9.5) was iodonated with 20 µl of 0.1 M I/0.2 M KI for 5 hrs at 4°C (12). For trinitrophenylation of ε-amino group of lysine, the protein was dissolved in 0.5 ml of 1 M borate buffer (pH 9.2), and then 50 µl of 0.02 M sodium 2,4,6-trinitrobenzene-1-sulfonate (TNBS) was added and the mixture was kept for 4 hrs at room temperature (13). Histidine residues were photoxidized with 10 µl of 0.5 % methylenblue in 0.5 ml of 0.1 M phosphate buffer (pH 7.3) under light (14).

RESULTS

Isolation of the monoclonal IgG3 protein (Kam). Protein Kam was separated from serum of the patient with myeloma by cryoprecipitation. The cryoprecipitate ability of this separated protein was less than that of IgG3 (Jir). The IgG3 Kam was identified as a lambda type IgG3 carrying G3m (b0,b3,b5,s,t) allotypes by immunodiffusion and Gm typing.

Isolation of the pFc' (Kam) fragment. The pFc' (Kam) fragment was isolated by gel-permeation chromatography (GPC) on a Hitachi HPLC apparatus (type 638). No differences between Jir and Kam could be detected in the first 7 amino-terminal residues of carboxymethylated pFc' fragment by a manual Edman degradation.

Sequence analysis of the Gm(s,t) specific peptides. The tryptic peptides from carboxymethylated pFc' (Kam) fragment were separated by reverse-phase chromatography. The chromatogram was identical to that of pFc' (Jir). The pooled fraction was carried out rechromatography under a acidic elution system. Amino acid composition of peaks was shown in table. Except for PT1 (Lys) and PT3 (Thr-Lys), 10 peptides were obtained on these chromatography. The two peptides might be eluted in the first peak on rechromatography. Peptide PT12 carrying G3m(s) specific residue was partially sequenced by manual Edman degradation and carboxypeptidase treatment; Trp-Gln- - - -Asx-His-Tyr-Thr-Glx-Lys. Similarly, peptide PT8 carrying G3m(t) was determined; Gly-Phe-Tyr-Pro-Ser-Asp-Ile-Ala-Met- - - -Glx-Asx-Tyr-Lys.

STRUCTURAL STUDIES OF ANOTHER HUMAN IgG3 MYELOMA PROTEIN (Kam) CARRYING THE ALLOTYPIC MARKERS Gm(s,t) AND ITS ALTERRATION INDUCED BY CHEMICAL MODIFICATION.

Hideo Matsumoto, Shigenori Ito, Tokiko Miyazaki, and Naoki Kawai
(Dept. of Legal Med., Osaka Medical School, Takatsuki, Osaka 569 JAPAN)

INTRODUCTION

Immunoglobulins of the IgG class can be divided into four isotypes, IgG1, IgG2, IgG3 and IgG4, on the basis of the amino acid differences in the heavy chain constant region, of variation in the position of attachment of light to heavy chain, and of the number and arrangement of inter-heavy chain disulfide bridges (1,2). These structural differences are reflected in unique antigenic determinants that allow their serologic recognition and in a series of allotypic determinants of gamma markers (Gm) that reflect genetically controlled polymorphisms (1-3).

A great number of genetic markers so far discovered in man are located in the constant region of the molecule. Fifteen allotypes have so far been described for the IgG3 subclass constant domain (4). G3m(s) and G3m(t) allotypes were first described in 1966(5), and they are now generally recognized as the characteristic amino acid markers of Mongoloid populations (5,6). Rechts et al.,(7) and van Loghem et al.,(8) described the differences in amino acid sequences found in the binding of IgG3 and other proteins to Staphylococcal protein A; they also described the differences in the residues found at position 435-436 in the CH3 domain between G3m(s) and G3m(u) proteins, using the same myeloma protein, Goe, which had the allotypic markers G3m(b0,b3,b5,s,t,v). The authors described the amino acid substitutions determining G3m(s) and G3m(t) specificities, which characterize Mongoloid populations, by sequence analysis of the Fc region of a myeloma protein (Jir) (9). By comparing the amino acid sequences of the IgG3 and the other IgG subclasses analysed to date, it was established that G3m(s) was an isoallotype specified by an amino acid substitution at position 435; i.e., whereas the subclasses IgG1, IgG2, and IgG4 had histidine in common, G3m(s-) had arginine in this position. This was also confirmed by the observation that the Fc fragment bound to protein A (10). It was also established that the amino acid at position 379 of G3m(t-) IgG3 and the other subclasses was valine, whereas methionine in this position was specific for G3m(t+).

The authors obtained an another monoclonal IgG3 protein (called Kam) derived from a Japanese female with essential cryoglobulinemia treated by plasmapheresis. By sequence analysis of the pFc' fragment of the protein Kam we reaffirmed that G3m(s) was an isoallotype specified by histidine instead of arginine at position 435 and G3m(t) specific substitution was methionine in stead of valine at position 379, as described previously (9). This paper also concerns the results of chemical modification to investigate the structural environment around the epitopes.

MATERIALS AND METHODS

Purification of myeloma protein (Kam). IgG3 protein was isolated from the serum of the myeloma patient (Kam) by precipitation in cold saline.

Gm typing. The classical hemagglutination inhibition test on microflocculation slide was used for allotype determination.

Preparation of the IgG3-pFc' fragment. IgG3(Kam) (10 mg/ml) dissolved in 0.1 M sodium acetate buffer (pH 4.5) was incubated with pepsin (Worthington, Freehold, NJ) at an E/S ratio 1;100 (w/w) for 1 hr at 37 ^{o}C. The reaction was stopped by addition of 0.1 g of Tris per 1 ml of the mixture.

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Nach IAGIF ergab sich überraschenderweise, daß die Variante B*10 einen mit B*3 identischen pI-Wert aufwies, also bei alleiniger Anwendung dieser Technik nicht als solche erkannt worden wäre (s. Abb. 2).

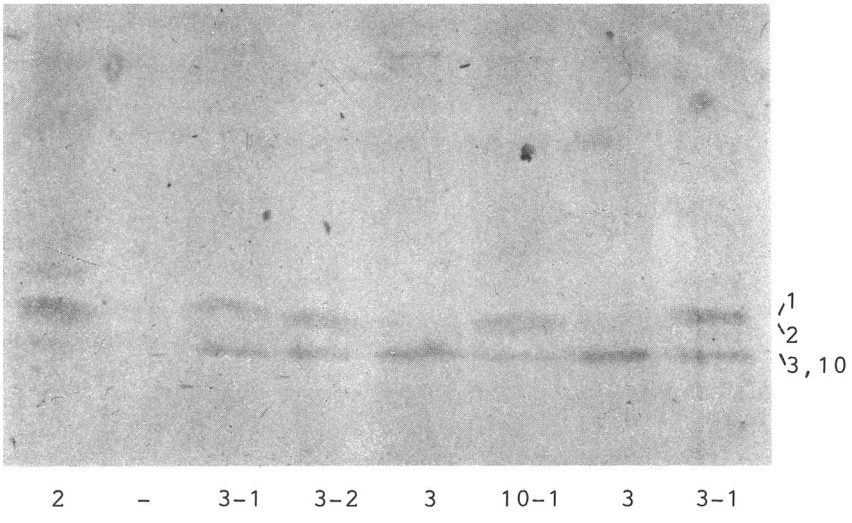

Abb.2. F13B-Allotypen nach IAGIF-Trennung im pH-Bereich 5-8.Das Genprodukt von B*10 weist einen mit B3 identischen pI-Wert auf.Die Anode liegt oben

Auch in anderen Blutgruppen-Systemen, wie z.B. dem GC- oder PGM1-System, ist ein ähnliches Verhalten einiger GC- und PGM1-Varianten bekannt. Dies hat zu der begründeten Forderung geführt, daß für die Anerkennung eines vermuteten neuen Allels mindestens zwei unterschiedliche Techniken zum Einsatz kommen müssen. Im F13B-System lautet jetzt die Reihenfolge der B-Allotypen in kathodaler Reihung nach IAGE: B*7, B*8, B*1, B*9, B*6, B*4, B*2, B*3, B*10 und B*5.

Der Informationswert des F13B-Systems in der forensischen Blutgruppenserologie liegt mit einem AVACH-Wert von 22,29 % sehr hoch (unter PGM1,GC,PI und HP-Subtypen). Der Nachweis seltener F13B-Produkte bei Kind und Putativvater bedeutet verständlicherweise im Einzelfall eine weitere, wesentliche Erhöhung des Vaterschaftswahrscheinlichkeits-Wertes.

F13B*10, ein neues Allel im System der Untereinheit B des Gerinnungsfaktors 13

Peter Kühnl und Willi Spielmann

Institut für Immunhämatologie der Universität und DRK-Blutspendedienst Hessen, Sandhofstr. 1, D-6000 Frankfurt/Main 71

Seit der Entdeckung eines genetischen Polymorphismus der Untereinheit B des Gerinnungsfaktors 13 im Jahre 1980 durch BOARD wurde neben den drei häufigen Allelen, B*1, *2 und *3, eine Reihe weiterer, bei Weißen seltener Varianten gefunden. Zur Darstellung dieses für blutgruppenkundliche Fragestellungen informativen Systems dienten dabei die Immunfixations-Agarosegel-Elektrophorese (IAGE) sowie die Immunfixations-Agarosegel-Isofokussierung (IAGIF) Neuraminidase behandelter Serumproben bei Verwendung monospezifischer Antikörper (Firma Behringwerke).

Wir konnten kürzlich im Rahmen einer Blutgruppenbegutachtung ein weiteres, neues Allel im F13B-System identifizieren, wobei die Variante in Fortführung der bisherigen numerischen Nomenklatur die Bezeichnung B10-1 erhielt. Das Genprodukt von B*10 lag dabei eindeutig unterhalb der B3-Bande (IAGE-Trennung) und - wie Vergleiche mit der 1983 von BOARD und CASTLE beschriebenen Variante B5 zeigten - oberhalb derselben (Abb. 1).

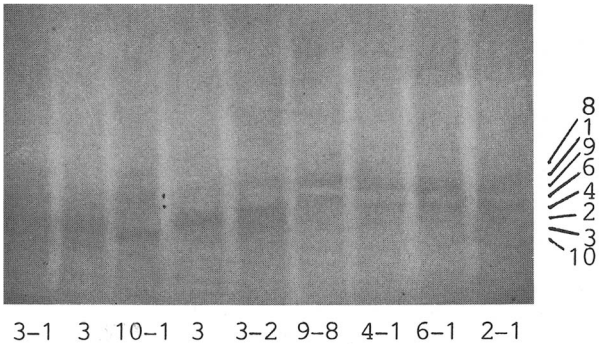

Abb.1. F13B-Allotypen nach IAGE-Trennung.Die Anode liegt oben

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The phenotype B 4-3 demonstrated in Fig. 2 shows a combination of the B 3- and the B 4-pattern.

The incomplete phenotype we named provisionally FXIIIB 1-D shows a deficient anodal band.

Table 1: Distribution of FXIIIB phenotpyes and allele frequencies in Southern Germany

Phenotypes	observed		expected		Allele frequencies
	n	%	n	%	
FXIIIB 1	371	58.61	370.2	58.48	FXIIIB 1 = 0.7647
2-1	94	14.85	90.2	14.25	
2	5	0.79	5.5	0.87	FXIIIB 2 = 0.0932
3-1	131	20.70	136.0	21.49	
3-2	14	2.21	16.6	2.63	FXIIIB 3 = 0.1405
3	16	2.52	12.5	1.98	
4-1	1	0.16	1.5	0.25	FXIIIB 4 = 0.0016
4-3	1	0.16	0.3	0.05	
Total	633	100.00	632.8	100.00	

$\Sigma X^2 = 0.0793$, df = 2; $0.3 > p > 0.2$

Table 1 presents the distribution of FXIIIB-phenotypes and the allele frequencies of 633 unrelated blood donors from Southern Germany. The theoretical exclusion rate is calculated to be 18.07 %.

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band pattern. This resolution can be used also as an indicator for the quality of the separation.

The rare phenotypes B 4-1 shows one band in the corridor between the type 1 and 3, the other band between the two B 1 bands.

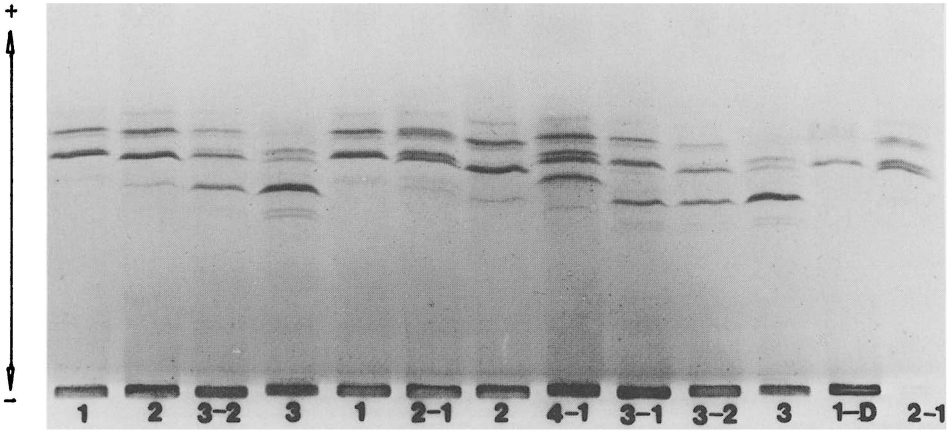

Fig. 1: Phenotypes of FXIIIB as attained by IEF on agarose followed by immunofixation. The alleles B*1, B*2 and B*3 determine six phenotypes; in addition B 1-D and B 4-1 are shown.

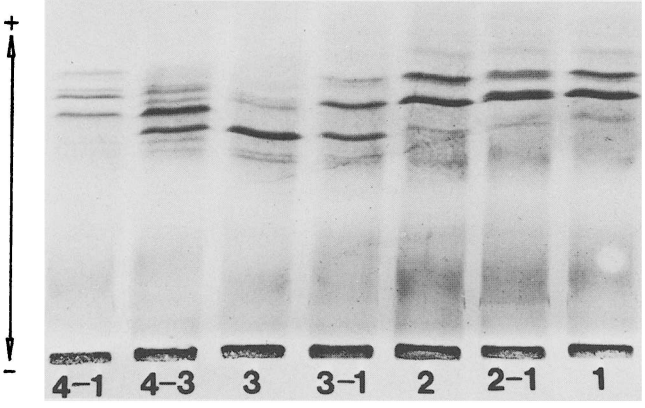

Fig. 2: Presentation of the rare phenotype FXIIIB 4-3 as resolved by IEF on agarose.

MATERIAL AND METHODS

The sera from 633 unrelated healthy blood donors from Southern Germany were examined, whereby the sample described previously (5) is included. Neuraminidase was used to treat the sera. Each 50 µl sample was incubated with 0.04 U enzyme for 2 hrs at 37 °C.

The agarose gels have a thickness of 0.5 mm and contain 0.8 % agarose IEF (Pharmacia), 0.8 % ACES and 10 % sorbitol dissolved in 18.8 ml distilled water. After degasing the gel solution were cooled to 75 °C then the ampholytes (LKB) were added (10 ml pH 5 - 8 and 0.2 ml pH 4 - 6.5). At a cooling temperature of 8 °C prefocusing is carried out for 30 min. 8 µl of the sample are applied to the cathodal side. The salts are then removed at 250 V for 30 min. Focusing is performed for 150 min at a setting of 1400 V, 20 mA, 10 W.

Immunofixation is done with 0.5 ml of a 1 : 2 diluted FXIIIB-antiserum (Behring) for 90 min at 37 °C.

After pressing, washing and drying the gel was stained with 0.5 % coomassie blue solution.

RESULTS AND DISCUSSION

Fig. 1 and Fig. 2 show the subunit B phenotyping of neuraminidase treated sera after isoelectric focusing and subsequent immunofixation.

In the homozygous phenotypes FXIIIB 1 and B 2 two main bands are observed. The bands of B 2 are slightly displaced towards the cathode. The phenotype B 3 is characterized by a single cathodal main band.

The heterozygous phenotypes are combinations of the homozygous phenotypes. B 2-1 is resolved in to a clear double

GENETIC FXIIIB-VARIANTS DEMONSTRATED BY ISOELECTRIC FOCUSING ON AGAROSE GELS

Leifheit H-J[1], Gathof AG[1], Cleve H[2]

1 Blood Service Center, Bavarian Red Cross, Munich, FRG *

2 Institute of Anthropology and Human Genetics, University of Munich, FRG

INTRODUCTION

The polymorphism of the B-subunit of coagulation factor XIII was first described by Board in 1981 who used agarose gel electrophoresis with subsequent immunofixation (1). He proposed a three-allele model which has been confirmed by other authors (4, 5, 7, 9, 10).

Isoelectric focusing (IEF) of FXIIIB is an improvement over electrophoresis because a clearer separation of the individual phenotypes is obtained with this method.

Agarose is used as a carrier medium due to the following advantages. It is a non-toxic medium, the gels are easier to prepare and less expensive, the separation time is short because the pH-gradient is established within 20 min, immunofixation can be done directly on the gel and there is no limitation by gel pore size for large proteins to penetrate the gel.

The analysis of FXIIIB by IEF on agarose we described earlier (5). In this communication further results are presented: The phenotype of FXIIIB 2 is demonstrated which corresponds to the phenotype predicted by us (5). In addition, the rare variants FXIIIB 4-1 and 4-3 are shown as well as an unusual phenotype named FXIIIB 1-D (D = deficiency).

* Herzog-Heinrich-Straße 2, D-8000 München 2

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Discussion

This method can be used in routine representation of haptoglobin subtypes for both paternity assessment and studies into population genetics. Serum preparation of about 40 serum samples, as can be separated in one operation with LKB multiphor hardware, takes about one hour. We feel that the favourable distribution of types, which resulted in a paternity exclusion chance of about 33 per cent in the region reviewed, is likely to justify the slightly increased consumption of time which proved to be absolutely comparable, for example, to the preparation of stroma-free haemolysates or thrombolysates. A comparison of results obtained from phenotyping by means of starch gel electrophoresis (three phenotypes) with those recorded from isoelectric focusing (15 phenotypes) revealed compatibility in all cases. The limitation must be added that this method is applicable only to typing of haemolysis-free sera, since, with the pH chosen for the ion-exchanger, the Hp-Hb complex,with its higher iso-electric point in comparison to free Hp, cannot be separated. This would eliminate the tracing function of haptoglobin heterogeneity.

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Results

The results obtained from iso-electric focusing of haptoglobin-α peptides, as have been prepared by our method, are depicted as diagram in Fig. 2 and in original in Fig. 3. The following allelic frequencies were recorded from the persons mentioned in the area of Berlin/GDR:
Hp *1F = 0.1472; *1S = 0.2500; *2FF = 0.0020; *2FS = 0.5757; *2SS = 0.0243.

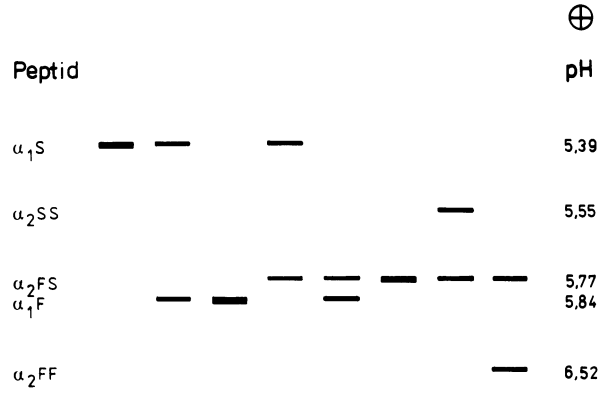

Fig. 2: Diagram of Hp-α peptide mobility in response to isoelectric focusing

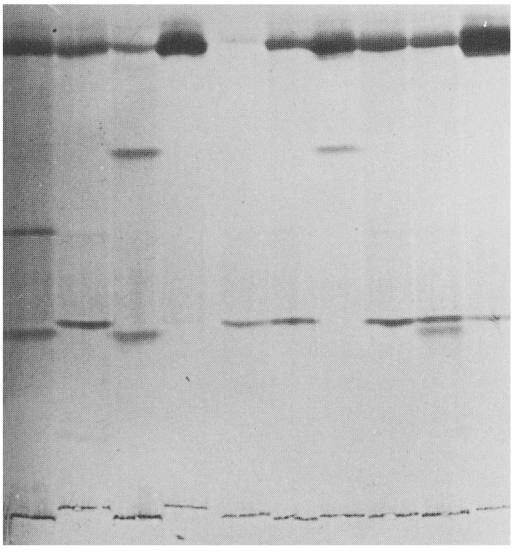

Fig. 3: Representation of Hp subtypes; types, from left to right, are: Hp 1F-2SS, 2FS, 1F-1S, 2FS? 2FS, 2FS, 1S, 2FS, 1F-2FS, 2 FS

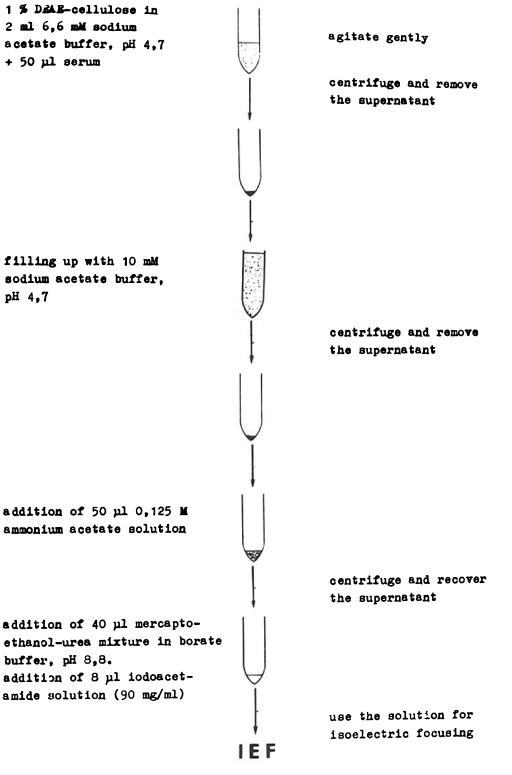

Fig. 1: Steps of haptoglobin preparation

Hp - Subtyping

Gel	polyacrylamide gel, T=5,5%, C=3% 260 x 125 x 0,5 mm (16 ml)
Carrier ampholytes (LKB ampholine)	2,2% (0,4 ml pH 3,5-10, 0,4 ml pH 5-7, 0,2 ml pH 6-8)
Electrode solutions	
anode	0,5 M H_3PO_4
cathode	0,5 M NaOH
Samples	ca. 10 µl purified and reductive cleavaged serum haptoglobin (paper pieces 10 x 5 mm)
Application area	at cathodic end of the gel
Maximum electric values	P = 10 W, U = 1600 V, I = 10 mA
Cooling temperature	10 °C
Prefocusing	30 min
Focusing with samples	30 min
Focusing without samples	2 h
Total time of focusing	3 h
Fixation and staining	simultaneously by formaldehyde-ethanol and Coomassie R 250 according Steck et al. (1980)
Destaining	as required

Table 1: Data for isoelectric focusing of Hp-α peptides

SIMPLE METHOD FOR HAPTOGLOBIN SUBTYPING

D. Patzelt and H. Schröder
Institute of Forensic Medicine, Humboldt-Universität,
GDR - 1040 Berlin

Hardly any forensic use is being made of the informative Hp subtype polymorphism because of the need for Hp purification which so far has been considered time-consuming. The introduction to forensic serology of highly sensitive iso-electric focusing encouraged some rethinking of the problem of Hp preparation. Proceeding from existing experience, we have succeeded in developing a practicable method which meets the demands on paternity assessment.

Material and methods

The technique described is primarily based on Smithies' specifications (Smithies et al., 1962) which were modified in line with suggestions made by Schössler et al. (1979) and Shibata et al. (1982). Serum haptoglobin is linked through a batch technique to a DEAE cellulose ion-exchanger which can be re-separated from the serum by centrifugation and subsequently washed. The amount of haptoglobin eluated from the ion-exchanger by addition of ammonium acetate solution (between ten and 15 per cent of the original quantity) is obtained from supernatant again by centrifugation and is then reductively decomposed by means of urea-mercapto-ethanol solution and carboxymethylated by addition of iodine acetamide. The preparations can then be cracked by iso-electric focusing either immediately or on the next day.

A diagram of the steps of Hp preparation is depicted in Fig. 1.

Focusing data may be seen from Table 1. More than 1,000 sera have so far been tested from clinically intact blood donors or persons involved in paternity assessment procedures in Berlin.

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Conclusion

The isofocusing/immunoblot method

1) detect isoelectric α- and β-chain variants

2) is well suited for large scale routine Hp-sybtyping

3) indicates approx. 30% paternity exclusion efficiency

4) indicates increased sensitivity compared with conventional starch gel electrophoresis

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Table 2. Haptoglobin allele frequencies in 606 unrelated individuals involved in cases of disputed paternity.

HP ALLELE FREQUENCIES

	1F	1S	2FS	2SS	2FF	
Olaisen et al (Norway)	0.13	0.21	0.63	0.03		(N=52)
Shibata et al (France)	0.139	0.245	0.547	0.045	0.012	(N=202)
Thymann (Denmark)	0.16	0.27	0.54	0.03		(N=208)
This material (Norway)	0.162	0.209	0.588	0.038	0.003	(N=606)

By looking at men excluded in other systems than Hp, it is possible to evaluate the exclusion efficiency of the Hp-system in a material of non-fathers. So far our material consists of 85 non-fathers. As seen from table 3, Hp excluded 11 of these men when typed by starch gel electrophoresis and 26 when subtyped by the isofocusing/immunoblot technique.(During a test period all serum samples arriving at the Institute in connection with cases of disputed paternity are Hp-typed with conventional starch gel electophoresis and with the new isofocusing/immunoblot method.) The results from this small preliminary study look promising regarding the usefulness of the new Hp-subtyping system in cases of disputed paternity.

Table 3. Haptoglobin paternity exclusion efficiency in a material of 85 non-fathers[1]

	Hp (starch-gel)		Hp (new method)	
	observed	(expected)	observed	(expected)
Number of Hp exclusions	11	(15.3)	26	(27.2)
% Hp exclusions	12.9	(18)	30.6	(32)

[1] *excluded in other systems*

One advantage exhibited by the new subtyping method is the small amount of serum necessary. Five µl serum is enough for at least 3 gel applications. Another advantage seems to be its ability to detect Hp in sera with rather low Hp content. Serum samples from infants are often small and low in Hp. So far only 42 infants less than 4 months old have been typed by both methods. In this small material, the isofocusing/immunoblot method typed 93% (n=39), and the starch-gel method 83% (n=35).

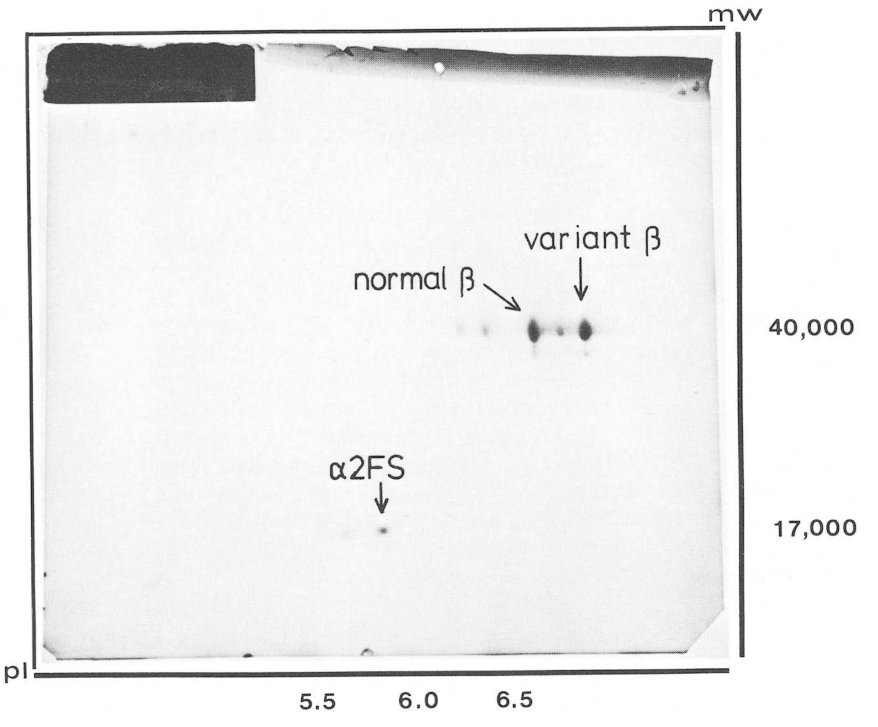

Figure 3. Two-dimensional blot demonstrating the new Hp β-chain variant.

Sixhundred and six unrelated individuals involved in paternity cases have been subtyped by the described isofocusing/immunoblot method. The haptoglobin phenotype distribution is presented in table 1, and the allele frequencies are presented in table 2. For comparison, the allele frequencies determined in three earlier works (1,2,6) have been included in table 2.

Hp-types	Observed	Expected
1S	23	26.5
1F	10	15.9
1F1S	43	41.0
2FS	207	209.5
2FS2SS	23	27.1
2FS2FF	2	2.1
2SS2FF	1	0.1
2SS	0	0.9
2FF	0	0.0
2FS1S	150	148.9
2FS1F	124	115.5
2SS1S	13	9.6
2SS1F	9	7.5
2FF1S	1	0.8
2FF1F	0	0.6
N=	606	606.0

Table 1. Haptoglobin phenotype distribution in 606 unrelated individuals involved in cases of disputed paternity.

Neuraminidase treated β-chains with different isoelectric points, can be detected by the described subtyping method. One such β-chain variant is haptoglobin Marburg (4). The difference in isoelectric points between normal neuraminidase treated β-chain and neuraminidase treated Marburg β-chain has been demonstrated earlier by two-dimensional electrophoresis (5).

A new β-chain variant has been detected with the described method during routine Hp-subtyping of serum in cases of disputed paternity. After neuraminidase treatment this β-chain variant has a more basic isoelectric point than both normal and Marburg neuraminidase treated β-chains. The new β-chain variant and Marburg are demonstrated in figure 2. Two-dimensional electrophoresis has verified that the observed variant is due to a variation in the β-chain; figure 3. The two-dimensional pattern shows that the new β-chain variant has the same molecular weight as normal β-chain. It further confirms the one-dimensional isofocusing observation; that the two β-chains have different isoelectric points. The starch gel electrophoretic Hp-pattern of serum containing the variant, was that of a normal Hp2.

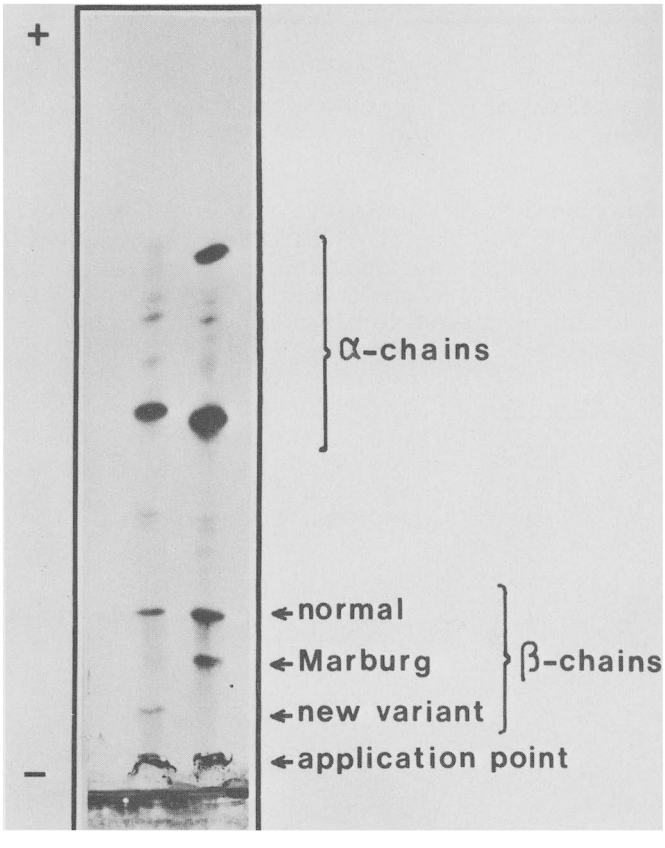

Figure 2. Haptoglobin isofocusing band patterns of two neuraminidase treated serum samples demonstrating β-chain variants.

Subtyping of haptoglobin

Brita Teige, Bjørnar Olaisen, Lilian Pedersen
Institute of Forensic Medicine, University of Oslo,
Rikshospitalet, 0027 Oslo 1, Norway

The haptoglobin molecule consists of α- and β-chains linked with disulfide bridges. The isoelectric heterogeneity observed in the β-chain is due to its content of sialic acid. The α-chain shows genetically determined structural polymorphism, and the most common subtypes 1S, 1F, 2FS, 2SS, 2FF and Johnson may be separated by isoelectric focusing (1,2). A subtyping method is developed which is well suited for large scale haptoglobin subtyping, and which requires no purification of the haptoglobin molecule prior to isofocusing (3): Five µl serum is treated with neuraminidase and reduced. Approximately one third of this mixture is subjected to polyacrylamide gel isoelectric focusing, and the remaining sample may be frozen and applied on a new gel if necessary. The neuraminidase treatment is included in order to diminish background staining, and to avoid interference between the isoelectric band patterns of the α- and β-chains. The Hp band pattern is visualized with an immunoblotting procedure using anti human haptoglobin as the first antibody and a peroxidase conjugated second antibody. In figure 1 is presented an immunoblot showing Hp-subtypes in serum samples from routine cases of disputed paternity.

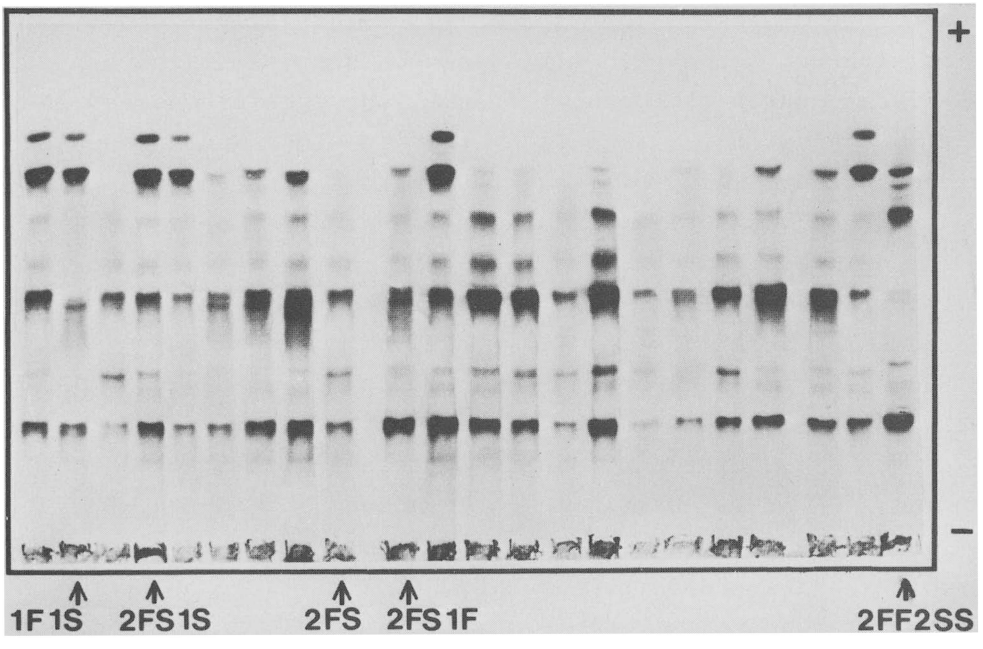

Figure 1. Haptoglobin isofocusing band patterns of neuraminidase treated serum samples. The method is described in the text.

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Neben den im linken Teil der Abbildung gezeigten Strukturvarianten,deren Genprodukte von dem anodennächsten Typ F bis zum kathodennahen Typ D reichen, wurde darüber hinaus ein stummes Gen ACP1*QO von HERBICH et al. beschrieben sowie eine Variante mit niedriger Aktivität, ACP1*GUAYMI. Sie steht in enger Beziehung zu erhöhten Glutathion-Reduktase-Spiegeln und wurde ebenso wie das bei den Ticuna-Indianern mit 0,11 polymorphe Allel ACP1*TIC bei amerikanischen Indianiden beobachtet. Messungen der GR-Aktivitäten wurden in unserem Fall nicht durchgeführt, doch spricht die bei der Mutter des Propositus beobachtete normale AB-Aktivität gegen einen ähnlichen Mechanismus als Erklärung für die reduzierte Aktivität des K-Genprodukts.

Ein Blick auf die heute verwendete Nomenklatur (Abb. 4) läßt erkennen, daß die Arbeitsgruppe NELSON et al. (1984) bezüglich der Allele *F, *G, *H und *I die von RADAM et al. (1982) sowie MARTIN et al. (1982) publizierten Varianten *F, *G, *H und *I unberücksichtigt lassen. So wurden die Bezeichnungen F und G zwei Jahre später (1984) erneut vergeben. Vergleichsuntersuchungen mit Austausch der entsprechenden Hämolysate unter den genannten Arbeitsgruppen erscheinen daher wünschenswert. Falls es hierbei zu einer Neubenennung einer Variante käme, bliebe der Buchstabe J verfügbar. Eine Interferenz von ACP1*K mit den vier genannten Varianten F, G, H und I besteht bezüglich der elektrophoretischen Mobilität in keinem Fall.
In dem hier geschilderten Fall ergab sich im Normgutachten (15 Systeme, jedoch ohne ACP1) sowie unter Hinzunahme des HLA-Systems eine kombinierte Vaterschaftsausschlußchance von 97,9 % und eine Vaterschaftswahrscheinlichkeit von 99,2 %. Unter Berücksichtigung der neuen Phasophatase-Variante ACP1*K würde sich das bisher erreichte verbale Prädikat "Vaterschaft höchstwahrscheinlich" auf "praktisch erwiesen" erhöhen.

Wiederholte Untersuchungen an neu entnommenen Blutproben bestätigten diese Aussage. Bei der Isoelektrofokussierung wurde gleichfalls die verminderte Aktivität des K-Genprodukts neben der normalen A-Aktivität deutlich (Abb. 3)

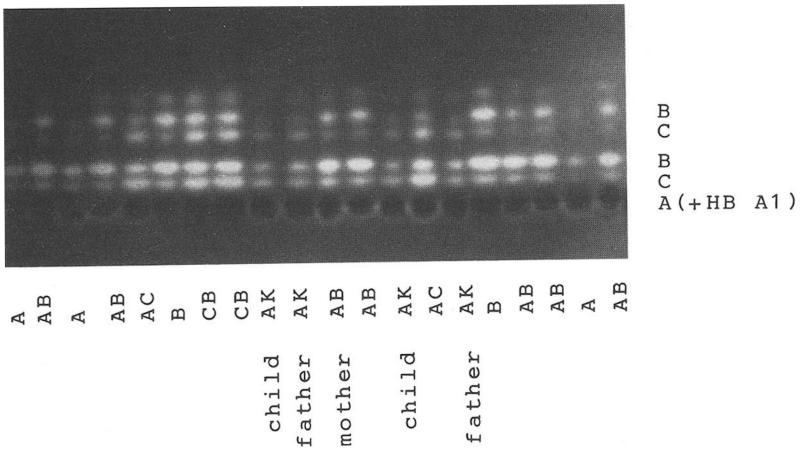


Abb.3. ACP1-Phänotypen nach PAGIF im pH-Bereich 3,5-9,5 und 4-MUP-Färbung.Bei Verwendung von Hämolysaten überlagert sich ACP1 A mit HB A1.Der Phänotyp AK ähnelt hier einem 'AC weak'. Die Anode befindet sich oben

Reiht man das neue Allel ACP1*K in die Gruppe der bisher beschriebenen ACP1-Varianten ein, so ergibt sich die in Abb. 4 gezeigte Reihenfolge.

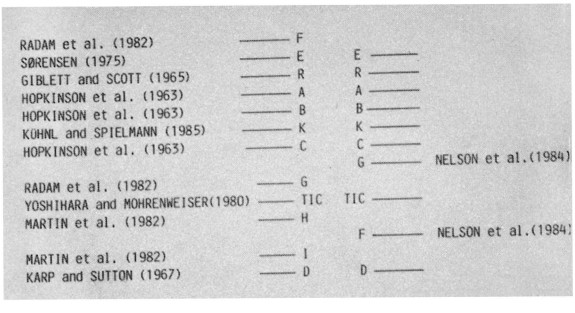
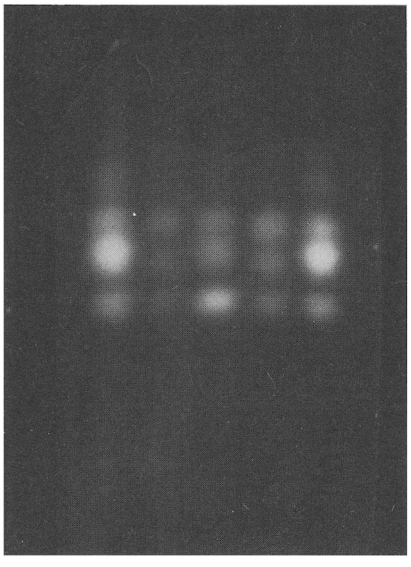


Abb.4. Übersicht der beiden verwendeten Nomenklaturen im ACP1-System.Die Genprodukte von *F,*G,*H und *I sind kontrovers bezüglich ihrer elektrophoretischen Position



AB AK AC AK AB

Abb.1. ACP1-Phänotypen nach CAF-Trennung und 4-MUP-Färbung (v.l.n.r.:AB-Kontrolle, Propositus,AC-Kontrolle,EvV, Mutter).Die Anode liegt oben

Es fällt auf, daß die Aktivität des ACP1*K-Genprodukts schwächer ist als diejenige der drei häufigen Allelprodukte und daß das kathodennahe Segment knapp oberhalb von C liegt. Dies ist in Abb. 2 schematisch dargestellt.

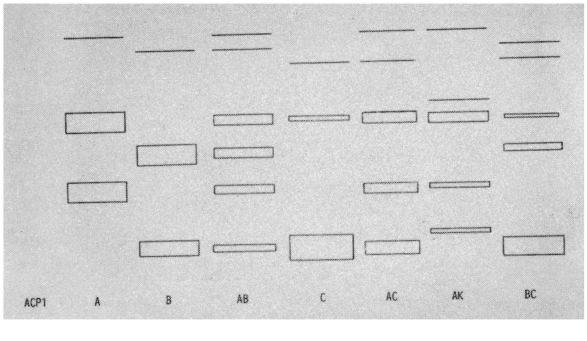


Abb.2. Die sechs häufigen ACP1-Phänotypen und der neue Phänotyp AK in Position 6

ACP1*K, ein neues Allel im System der sauren Erythrozyten-Phosphatase

Peter Kühnl und Willi Spielmann

Institut für Immunhämatologie der Universität und DRK-Blutspendedienst Hessen, Sandhofstr. 1, D-6000 Frankfurt/Main 71

Seit 20 Jahren wird das System der sauren Erythrozyten-Phosphatase (ACP1) in der Abstammungsbegutachtung eingesetzt. Neben den drei häufigen Allelen ACP1*A, *B und *C, die als häufige Allele (0,35, 0,59 bzw. 0,06) den hohen Informationswert der ACP1 in der Abstammungsbegutachtung begründen, wurden seitdem eine Reihe weiterer, bei Weißen seltener Allele identifiziert.

Wir möchten an dieser Stelle über einen neuen Phänotyp im System der ACP1 berichten, der uns im Rahmen eines Abstammungsgutachtens begegnete. Die Untersuchung der Hämolysate aus frisch entnommenen Blutproben eines Kindes und des Putativvaters zeigten ein Isoenzym-Muster, das zunächst dem des Phänotyps ACP1 AC ähnelte. Während die Mutter, die, ebenso wie Kind und PV, Deutsche war, eindeutig den Phänotyp AB aufwies, zeigten wiederholte Untersuchungen der Hämolysate mittels CAF-Elektrophorese, Isoelektrofokussierung im pH-Bereich 3,5 bis 9,5 sowie mittels Agarosegel-Elektrophorese (4-Methyl-Umbelliferyl-Phosphat-Färbung, Methodenbeschreibung s. SPIELMANN und KÜHNL, 1982) einen Phänotyp, den wir in Fortführung der bisherigen Nomenklatur als AK bezeichneten.

Abb. 1 zeigt das ACP1-Zymogramm des Propositus, seiner Mutter sowie seines Putativvaters neben zwei Kontrollen des Typs AB und AC.

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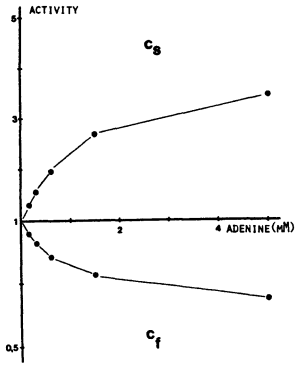


PHENO-TYPE	MAX. ACTIVATION/INHIBITION ADENINE S FORMS	ADENINE F FORMS	HYPOXANTHINE S FORMS	HYPOXANTHINE F FORMS
A	3,8	0,61	1,0	5,0
B	4,2	0,60	1,0	4,8
C	4,1	0,62	1,0	5,0
MEAN	4,0	0,61	1,0	4,9

Fig. 2. Activation/inhibition of purified ACP_1 allozyme forms by purines. Substrate: p-nitrophenyl phosphate (10 mM) at pH 6.0, 30°C.

PHENO-TYPE	FORM	CONTENT OF S AND F(%)	NORMAL ACTIVITY(%) CONTRIBUTION (SP. ACT.,S:72,F:51)	S+F	MODULATION BY PURINES ADENINE MODUL. FACTOR	ADENINE ACTIVITY(%)	ADENINE S+F	HYPOXANTHINE MODUL. FACTOR	HYPOXANTHINE ACTIVITY(%)	HYPOXANTHINE S+F
B	S	17	22	100	4.0	88	135	1.0	22	404
	F	83	78		0.6	47		4.9	382	
A	S	27	34	100	4.0	136	176	1.0	34	357
	F	73	66		0.6	40		4.9	323	
C	S	80	85	100	4.0	340	349	1.0	85	159
	F	20	15		0.6	9		4.9	74	

Table 3. Prediction of modulation characteristics of hemolysates in relation to the ACP_1 phenotype. Column denoted "Content of s and f (%)" gives the proportion between s and f protein. Column denoted "Normal activity (%)/contribution" gives the proportion between s and f activity as corrected for differences in specific activity.

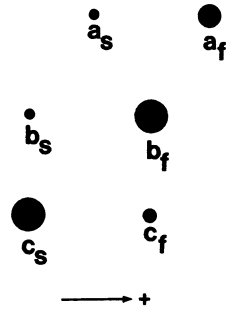
Electrophoretic pattern	Phenotype	Activity (rel) in red cells	Modulation: Adenine	Modulation: Hypoxanthine
	A	50	1.4	2.0
	B	75	1.2	2.1
	C	100	2.3	1.2

Fig.1. Electrophoretic patterns (in citrate/phosphate buffer, pH 5.9), relative activity levels (1) and modulation characteristics (2) of ACP_1.

Phenotype	Enzyme protein S:F	Enzyme protein (relative quantity)	Activity (relative)
A	1:3	40	45
B	1:5	70	77
C	4:1	100	100

Table 1. Quantitation of ACP_1 allozymes in hemolysates by crossed immuno-electrophoresis (3). Activity levels were determined at pH 6.0, 30°C using p-nitrophenyl phosphate (10 mM) as substrate.

Phenotype	Km (mM) S forms	Km (mM) F forms	Specific activity S forms	Specific activity F forms
A	0.51	0.13	75	53
B	0.48	0.14	70	46
C	0.48	0.14	72	55

Table 2. Comparison of enzymatic properties of purified ACP_1 allozyme forms. Substrate: p-nitrophenyl phosphate at pH 6.0, 30°C. Specific activity: /umoles/(min.x mg).

forms. It will be seen that the predicted order of increasing modulation for hemolysates of the three types is either B, A, C or C, A, B, which corresponds to the experimental results on hemolysates (2). Hence differences in modulation of the various phenotypes seem to be due to the different proportions of the s and f forms in these phenotypes.

Preliminary results on immunogenic and stability properties also show a similarity between the s forms and between the f forms, and a difference between the s and f forms.

In conclusion it appears that from a biochemical point of view only two different enzyme species exist: a "s-enzyme" and a "f-enzyme". The different proportion of these within the various phenotypes determines the biochemical differences between the phenotypes, whereas genetically determined structural differences between the a, b and c enzymes may determine how much of the enzyme that will occur as s-enzyme and how much as f-enzyme.

References:

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4) Dissing, J., Dahl, O., Svensmark, O.: phosphonic and arsonic acids as inhibitors of human red cell acid phosphatase and their use in affinity chromatography. Biochem. Biophys. Acta 569 159-176 (1979).

The activity of the enzyme forms in the presence of various concentrations of p-nitrophenyl phosphate was determined at pH 6.0, 30°C and the Km values were calculated using a cumputor program (4). Similar Km values were found for all the three s forms (table 2). The three f forms also showed identical Km values, but they were significantly lower than those of the s forms. Also with respect to specific activity the s forms were similar and the f forms were similar, whereas there was a significant difference between the s and f forms. Hence the three s forms seem to be enzymatically similar - in spite of their genetic difference, and the three f forms are similar, whereas the s forms are different from the f forms.

The modulation of the enzyme forms by adenine and hypoxanthine was investigated as sketched for the c_s and c_f forms (fig.2). It will be seen that adenine activated the s form and inhibited the f form. The data fitted a hyperbolic function and the maximum activation or maximum inhibition was calculated using the cumputer program. All the three s forms were activated by adenine to about the same degree (aproximately 4 times the unmodulated activity) and the three f forms were all inhibited by a factor of about 0.6. In contrast with adenine, hypoxanthine showed a 5 fold activation of all the three f forms, whereas the s forms were not affected. Concequently, also with respect to modulation by purines the three s forms seem to be enzymatically similar as well as the three f forms seem to be similar, whereas the s and f forms are different.

From these findings on the separated s and f forms one can predict the modulation characteristics of hemolysates of the various phenotypes as shown for the three homozygous types (table 3). The unmodulated activities of the hemolysates are set to 100 and one gets to the modulated activity from the unmodulated activities of the s and the f forms using the respective modulation factors. For example estimation of the effect of adenine on hemolysate of type B gives: Unmodulated s-activity: 22, modulated s-activity: 22x4 = 88; unmodulated f-activity: 78, modulated f-activity: 78x0.6 = 47; modulated activity of B-hemolysate: s+f= 88+47= 135; hence the result is a slight net activation. The A-type is activated more because of its greater content of s form and the C-type is consequently activated to the highest degree. The reverse situation is found with hypoxanthine, which activates the f

RED CELL ACID PHOSPHATASE: ONLY TWO DIFFERENT ENZYMES - THE "SLOW" AND THE "FAST" ENZYME - DETERMINE DIFFERENT BIOCHEMICAL PROPERTIES OF THE SIX COMMON PHENOTYPES.

J.Dissing (Institute of Forensic Genetics, University of Copenhagen, Copenhagen, Denmark).

Red cell acid phosphatase is well known as a valuable genetic marker. Another feature, however is its intriguing biochemical properties. Spencer, Hopkinson and Harris (1) found over 20 years ago that each of the three common alleles (P^a, P^b, P^c) give rise to two electrophoretically different enzyme forms: a "slow" (s) form and a "fast" (f) form. The staining intensities of these forms as well as the overall enzyme activity in the red cell depend on the phenotype, the order with respect to increasing activity being A, B and C (fig. 1). Mansfield and Sensabaugh (2) showed subsequently that the enzyme is either activated or inhibited by some purines, that this modulation also depends on the phenotype, but that the order is not A, B, C but B, A, C or C, A, B. Examples for adenine and hypoxanthine are given in fig. 1. Little is known, however, about the molecular basis of these properties and the following question can be raised: Are the phenotype dependent differences in activity and modulation due to 1) catalytic differences between the a, b and c allozymes or 2) differences in the contents of the s and f forms in the various phenotypes?

To answer this question the quantity of enzyme protein in red cells of the different phenotypes were determined by crossed immuno electrophoresis using specific antibodies (3). It was found that the ratio between the s and f forms was dependent on the phenotype as shown for the three homozygous types (table 1). The A-type had three times more f protein than s protein, the B-type 5 times more f protein than s protein, whereas the C-type had 4 times more s protein than f protein. Hence the distribution between s and f protein resembles the well known distribution between s and f activity. The total quantity of enzyme protein varied also; hemolysates of type A contained the least enzyme and those of type C contained the most. Again the protein quantities correspond well with the activity levels observed. Therefore the enzyme activity and electrophoretic patterns seem to be related to the quantities present of the s and f forms.

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are statistically significant ($p < 0.001$). The lower value for the C type may be accounted for in part by the preponderance of the cathodal, low transferase activity, isozyme in this type.

TABLE III

PHENOTYPIC VARIATION IN PHOSPHOTRANSFERASE ACTIVITY IN 2 M GLYCEROL

Phenotype (n)		Relative Activity Mean	S.D.
B	(23)	4.74	0.25
BA	(22)	4.49	0.46
A	(25)	4.38	0.22
CB	(8)	3.94	0.33
CA	(10)	3.74	0.34
C	(1)	3.43	

CONCLUSIONS. The addition of glycerol or other acceptor polyols to gel staining solutions or to gel media can enhance significantly the sensitivity of the staining reaction. The effect is greatest in gel buffers not containing phosphate. The differential effect on isozyme enhancement must be taken into account when typing but the phenotype dependent differences in transferase activity levels should not affect typing.

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4. Sensabaugh GF, Golden VL (1976) "Erythrocyte acid phosphatase typing on starch gels containing glycerol." Forensic Serology News 2: 1-2.

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ISOZYME DIFFERENCES. Each ACP_1 allele product is represented electrophoretically by two isozyme bands. Analysis of the chromatographically separated isozymes from a B homozygote indicated that the anodal isozyme had more than twice the phosphotransferase activity of the cathodal isozyme. Comparison of staining patterns from replicate gels stained in the absence and presence of glycerol indicates that glycerol enhances the intensity of the anodal band relative to the cathodal band with all the phenotypes. This effect is illustrated for the B and CB types in figure 1. It is obvious that this effect must be recognized if typing problems are to be avoided.

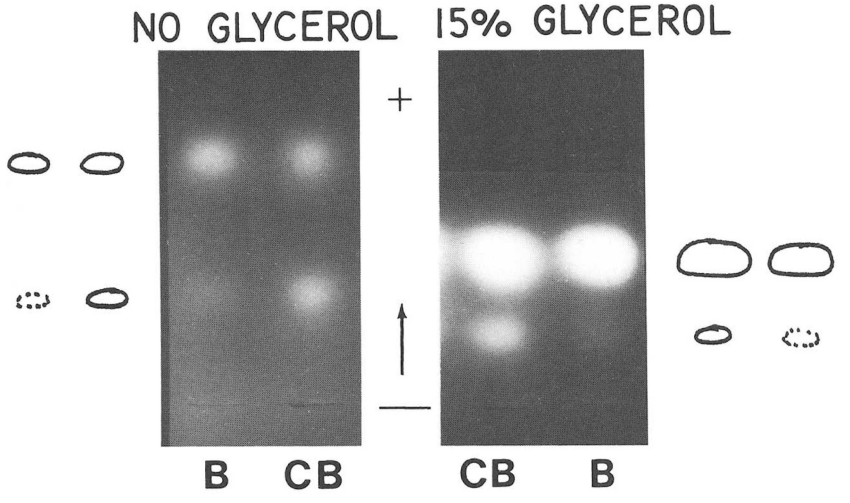

Figure 1. Electrophoretic patterns of ACP_1 in the presence and absence of glycerol. Electrophoresis was done on starch gels using the phosphate-citrate buffer system of Swallow, et. al. (5); run time was 17 hrs at 11 ma/gel. The gel shown on the right contained 15% glycerol; in this solvent, mobility is retarded. The stain on both gels was 2 mM methylumbelliferone phosphate and both were photographed after 10 minutes of staining.

PHENOTYPE DIFFERENCES. Assay of dialysed hemolysates of different types in the presence of 2 M (ca. 15%) glycerol showed a distinct phenotype dependence in phosphotransferase activity. The distributions of activities are summarized in table III. The phenotypes show an ordering B > BA > A > CB > CA > C; the differences between the means

TABLE I

INCREASE IN p-NITROPHENYL PHOSPHATASE ACTIVITY IN THE PRESENCE OF GLYCEROL

Glycerol % (v/v)	0	5	10	20	30	40	50
Relative Activity	1.0	2.5	3.8	5.2	5.6	5.2	4.2

Activity was determined spectrophotometrically in an assay mixture containing hemolysate, 2 mM p-nitrophenyl phosphate, and 100 mM acetate buffer pH 5.5.

Chemical analysis of the reaction products shows that phosphate production from step 2a is unchanged in the presence of alcohols. Thus the increase in the overall reaction rate is due entirely to the transferase reaction 2b. This also indicates that although 2b is faster than 2a, the two pathways are not competitive.

ACCEPTOR SPECIFICITY. Table II shows the effect of several alcohols on ACP_1 activity. Polyols such as glycerol are better acceptors than the corresponding simple alcohols; of the polyols, ribitol is clearly the best acceptor. There is a requirement for linear chain conformation; despite their polyol structure, sugars in ring conformation are not good acceptors. Comparison of the several 5 carbon polyols gives evidence of configuration and stereo specificity.

TABLE II

ACCEPTOR ACTIVITIES OF ALCOHOLS, POLYOLS, AND SUGARS

Compound (0.5 M)	Relative Activity
Methanol	1.42
Ethylene Glycol	1.74
1-Propanol	1.43
2-Propanol	1.05
Glycerol	2.40
Erythritol	2.80
D-Arabitol	2.38
L-Arabitol	1.32
Ribitol	4.11
Mannitol	2.83
D-Ribose	1.52
D-Arabinose	1.55

CHARACTERIZATION OF THE PHOSPHOTRANSFERASE ACTIVITY OF RED CELL ACID PHOSPHATASE (ACP_1).

Vivian L. Golden and George F. Sensabaugh

University of California, Forensic Science Group, School of Public Health, Berkeley, CA 94720 U.S.A.

Red cell acid phosphatase (ACP_1) is well established as a useful genetic marker in population studies, in paternity testing, and in the forensic analysis of bloodstains. One interesting biochemical property of this marker is its striking activity increase in the presence of certain alcohols (1-3). This property has been exploited to enhance the sensitivity of ACP_1 detection in electrophoretic typing systems (4). We describe here important features of this enhancement phenomenon.

MECHANISTIC CONSIDERATIONS. There is good evidence that the reaction mechanism of ACP_1 involves two steps. Step 1 is the reaction of enzyme with substrate and results in the formation of a phospho-enzyme intermediate with the concomitant release of the donor group from the substrate.

(1) $E + ROP \longrightarrow E\text{-}P + ROH$

The second step involves the transfer of the phosphate from the intermediate to water yielding inorganic phosphate (2a) or to an acceptor alcohol yielding a new phosphate ester (2b).

(2a) $E\text{-}P + H_2O \longrightarrow E + Pi$

(2b) $E\text{-}P + ROH \longrightarrow E + R'OP$

The overall reaction rate is determined by the slower step in this two step sequence. In the presence of certain alcohols, the apparent reaction rate (as measured by the release of the phosphate donor) is greatly increased; this is illustrated with glycerol as the acceptor in table I. These results indicate that the second step of the reaction sequence is the rate limiting step. The rate increase is suppressed by phosphate; this is important to note since many ACP_1 electrophoresis buffers contain phosphate.

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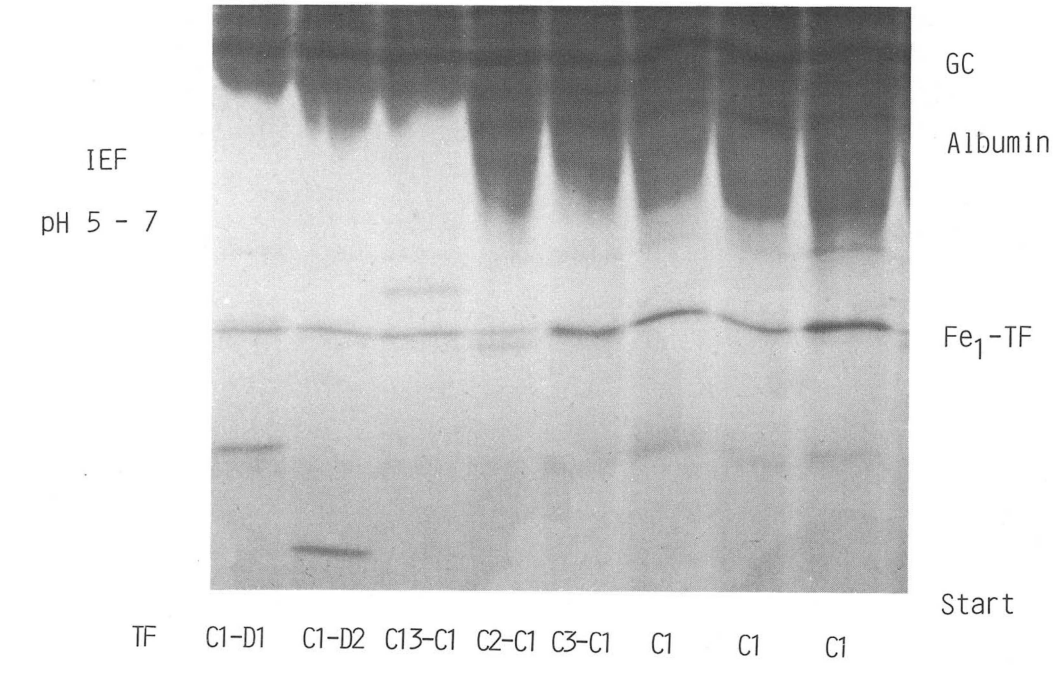
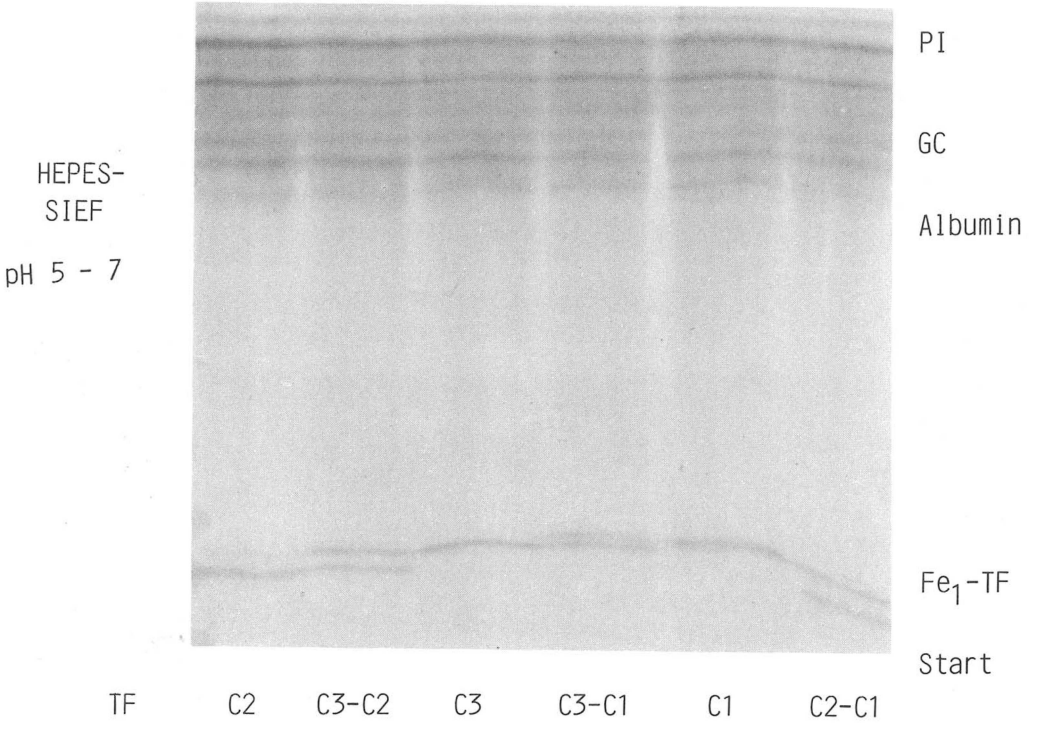
HEPES in a total concentration of 1.4% was found to be superior to the other substances added in comparable quantities (ACES, MES,EPPS and MOPS).ACES and EPPS led to drastic flattening of the area cathodal to the application zone and was found unsuitable for TF D differentiation;albumin,GC and TF were compressed to a gel area of 2 cm cathodal of the anode strip (ACES 0.06 g/gel) or even 1 cm (EPPS 0.06 g/gel), permitting no TF C1/C2 analysis.If MES was added in a concentration of 1 g/ 0.5 mm gel pH 5-7,albumin,GC, and TF were shifted to the cathodal half of the gel.The width of the C2-1 corridor reaches 2 mm,but no clearcut C3-1 types were seen.
Technical problems and drawbacks occurred due to inadequate maximum settings,salt concentrations and drying up of the ultra-thin gels during the application of the filter paper samples in the initial phase of our experiments.There were outweighed by considerably better separation results of TF C subtypes,smaller quantities of carrier ampholytes required and by shorter staining/destaining times.The results of the neuraminidase-treated samples were less satisfactory.Desialyzation led to the known cathodal shift of Fe_1-TF molecules,close to the application area.The sharpness of C-bands,in particular the C3-1 corridor however was second to that of the SIEF procedure described above.

Further improvements of the SIEF technique may render it an alternative to immobiline gels,which yield wider corridors between less distinct C subtype bands in our hands.Meanwhile routine and reference typing in our laboratory confirmed the existence of at least 16 different C subtypes,whose sequence in cathodal direction now is as follows: C14,13,15,12,9,4,11,1,8,3,5,2,10,6,7,and 16 (KORNHUBER et al.,1985,in preparation).Only three of them were confirmed to exist at polymorphic frequencies in the Caucasoid populations (Hessen/FRG,n=2000 : TF*C1= 0.76975;*C2=0.16350;*C3=0.05650),whereas *C6,*C7,and *C9 were only encountered as rare variants (0.00100,0.00025,and 0.00025 respectively. Based on these data and a combined allele frequency of non-C (B,D) of < 0.01,a single exclusion chance for non-fathers of 19.4% is calculated for the TF system,if sensitive and reproducible isofocusing procedures are applied.





ment,Macrodrive 5 power supply,Multitemp II thermostat and the Ultromould gel casting apparatus.For the pH ranges 3.5-9.5,4-6.5,and 5-7 Ampholine PAGplates were used;own gels were prepared with the following specifications:T=5%,C=3%,ampholyte concentrations 3%;gel dimensions 245x110x0.5mm; ampholytes were chosen for the gradients pH 4-6,5-7 with 10% 3.5-10 (LKB), and 4.2-4.9 (Pharmalyte).Numerous modifications were tested for optimal separation conditions in separator isofocusing (SIEF).For standard gels of 0.5 mm thickness,1600V,25mA,and 20W were found suitable;for HEPES-SIEF, the combination which yielded the best separation of TF isoproteins,the following maximum settings and IEF parameters were selected:2000V,25mA,and 13W at a cooling temperature of 5°C for 5hrs total focusing time ; 30 min prefocusing,45 min with sample filter papers on the gel, and 225 min without filter papers.The size of the Whatman #1 filter paper samples was 7x10 mm,15 mcl of diluted serum were applied with a microliter syringe.Electrode wicks were soaked with 1% acetic acid as anolyte, and 1% ethanolamine as catholyte (Whatman #17 strips).The samples were applied in the center of the gel in a distance of 4.5 cm to each electrode.

The fixing of the gels after separation was performed in a PAG solution, consisting of 30 g of sulfosalicylic acid,330 ml of methanol, aq. dest. ad 2000 ml,for 10 min.The gels were then stained with a 0.1% Coomassie Brillant Blue R 250 dye dissolved in destaining solution (700 ml of 70% ethanol,160 ml acetic acid 96%,aq. dest. ad 2000 ml for 15 min.The subsequent destaining was oriented to the optimal visualization of the Fe_1-TF bands with a pI of approximately 5.9.

Compared with the standard IEF separation on commercially available 1mm gels or 0.5 mm gels cast on the Ultromould (Fig.1),wider corridors were obtained in the HEPES-SIEF ultra-thin-layer gels for the phenotypes C2-1 and in particular C3-1 (Fig.2). Whereas there is only a blurred,broad zone of Fe_1-TF activity,the corresponding isoprotein fractions are clearly distinct due to the flattening of the gradient caused by the biological buffer.Another remarkable side effect of these gels is the fact,that the area anodal to the region of the common C subtypes is devoid of the albumin trail,which reaches the TF B1-2 bands and may cover variants with even lower pI values than B1-2.

SEPARATOR ISOELECTROFOCUSING : THE INFLUENCE OF BIOLOGICAL BUFFERS ON THE IEF PATTERNS OF TRANSFERRINS

Bernhard Kornhuber and Peter Kühnl

Institute of Immunohematology ,University Frankfurt,Sandhofstrasse 1, D-6000 Frankfurt/Main 73,FRG

Genetic variation of human transferrin (TF) has remarkably increased, since isoelectrofocusing (IEF) procedures were applied for the investigation of this polymorphism in 1977.Until 1985, TF*C with a frequency of 0.99 in Caucasoids was split into 15 subtypes,C1 to C15,with C1,C2 and C3 encountered as common alleles.The identification of rare TF C subtypes, whose bands have pI values close to that of C1,C2 or C3 is complicated by too narrow corridors of heterozygotes in standard separation systems.

To overcome this problem,we applied the following five biological buffers ('separators') to polyacrylamide gels of various pH ranges:

1.ACES (N-2-acetamido-2-aminoethane-sulfonic acid ; pK 6.8)
2.HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid ; pK 7.5)
3.MOPS (3-(N-morpholino)-propane sulfonic acid ; pK 7.2)
4.MES (2-N-morpholino)ethane sulfonic acid ; pK 6.1)
5.EPPS (N-(2-hydroxyethyl)-1-piperazinepropane-sulfonic acid ; pK 8.9)

The total concentration of buffers ranged from 0.5 to 6.5 % (w/v).Sera were obtained from random blood donors from Hessen/Germany,from individuals tested in blood group expertises,and for reference typing purposes. Pretreatment of sera was performed with 0.15% ferrous ammonium sulphate 1:7 (v/v),incubated for 18hrs at 4^{o}C in a sealed plastic tube for Fe-saturation of TF.Neuraminidase treatment was used for desialysation of the TF molecule (clostridium perfringens neuraminidase type V,Sigma Co.),1:3 (v/v) in Fe-untreated or 1:9 in Fe-treated sera solutions.

Agarose gel electrophoresis (AGE) was performed with a DESAGA 202000 electrophoresis equipment in the barbital/sodium barbital/calcium lactate buffer system of pH 8.6 as originally designed for C3 typing by TEISBERG (1970).Isoelectrofocusing (IEF) procedures were carried out according to previously described issues with Multiphor/Ultrophor (LKB) equip-

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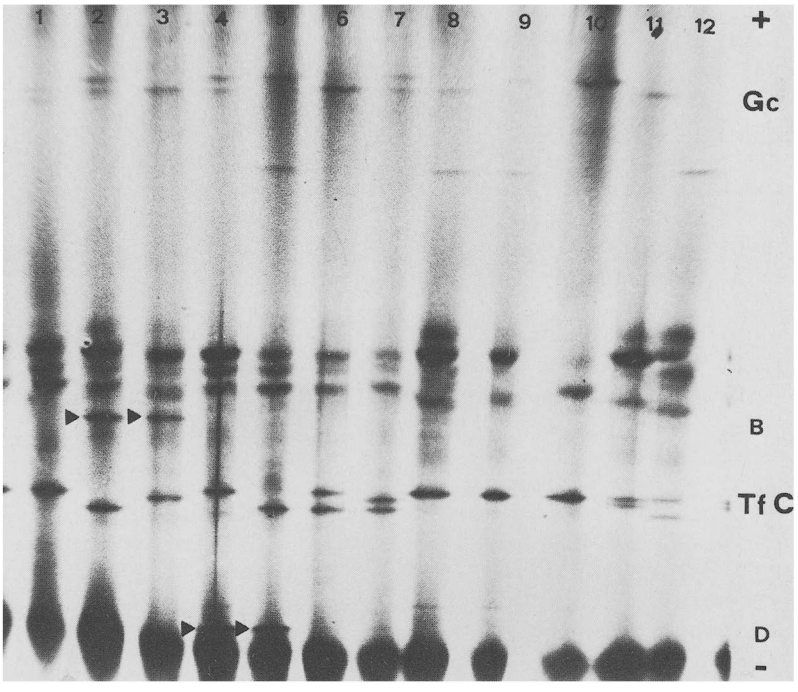

Fig. 1:C1/1S-1C3;2:C2-B/1S-1F;3:C3-B/1S;4:C1-D/1S-1F;5:C2-D/2-1F; 6:C2C1/1S;7:C3C2/1S-1F;8:C1/2-1S;9:C1/2-1F;10:C1/1F;11:C3C1/1S; 12:C2C1/2

2.2. Gel Casting
According to "LKB Application Note No. 321". The slot former is situated 1,5 cm from the cathodical edge of the glass plate.

2.3. Sample Preparation
Add 3 ul of 0.01 M FeCl3 (27 mg/10 ml) to 50 ul serum diluted with 150 ul bidest. water and leave for 4-6 hours.

2.4. Sample Application
Pipette 5 ul of sample into the gel slots

2.5. Running Conditions
2500 v, 25 mA (max),10 W (max), 10 C, overnight
Filter stripe solutions: Anolyte: 0.1 M H3PO4 ;
Catholyte: 0.1 M NaOH

2.6. Fixation and Staining
a) Fixation: TCA (6M):Sulfosalicylacid :Methanol:H2O=5:15:30:50
b) Staining: leave for 10-20 Minutes at 60oC in a solution of Methanol:TCA: H2O=45:12:43. Add 3 g of PAGE Blue 83 per liter staining solution (BDH Chemicals Ltd Poole England)
c) Destaining: Solution of Methanol:TCA:H2O=45:12:43

3. Results and Discussion

The Tf bands show up on the cathodical half of the gel, those of Gc on the anodical side. An anti-Gc serum is not necessary since the bands appear umambiguously after fixation and staining with page Blue. Variants are easily detected and no extra bands appear in this part of the gel.The phenotyping Tf C3C1 and C3C2 is always clear-cut. Rare Tf-D variants, lying cathodically, could escape detection and simulate a second order exclusion . The Tf-B variants lie more anodically and must not be confused with extra-bands appearing at this location (as double-check in complement C3 typing transferrin D- and B variants are also detectable!).

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DETERMINATION OF TRANSFERRIN (Tf) AND GROUP-SPECIFIC COMPONENT (Gc) SUBTYPES ON A SINGLE POLYACRYLAMIDE GEL WITH AN IMMOBILIZED pH GRADIENT

V. Biedermann and W. Bär, University of Zürich, Switzerland, Institute of Forensic Medicine

1. Introduction

With isoelectric focusing the simultaneous determination of multiple systems - routinely done in starch gel electrophoresis - is usually not possible. The consequent increase in labor and costs is a non-neglectable fact for many laboratories. The ease in generating stable, linear and ultranarrow pH gradients with Immobiline is helpful to develop methods for the simultaneous typing of focusing systems that have isoelectric points that lie within a half of a pH unit. The allelic products of Gc and Tf have iPs between 4.95 - 5.4. A method using immobilized pH gradients to type the complete sets of phenotypes of Tf and Gc in one run on polyacrylamide is presented.

2. Methods

2.1. Gel Composition

Starting Solution:	light, basic	dense,acidic
Acrylamide/Bis (16,2%,0.5%)	3 ml	3 ml
Glycerol 87%	3 ml	3 ml
Bidest. water	3 ml	6 ml
Immobiline pK 4.6 (0.2M)	0.5 ml	0.5 ml
Immobiline pK 6.2 (0.2M)	0.5 ml	0.5 ml
Total	10 ml	10 ml
Adjust pH with Immobiline pK 3.6 ->pH 5.00		w. pK 9.3 -> 5.90
Add after pouring 9 ml of each solution in the gradient mixer:		
TEMED	7 ul	7 ul
APOS 30%	10 ul	10 ul

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Transferrin

	Münster (N=3255)	Weidinger (N=184)
C^1	0,79	0,78
C^2	0,15	0,15
C^3	0,05	0,07
B	0,008	0,005
D	0,001	-

Fig.1: Tf gene frequencies in Germany

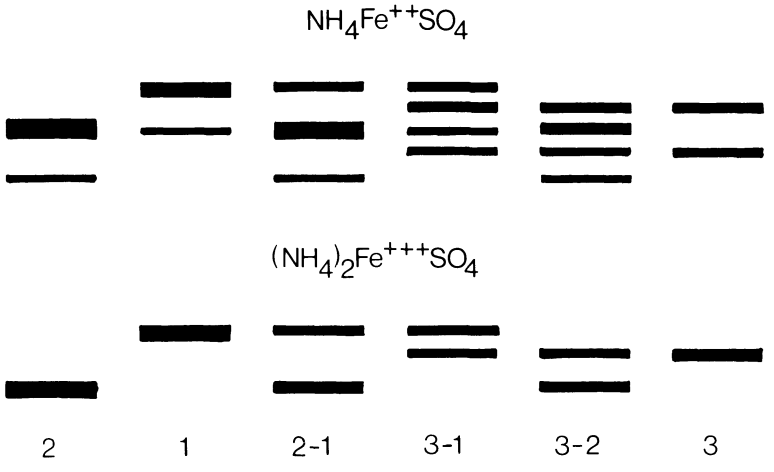

Fig. 2: Diagrammatic representation of Tf band patterns after pretreatment

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heterozygote, it was sometimes possible to identify a 1 band from a 2-1 phenotype but with no activity in the 2 region. As no reference could be made to the relative band intensity this would pass unnoticed and a false result obtained. Although this was not often the case, it was more noticeable in older stains and occured occasionally in fresh stains.

Treatment with Mercaptoethanol did not improve the band resolution. It was also found that in stored alcohol samples bandswere sometimes observed which could be misinterpreted as B or D variants.

These observations show that although stain grouping was possible and in most cases the correct phenotype identified, errors could be made in the interpretation. Until this is resolved it would be better to use this system cautiously in casework.

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investigated using ultra-thin (0,15 mm) polyacrylamide gel isoelectric focusing (pH 4-6).
Fixation in sulphosalicylic acid was followed by staining in Coomassie Blue. Serum samples were diluted 1:9 with the extraction solutions, ferrous and ferric ammonium sulphate (0,35 %) and 0,18 M 2-Mercaptoethanol and incubated overnight at 4° C.

Results

Pretreatment with ferrous and ferric salts produced clear results in both cases but the bands were always more intense using the ferrous salt.

The band pattern was also observed to be different (fig.2). The ferrous salt produced two bands for each allele, a stronger anodal and weaker cathodal band, whereas the ferric salt showed activity only in the cathodal region. The identification of homozygotes from serum samples typed after ferrous salt extraction gave no problems. Heterozygotes however were sometimes difficult to interpret because the cathodal band of the 2 allele, in 2-1 or 3-2 phenotypes was always weak and sometimes absent. In these cases the relative intensity of the other bands was of great importance, and provided the only means of avoiding a false interpretation.

After storage at -20° C serum samples could, in most cases be typed without loss of activity for two to three months.

The typing of bloodstains proved to be not so straightforward because this relative intensity of the bands was not consistent. Consequently identification of the phenotype was only possible by reference to the presence or absence of bands. As the 2 band was always weaker in a

OBSERVATIONS ON THE USE OF ISOELECTRIC FOCUSING FOR SUBTYPING IN THE TRANSFERRIN (Tf) SYSTEM

Steven Rand[1], Anke Kofahl, Bernd Brinkmann (Institut für Rechtsmedizin, Münster/Germany. [1]On loan from the Metropolitan Police Laboratory, London)

In 1978 Kühnl and Spielmann, and simultaneously Thymann, identified 2 alleles of TfC by the application of isoelectric focusing. Further work by Kühnl and Spielmann (1979) revealed the TfC^3 gene, a further subdivision of TfC^1, and pretreatment of serum samples with ferric chloride by Weidinger (1980) improved the visualisation of the bands. This gave a possible total of 6 phenotypes at the TfC locus. Weidinger also calculated the theoretical exclusion rate to be approximately 19 % and the system is now widely used for paternity testing.
In this laboratory, over the past 2 1/2 years a total of 3255 serum samples have been tested using ammonium ferrous sulphate pretreatment (Constans 1980).
From this survey (fig. 1) gene frequencies were calculated as approximately TfC^1 0,79, TfC^2 0,15, TfC^3 0,05, TfB 0,008 and TfD 0,001 which agree well with other published figures.

This distribution and the relative stability of the system observed in serum samples, would prove useful for blood stain identification if typing were possible.

In 1979 Hoste reported disappointing results but used no pretreatment of stains and in 1983 Pascali reported increased sensitivity after pretreatment of sera with 2-Mercaptoethanol. These solutions, i.e. Mercaptoethanol and ferrous and ferric ammonium sulphate, were investigated as a possible means of bloodstain typing.
Parallel studies on 230 paired sera and bloodstains were

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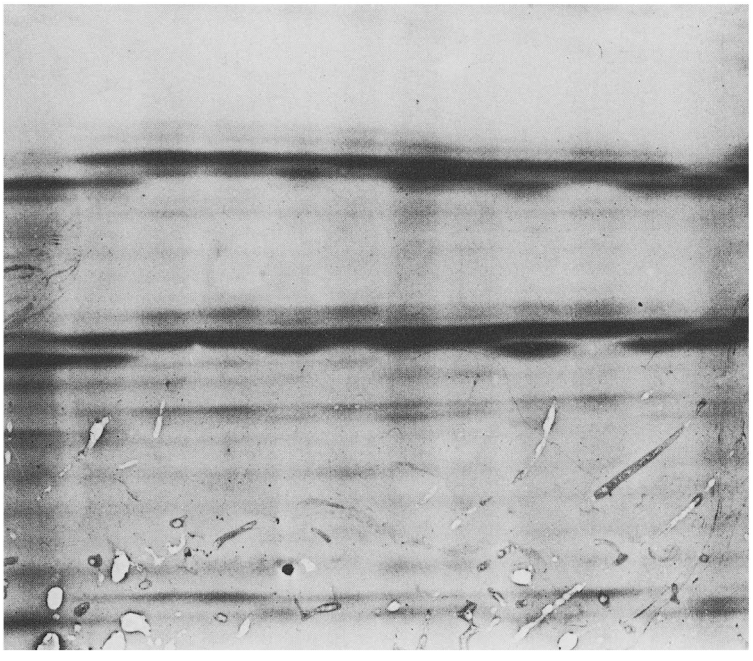
Fig.1. Resolution of Antitrypsin subtypes on a shallow immobilized pH gradient (interval of pH 4.35-4.85, anode on top). From left: M2, M1M2, M1, M1, M1M3, M1, M1M3, M1M3, M1M2, M1, M1M2, M2M3



bility in gels and in solution.

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VLP and AC thankfully acknowledge Prf.E.Villanueva and Prof. A.Fiori, who encouraged a collaboration of the two lab.staffs during the Vth Course of Instrumental Techniques applied to Forensic Sciences (University of Granada, Feb.1985).

suggested us some comments.
1)On a methodological standpoint, the strategy to interpolate shallow gradients from larger is a convenient shortcut to Henderson-Hasselbach equation. Precalculated broad gradients on which to rely are numerous and interpolated gradients have accurate endpoints and satisfactory buffering properties. All our gels contained sucrose instead of Glycerol as density medium. That would allow lessticky gels and prevent water exudation. Moreover sucrose additioned gels would impart counteract the side broadening of band patterns, provided that the preliminar gel washing be avoided. In order to optimize the polyacrylamide-Immobiline bond we found useful to replace water with a Tris/Gly buffer(50 mM, pH 8.3), and to increase the amounts of TEMED and Ammonium Persulfate, as suggested by Righetti et al.(6).
2) Large IPGs of one point of pH do not notably improve PI resolution yet given by properly drifted CA gradients. But, quite obviously, they are undefinitely stabler and more reproducible than CA gradients. Shallower intervals provide a far more powerful way to separate PI, which,this way,splits easily all M mutants, including the currently mistyped M4. But shallow IPGs are not shortcoming-free. First they must overrun many more hours(about 15) and thereby a side broadening occurs. Even worse, resolution increases at the expenses of band sharpness (the shallower interval the fainter its pattern). We cannot presently avoid thinking of that as a general mishap of ultranarrow IPGs, whose removal needed proband sera amounts to be drastically increased. We could estimate that 30 to 40 µl serum still mantain the PIM pattern within the range of Coomassie stain sensitivity. Neverthless the problem is not solved for the Z heterozygous whose faint proteic activity still risks mistyping. Therefore a two step procedure should be adopted whenever the MZ diagnosis is the case: firstlarge CA IEF, then narrow IPGs for M subtyping.
3)We shall not leave this topic without hinting the most cumbersome problem which hinders the full introduction of IPGs in the routine, say the high instability in solution. Whoever dealt sufficiently long with IPGs must have been deeply disappointed by at least one expired batch of expensive chemicals. Our personal experience was made on an Immobiline pK 9.3 which had most likely lost its ability to bind the polyacrylamide by still keeping titrating properties.
In summary, our current use of IPGs is in solving some special problems of stressed resolution, such as in PIM4 typing. We do expect for the future that such a powerful tool be mended from its mistrustful features: lowest conductivity and strong defocusing effect of narrow gradients, chemical insta

IMPROVED DIAGNOSIS OF ANTITRYPSIN SUBTYPES BY ULTRANARROW IMMOBILIZED pH GRADIENTS

Vincenzo L.Pascali(*), Gabriella Conte(*), Pietro Auconi (+) and Angel Carracedo (°)
(*)Istituto di Medicina Legale,Università Cattolica del S.Cuore, Roma, and (+) Dip.Pediatria, Ospedale,Arezzo,Italy;(°) Departamento del Medicina Legal,Santiago de Compostela,Spain

The ability to discriminate isotypes whose spectra lie very close one to another is one of the today's compelling tasks to every workers applying their efforts on the electrophoresis of the genetic markers of blood. This statement applies well to acid glycoprotein systems of human serum, whose pherograms are growing overcrowded.
As a consequence, the adoption of high resolution electrophoretic methods is presently a must, and the technique of IEF on ultranarrow pH gradients (IPGs) promises to be providential for this purpose (1).
Like other groups of Forensic Serology area, our laboratory unit is planning to introduce IPGs in research programs and in routine paternity casework. As a first system with which to refine our skilfulness with IPGs we choose Antitrypsin(PI) ,that for at least one good reason. PI resolution is not quite easy with carrier ampholytes (CA). The resolving power of CA is barely reproducible from run to run, and whenever accurate Volt/hs coefficient be applied, uneven batch-to-batch outcome will ensue. The efficiency of IPGs in improving PI separation has been repeatedly stressed by the group of Munich (2)(3). We have recently summarized a method for PI separation by IPGs on a paper in press (4). Here we shall outline our procedure,with some further improvements, and deal with current problems of introducing IPGs in the routine.

Materials and Methods

Ultrathin layers IPGs were performed either as described previously (4) or as advised in Ref.(5). Samples of serum previously typed for PI in Santiago and Rome labs by CAIEF were first tested on broad IPG pH 4.1-5.1 (buffering/titrating amounts as in (5)). Twofold (or more) deeper intervals were then derived from such gradient by a simple linear interpolation. Intervals of pH 4.35-4.85 (dense solution:0.368 ml pK 4.6 and 0.150 ml pK 9.3 Immobiline; light solution:0.390 pK4.6 and 0.270 ml pK9.3) and pH 4.40-4.80 (dense solution: 0.370 ml pK4.6 and 0.160 pK9.3; light solution: 0.389 ml pK 4.6 and 0.258 ml pK 9.3) were especially experimented.

Results and Discussion

The routine implementation of the above described intervals

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Other PiM-variants were not observed.

For practical purposes at parentage problems of disputed paternity it seems to be advisable to employ APAGIF with carrier ampholytes within a range of pH 4.2 - 4.9 as basic method. IPAGIF with Immobilines (pH 4.5 - 4.7) may be used simultaneously in order to distinguish PiM-subtypes. After having acquired sufficient manual practice in handling and preparation of Immobiline-gels, this method is characterized by high reproducibility and technical stability.

For paternity tests the Pi-system should be highly recommended because of its efficiency: The general exclusion chance of the Pi-system based on the figures given above gave as a result 29.8 %.

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The particular value of IPAGIF as complementory method for Pi-subtyping may be demonstrated on Fig. 1 and Fig. 2.

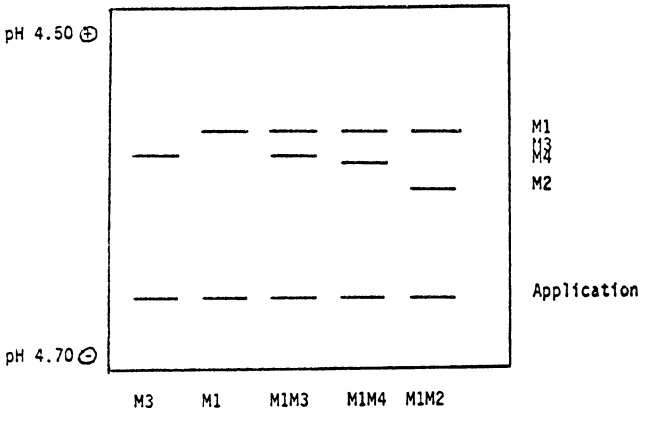

Fig. 1: Pi- Phenotypes (scheme)

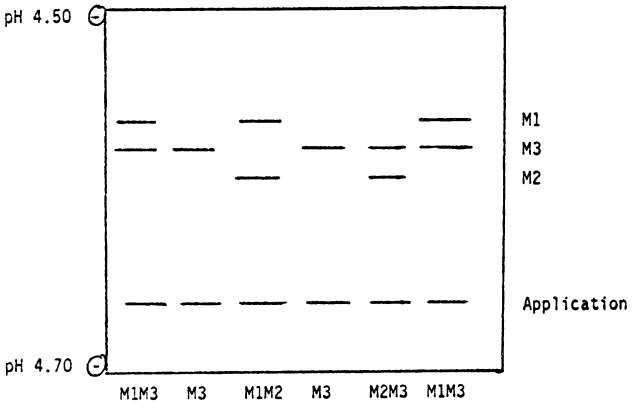

Fig. 2: Pi-Phenotypes (scheme)

The different bands of the PiM-subtypes are clearly separated by wide spaces. However due to the narrow pH gradient, cathodal resp. anodal Pi-types as PiS, PIZ and other variants are not visible. The PiM4-subtype identified by Constans et al. (5) could be found in our randomized sample of 900 sera only once (Fig. 1). Because of the narrow distance between the PiM3 and PiM4 bands PiM4 cannot be reliably identified in all cases, despite of the optimal resolution of IPAGIF. It seems to be easy to mis-interprete PiM4 as PiM3. Therefore gene frequencies of Pi*M4 published so far have to be cautiously evaluated.

Results and Discussion

The gene frequencies for the North German population were calculated from the observed phenotypes:
Pi*M1 = 0.7033; Pi*M2 .....

The observed frequencies are in agreement with the Hardy Weinberg equilibrium (x^2 = 2.4791; d.f. 5). Compared to other European frequencies no significant differences could be stated (Table 3).

Population	n	M1	M2	M3	M4	S	Z	VAR	
West-Japan	1.000	0.7065	0.2390	0.0480	0.0015	-	-	0.0050	*
Toscana	965	0.6772	0.1814	0.1073	-	0.0245	0.0410	0.0047	
Österreich	939	0.6731	0.1741	0.1097	-	0.0218	0.0138	0.0075	
Süddeutschland	752	0.6894	0.1649	0.0904	0.0179	-	-	0.0374	*
Niederlande	357	0.6793	0.1471	0.0812	0.0476	-	-	0.0448	*
Zentralspanien	103	0.6650	0.1699	0.0679	0.0146	-	-	0.0825	*
Süddeutschland	347	0.6917	0.1686	0.0865	-	0.0230	0.0187	0.0115	
Hessen (BRD)	282	0.6879	0.1720	0.0957	-	0.0284	0.0106	0.0053	
Toulouse (Frankreich)	163	0.6260	0.0920	0.1040	0.0370	-	-	0.1410	*
Niederlande	131	0.75	0.05	0.13	-	0.04	0.03	-	
Finnland	136	0.79	0.12	0.08	-	0.042	0.013	-	
USA-Weiße	240	0.64	0.19	0.11	-	-	0.01	0.005	
USA-Schwarze	304	0.903	0.028	0.054	-	0.005	0.003	0.007	
Norddeutschland	900	0.7033	0.1467	0.1089	-	0.0172	0.0167	0.0072	

* This value contains variants including PI S and PI Z

Table 3: Frequencies of PI-Alleles of different Populations

Acid Solution pH 4.50 (High Density)		Basic Solution pH 4.70 (Low Density)	
Immobiline pK 4.6	1,50 ml	Immobiline pK 4.6	1,50 ml
Immobiline pK 6.2	0,62 ml	Immobiline pK 6.2	0,88 ml
Stock solution	2,50 ml	Stock solution	2,50 ml
(29,1 % Acrylamide, 0,9 % Bis)		(29,1 % Acrylamide 0,9 % Bis)	
Glycerole (87 %)	4,20 ml		
Aqua bidest. ad	15,00 ml	Aqua bidest. ad	15,00 ml
7,5 ml into mixing chamber of mixer		7,5 ml into reservoir chamber of mixer	
TEMED (10 %)	60 µl	TEMED (10 %)	60 µl
Ammoniumpersulfate (10%)	30 µl	Ammoniumpersulfate (10%)	30 µl

Table 1: Composition of acid and basic solution for the preparation

The gels are ready for use after a polymerization time of 1 hrs without prefocusing. The serum amount of 25 µl has to be applied on sample application pieces (LKB 1850-901). Equipment, running conditions and staining is summarized on table 2.

Equipment: LKB Ultrophor 2217-001
LKB Power Supply 2197
LKB Gradient Mixer 2117-902

Running Conditions: Voltage 2000 V
Current 25 mA
Power 10 W

Electrofocusing time (total) 15 hrs (over night)
Electrofocusing with Filter paper pieces: 45 min.

Staining: Fixation: (57,5 g Trichloroacetic acid; 17,25 g Sulfosalicylic acid; 150 ml Methanol; 350 ml Aqua dest.) ca. 30 min.
Staining: 1,15 g Servaviolett in 1000 ml Destaining solution (60°C): ca. 60 min.
(Destaining Solution: 50 ml Methanol; 20 ml Acetic acid; 130 ml Aqua dest)

Table 2: Equipment, Running Conditions and Staining

Alpha-1-Antitrypsine (Pi) Polymorphism. Improved Pi-M-Subtyping by PAGIF with Immobilized Gradients.

F. Ferstl and H. H. Hoppe
Zentralinstitut für Transfusionsmedizin, Hamburg, D-2000 Hamburg
Filbektal 111

The demonstration of the genetically determined alpha-1-antitrypsine (PI) phenotypes may be achieved generally by isoelectrofocusing (IEF) on polyacrylamide gels (PAGIF) with carrier ampholytes. This method which originally was applied at a pH-gradient pH 3.5 - pH 6 does not produce a sufficient resolution quality though recently carrier ampholytes with a pH gradient pH 4.2 - 4.9 are offering an improved resolution (3).

The reason for this problem is the poor resolution of the PiM-gene products which show a narrow difference of about 0.01 pH only.

However a substantial improvement can be achieved by IEF with immobilized pH gradients. This method is based on a new concept of the preparation of linear pH-gradients. Compared to pH-gradients achieved by carrier ampholytes, Immobilines are covalently fixed on the polyacrylamide gel matrix during polymerization. So Immobilines incorporated into the gel offer a stabile buffering capacity.

The advantages of isoelectrofucusing with immobilized pH gradients are above all the application of extremely narrow pH gradients. Furthermore some problems have been solved, e.g. the plateau-phenomenon (1, 2).

Simultaneously PAGIF with carrier ampholytes (APAGIF) and Immobilines^{R} (IPAGIF) were carried out. The evaluation of the investigation described below has the following objects in view:

- Pi-Gene Frequencies of the North German Population
- Estimation of PiM-Variants
- Evaluation of Pi-tests by PAGIF with immobilized pH-gradients in paternity testing.

Material and Methods

600 serum samples of blood donors and 300 serum samples of not related persons involved in cases of disputed paternity (mothers, presumptive fathers) were tested.

Simultaneously APAGIF with carrier ampholytes for basic Pi-typing according to Dr. Martins method (3) and IPAGIF with immobilized pH-gradients (6) was carried out.

IPAGIF (pH 4.5 - 4.7) is performed on polyacrylamide gels 0.5 mm. The preparation of the gels follows the modified method originally described by Görg et al. (4).

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between M1 and M5.

The newly observed subtype, Pl M5, is located between M1 and M3. It was noted in a mother and her child who had the phenotypes M1M5 and M5S, respectively (Fig. 2 No.8 and No.7). The defendant of this case of disputed paternity had the type Pl M1. He was excluded in the Pl system as well as in five other blood group systems. The distance between M1 and M5 bands is approximately 0.006 pH-units only. If a gradient as narrow as 0.3 pH-units is employed M1M5 can be noted as a double band pattern.

Figure 2 shows a further new subtype, previously called Pl M7. The allele products of Pl*M7 are located between M2 and M4. The inheritance was confirmed by family study.

Figure 3 gives a simplified schematic representation of different Pl M subtypes, including the new type M1M5. The number of subtypes has now reached seven; their sequence from anode to the cathode is in the order: M6, M1, M5, M3, M4, M7, and M2.

In conclusion, we feel that IEF with immobilized pH gradients offers certain advantages over several procedures which have been used for Pl subtyping. The possibility to produce very narrow stable pH gradients ensure high resolution and provide, thus, a powerful tool for the study of genetic variability.

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has been a problem.

Recently, IEF with immobilized pH gradients (IPG) has been employed for the analysis of PI variants (14). By using a special IPG an improved resolution of PI M subtypes was obtained (11). This modified procedure

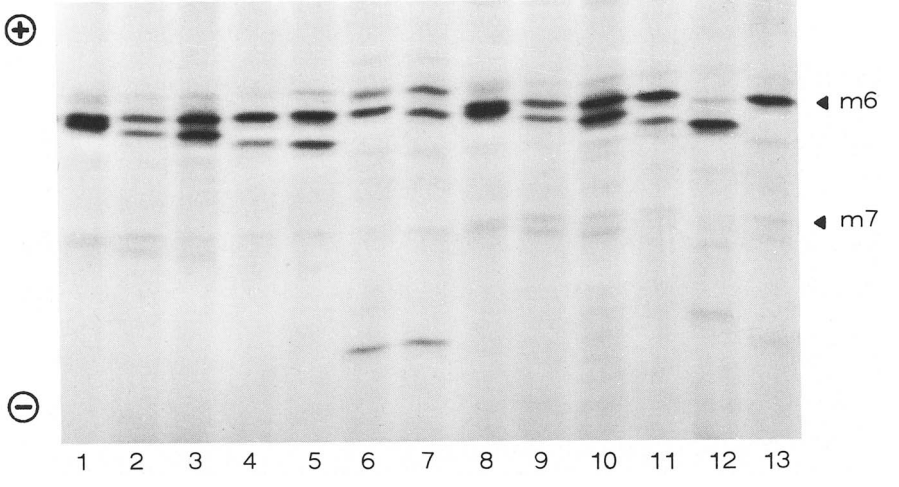

Fig. 2. Demonstration of PI M phenotypes as analyzed by IEF with immobilized pH gradients (pH-range 4.45 - 4.75). From left to right: (1) M1M5, (2) M1M3, (3) M1M4, (4) M1M7, (5) M1M2, (6) M1 S, (7) M5 S, (8) M1M5, (9) M1M3, (10) M1M4, (11) M1M7, (12) M2 Z, and (13) M1 Zaug

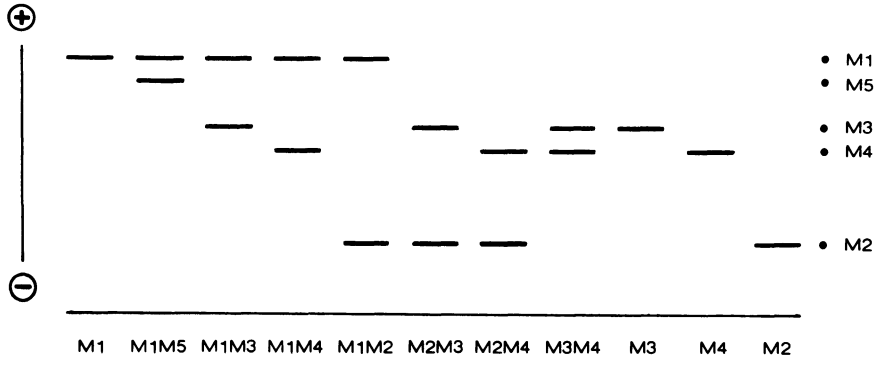

Fig. 3. Schematic representation of PI M subtypes

permitted the distinction between M3 and M4, and quite recently, also

1. IEF experiments with ampholytes were carried out on thin-layer polyacrylamide gels with the pH-range of 4.2 - 4.9 according to Weidinger et al. (12).
2. IEF with an immobilized pH gradient was performed with a pH-range of 4.45 - 4.75 (11).

Results and Discussion

Classification of PI phenotypes is routinely carried out by IEF with ampholytes. Figure 1 shows several PI types as obtained by IEF with Pharmalytes (pH 4.2 - 4.9). Separation with this pH-range reveals the different PI m-regions called m4, m6, and m8 (13). The phenotypes of

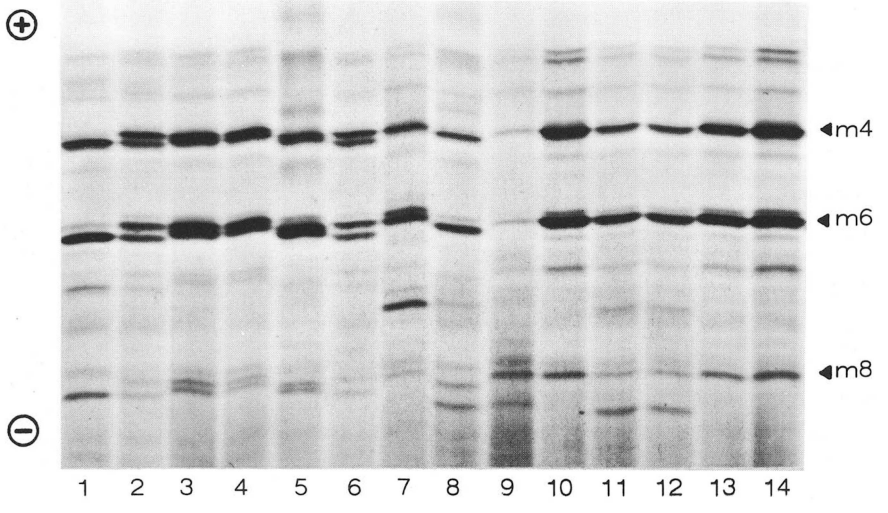

Fig. 1. Demonstration of PI phenotypes as analyzed by IEF with carrier ampholytes (pH-range 4.2-4.9). From left to right: (1) M2, (2) M1M2, (3) M1M4, (4) M1M3, (5) M2M3, (6) M1M2, (7) M1 S, (8) M3 Z, (9) M1 Z, (10) M1, (11) M1 Zaug, (12) M1 Zaug, (13) M1, and (14) M1

two persons with the deficient allele PI*Z augsburg (PI*Zaug) are also illustrated and compared with the common PI Z. Although the six common PI M subtypes (M1, M1M2, M2, M1M3, M2M3 and M3) can readily and reliably classified, the differentiation of M3 and M4 by conventional IEF

PI M5: AN ADDITIONAL ALPHA-1-ANTITRYPSIN PI M SUBTYPE REVEALED BY IEF WITH IMMOBILIZED PH GRADIENTS

S. Weidinger and F. Schwarzfischer (Institute of Anthropology and Human Genetics, University of Munich, Richard-Wagner-Str. 10/I, D-8000 München 2, Federal Republic of Germany)

Introduction

Alpha-1-antitrypsin (α_1AT) is an important protease inhibitor (PI) capable of inhibiting a wide variety of serine proteases. Since the first description of genetic variation in 1963 (1) more than 60 genetic variants of this protease inhibitor have been observed (2). For classification of PI variants isoelectric focusing (IEF) has been employed since ten years. IEF has been particularly useful in identifying subtypes of the most common variant PI M (3-9).

For generating stable pH gradients a modification of the IEF method has been developed (10). Immobilized pH gradients (IPG) represent an entirely new concept in electrofocusing. Unlike Ampholine carrier ampholytes, the Immobilines are not amphoteric, so they cannot migrate electrophoretical to an equilibrium position in IEF. Since in IPG the pH drift is completely eliminated it is possible to produce extremely narrow pH ranges (0.01 pH-unit/cm) in polyacrylamide gels by mixing Immobilines with different pK values.

The application of this technique to the PI system has permitted the distinction of ten common PI M subtypes (11). In this report, an additional subtype will be described.

Materials and Methods

For classification of PI phenotypes fresh sera or sera stored at -20 °C were used. Two different IEF-methods were employed:

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been reported, nonexpression of B obviously acts as a lethal factor. All hemizygous deficient individuals of this family were apparently healthy. Thus BF heterozygous deficiency seems to be without any disease risk.

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The BF*QO allele is carried by haplotype a. As shown in the pedigree the silent BF allele came from the deceased grandfather (I-1) and existed in altogether five individuals (II-1, II-2, II-4, III-1, III-2).

Immunochemical levels of factor B protein and factor B hemolytic activity (relative and absolute) are given in Table 1.

Table 1. Immunochemical and functional levels of factor B

Family member	BF-type	Factor B protein (mg/l)[a]	Factor B hemolytic activity	
			relative (U/mg)	absolute (U/l)
I-2	FS	113.7	1.218	138.5
II-1	SQO	68.6	1.914	131.3
II-2	SQO	97.5	1.222	119.1
II-3	S	126.5	1.095	138.5
II-4	SQO	85.3	1.581	134.9
II-5	FS	115.7	1.067	123.4
III-1	SQO	80.9	1.570	127.0
III-2	FQO	68.1	1.435	97.7

[a] Normal range 170–420 mg/l

While the protein level was markedly decreased in the five BF*QO carriers, the absolute hemolytic activity of factor B was normal in four out of them. Obviously the lack of an expressed BF gene is compensated by the functionally normal BF*S of BF*F allele on the other chromosome. This compensating effect is documented by the relative increase of hemolytic activity in the heterozygous BF deficient individuals.

An association of BF*QO with special alleles of other MHC loci, as described for rare alleles of the BF system (12), does not seem to exist (Table 2).

Table 2. BF*QO MHC haplotypes

Loci									Reference
HLA-A	HLA-C	HLA-B	C4A	C4B	BF	C2	HLA-DR	GLO	
3	n.t.	7	n.t.	n.t.	QO	n.t.	n.t.	2	Weidinger et al. (1979)
2	w3	15	n.t.	n.t.	QO	B	4	1	Suciu-Foca et al. (1980)
w24	w1	w54	3	6	QO	C	w8	2	Tokunaga et al. (1984)
3	w3	w60	3	2	QO	B	1	2	This study

n.t. = not tested

It is of interest, however, that BF*QO may possibly be associated with rarer alleles of the C4B locus (C4B*2, C4B*6) and the C2 locus (C2*B).

Since among numerous cases of single or combined complement component deficiencies complete factor B deficiency has not

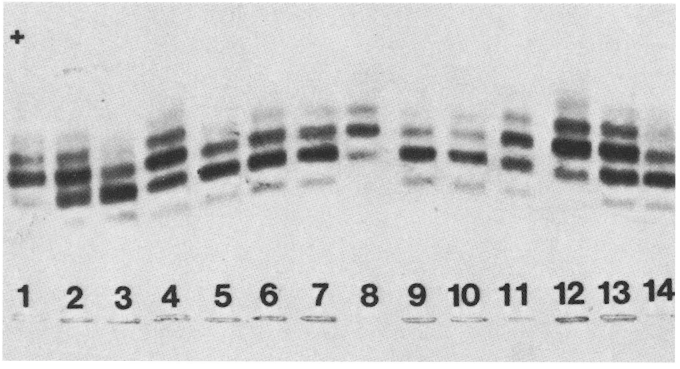

Fig.1 BF phenotypes after agarose gel electrophoresis and specific immunofixation: 1=control BF FF, 2=control BF FS, 3=control BF SS, 4=grandmother I-2, BF FS, 5=brother II-2, BF S(QO), 6=sister II-3, BF SS, 7=sister II-1, BF S(QO), 8=son III-2, BF F(QO), 9=son III-1, BF S(QO), 10=mother II-4, BF S(QO), 11=father II-5, BF FS, 12=control BF FF, 13=control BF FS, 14=control BF SS

In the pedigree of this family, given in Fig.2, heterozygous factor B deficient individuals are indicated by half black symbols. Haplotypes of the MHC region are marked by letters a,b,c,d,e,f. The given order of MHC loci is HLA-A, HLA-C, HLA-B, C4A, C4B, Bf, C2, HLA-DR, GLO.

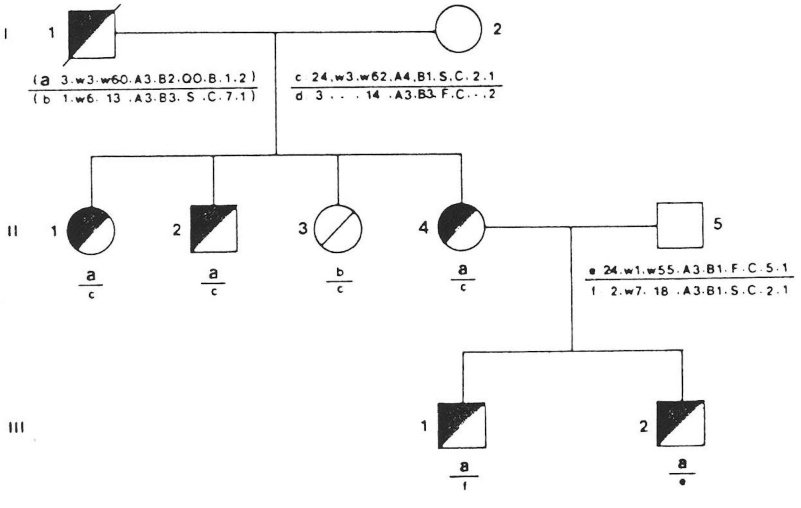

Fig.2 Pedigree of the family with five BF*QO carriers

A SILENT ALLELE OF PROPERDIN FACTOR B POLYMORPHISM (BF*QO) IN FIVE FAMILY MEMBERS:

J. Bertrams[1] and G. Mauff[2] (Abteilung für Laboratoriumsmedizin Elisabeth-Krankenhaus Essen[1], Hygiene-Institut, Universität Köln[2], FRG)

Introduction

Only three families with an apparently silent allele of the properdin factor B polymorphism (BF*QO) have been described until now (1-3). We found another family with altogether five BF*QO carriers during a disputed paternity case by a seemingly inverse homozygosity of the BF alleles S and F between mother and child (4).

Material and Methods

BF typing was performed by thin layer agarose gel electrophoresis and immunofixation with BF-specific antisera (5) (Atlantic Antibodies) and in addition by a hemolytic overlay after isoelectric focussing of BF allotypes (6). Factor B protein concentration was measured by radial immunodiffusion, functional levels of factor B by radial diffusion hemolysis (7). HLA-A,B,C,DR typing was performed by the standard lymphocytotoxicity technique (8). C2,C4 and GLO typing was done as previously discribed (9-11).

Results

The apparent inverse homozygosity of BF alleles S and F between mother (II-4) and child (III-2) is clearly demonstrated in Fig. 1 and could also be shown by the hemolytic overlay after isoelectric focussing of BF allotypes.

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Literature:

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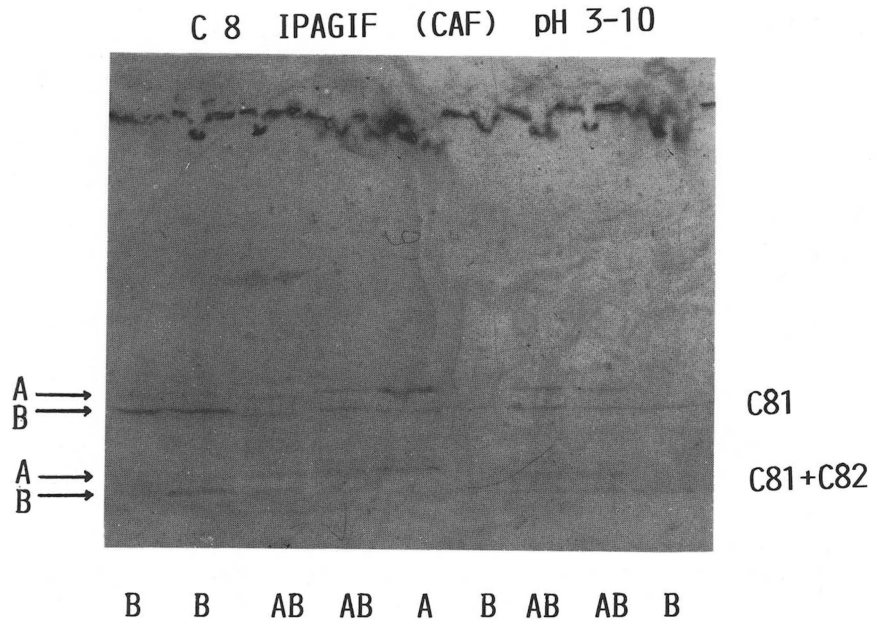

Fig. 2b: C8 IPAGIF (CAF strip) at pH 3-10

Concerning the population data, RAUM et al. published a C81*A frequency of 0.649 (n=165, US Whites); MEVAG et al. (1983) reported a frequency of 0.6 in Norwegians, RITTNER et al. (1984) found a frequency of 0.5536 for C81*A, 0.4286 for *B and 0.0178 for *A1. Our limited population data do not differ significantly from the latter figures (0.57, 0.41 and 0.02, respectively for the alleles C81*A, *B and *A1). Family data support the autosomal codominant inheritance of these C81 alleles.

According to the data of the Human Gene Mapping Conference, Los Angeles 1983, the C8 locus is on the short arm of chromosome # 1 in a distance of 6 centimorgans to PGM1 and 10 cM from the UMPK locus in direction to the centromere . These figures imply no restriction of the informative value of this genetic marker for forensic serological purposes. The exclusion chance for non-fathers hence may be estimated at 19.2 %. Further data will be needed to corroborate the hitherto published allele frequencies, family data and in particular the issue of the C82 system, where frequencies of 0.952 (C82*A), 0.044 (C82*B) and 0.004 (C82*A) were reported by ALPER et al. in 1983.

The question, if the contribution of the β chain to the microheterogeneity of the B region can be looked at independently, remains to be investigated in more detail.

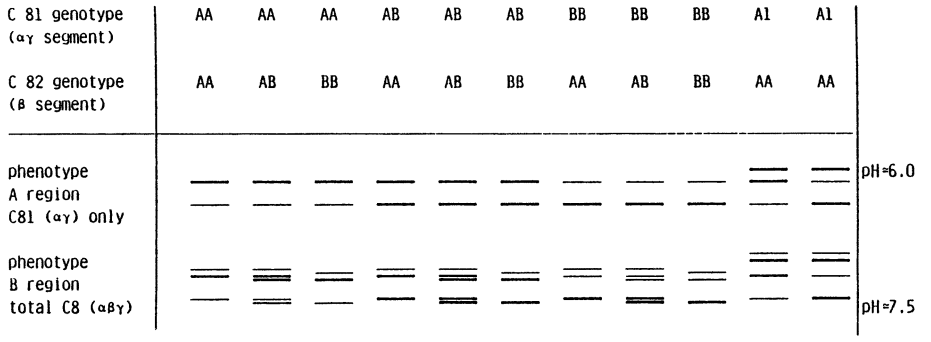

Fig.1: Model of C8(C81,C82) allotypes after IPAGIF at pH3-10

The separation of C8 phenotypes by IAGIF and IPAGIF is shown in fig. 2. The C8 IAGIF in fig. 2a shows the two rare phenotypes A1A and A1B besides the common types AB and B. In the broad pH range of 3-10 the C8 IPAGIF replica (CAF strip soaked with C8-antibody) reveals the two regions A (C81) and B (C81+C82) in a more cathodal position. The arrows at the left side of the figure point at the main fractions which become visible by this technique.

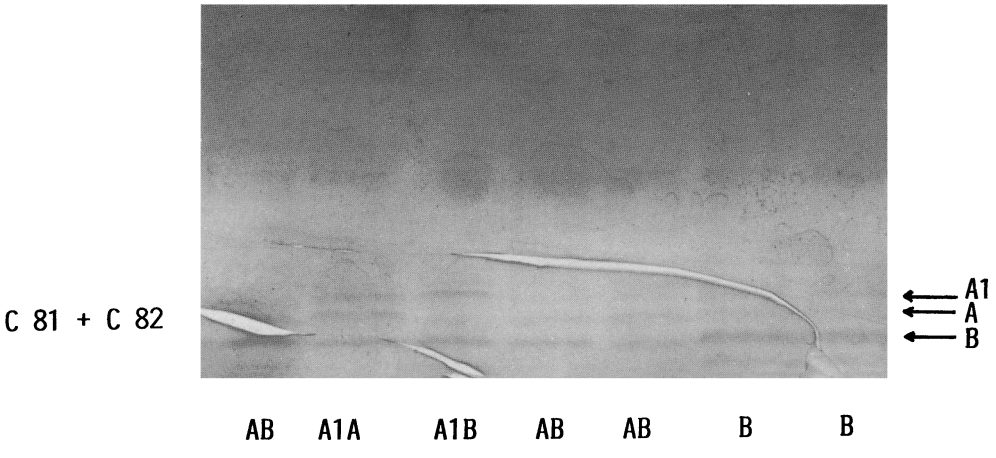

Fig. 2a: C8 IAGIF at pH 5-8

INVESTIGATIONS ON THE C8 SYSTEM BY IMMUNOFIXATION ISO-ELECTROFOCUSING

Peter Kühnl, Wolfgang Lenz and Willi Spielmann

Institute of Immunohematology, University Frankfurt, Sandhofstr. 1, D-6000 Frankfurt am Main

A genetic polymorphism of the eighth component of human complement (C8) was detected in 1977 by RAUM et al. who used a functional, hemolytic detection system. In the following years the three-chain structure of C8 (C8 α,β,γ) posed numerous questions concerning the formal genetic model. The interpretation of C8 phenotypes was complicated by the fact that activity zones were present at different pI values. In 1983 ALPER et al. reported on a C8 β-subunit polymorphism, subsequently called C82 in contrast to the C81 (α-γ) polymorphism detected 6 years ago. Population data on the C8 systems are scarce.

The aim of our study was the evaluation of various isofocusing parameters for C8 phenotyping of German blood donors by a combined immunofixation agarose gel isofocusing (IAGIF) and immunofixation polyacrylamide gel isofocusing (IPAGIF). In view of a possible replacement of the time-consuming hemolytic assay by a combined immunological/IEF detection system (immunoprint) two pH ranges were employed: pH 3-10 for IPAGIF and pH 5-8 (IAGIF). For the separation of C8 allotypes by IPAGIF fresh serum samples from random blood donors and families were examined on polyacrylamide gels (265x125x1mm; T=5%; C=3%; ampholyte concentration 2.5% w/v). At maximum settings of 1,200 V, 40 mA and 20 W and a cooling temperature of 4°C, 1M H_3PO_4 was used as anolyte, 1N NaOH as catholyte. After 20 min prefocusing, 30 µl serum were applied on 5x15 mm filter papers (Whatman #1) 1 cm from the anodal edge of the gel, they were removed after 70 min, and focusing was completed after 2.5 h. A CAF was soaked with a 1:2 diluted anti-C8 (C81) serum (ATAB) and placed on the gel surface for 20 min. After washing in saline overnight, the foil was stained in 0.1% CBB R 250 and evaluated.

A model of the C8 (C81, C82) allotypes after IPAGIF at pH 3-10 in different gel regions is shown in fig. 1. The main band patterns of the alleles C81*A and *B (gene products) are present in both A and B regions. In difference, the "total" C8 (α,β,γ = C81 + C82) molecule discloses further heterogeneity in the B region, according to a pH value of 7.0-7.5.

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Discussion

Our analysis make it possible that instead of the 3 phenotypes BfF,BfS and BfFS , reported by Alper et al.[2] now 6 phenotypes such as BfF' , BfF'F'' ,BfF'' ,BfF'S, BfF''S and BfS can distiguished. This fact leads to the increased validity of Bf subtyping for paternity testing.

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Table 3. Bf types in Japanese families . F' and F'' are included.

Parental Types	cases	Children F'	F'F''	F'	F'S	F''S	S	cases
F' x S	1	-	-	-	2	-	-	2
F'F''x S	1	-	-	-	2	-	-	2
F'S x F'S	4	2	-	-	6	-	3	11
F'S x S	3	-	-	-	3	-	4	7
F''S x F'S	6	-	3	-	3	3	3	12
F''S x F''S	4	-	-	2	-	4	1	7
F''S x S	8	-	-	-	-	10	11	21
Total	27							62

In this studies clear results were obtained by means of the electrofocusing technique followed by immunofixation with anti-properdin factor B serum (figure 1).The Bf gene frequencies in the populations tested (Table 1 and Table 2) are calculated:

Japanese population : $Bf^{F'} = 0.0778$
$Bf^{F''} = 0.1007$
$Bf^{S} = 0.8215$

Italian population : $Bf^{F'} = 0.0571$
$Bf^{F''} = 0.1219$
$Bf^{S} = 0.8210$

The gene frequencies in both populations observed are therefore quite similar.

The observed and expected values assuming the HARDY-WEINBERG equilibrium were in good agreement.

To elucidate a genetic basis of the new BfF patterns we studied 27 families with 62 offsprings.The result of family investigations are not in contradiction to the assumption of codominant inheritance of the new BfF subtypes $Bf^{F'}$ and $Bf^{F''}$ alleles at a single locus. (Table 3).

Results

Figure 1 . Schematic representation of Bf allotypes after isoelectric focusing with immunofixation

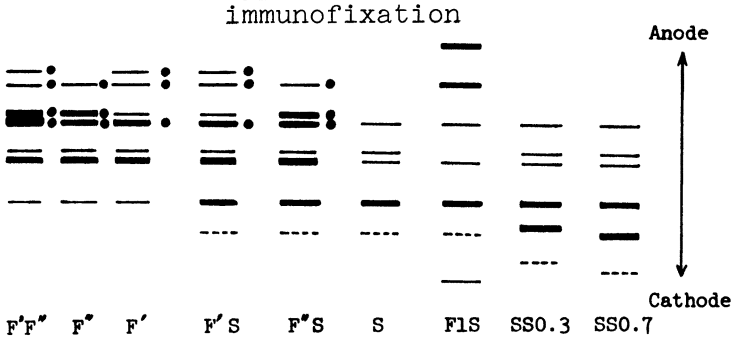

Table 1 . Bf phenotypes of Japanese population

Type	obs. No.	obs. %	exp. No.	exp. %	Gene Frequencies
F'	4	0.5	4.5	0.6	$Bf^{F'}$ 0,0778
F'F''	12	1.6	11.7	1.6	
F''	7	0.9	7.5	1.0	$Bf^{F''}$ 0,1007
F'S	96	12.9	95.2	12.8	Bf^{S} 0.8215
F''S	124	16.7	123.3	16.5	
S	502	67.4	5o2.8	67.5	
Total	745	100.0	745.0	100.0	

Table 2. Bf phenotypes of Italian population

	obs. No.	obs. %	exp. No	exp. %	
F'	1	0.2	1.9	0.3	$Bf^{F'}$ 0.0571
F'F''	10	1.7	8.3	1.4	
F''	9	1.5	8.8	1.5	$Bf^{F''}$ 0.1219
F'S	56	9.4	55.8	9.4	Bf^{S} 0.8210
F''S	117	19.7	119.1	2o.o	
S	4o2	67.5	401.1	67.4	
Total	595	100.0	595.0	100.0	

ISOELECTRIC FOCUSING IN THE STUDY OF THE Bf SYSTEM : EXISTENCE OF TWO COMMON SUBTYPES OF THE Bf^F ALLELE IN JAPANESE AND ITALIAN POPULATIONS

T.Nagai[+] , G.Rossi[++] ,M.Salviati[++] and O.Prokop[+++]

+ : Department of Legal Medicine,Teikyo University School of Medicine,Tokyo-173,Japan

++: Laboratory of the Center for Blood Transfusion and Human Genetics,Hospital of Vicenza , Vicenza 36100 ,Italy

+++: Department of Forensic Medicine,Humboldt University , 1040 Berlin,GDR

Summary

The Bf gene frequencies including $Bf^{F'}$ and $Bf^{F''}$ alleles in the Japanese and Northern Italian populations were studied using the PAGIF method. The results showed Bf gene frequencies in these populations :

Japanese population:	$Bf^{F'} = 0.0778$	$Bf^{F''} = 0.1007$
	$Bf^{S} = 0.8215$	
Italian population :	$Bf^{F'} = 0.0571$	$Bf^{F''} = 0.1219$
	$Bf^{S} = 0.8210$	

Introduction

Geserick et al.[1] developed the PAGIF method for subtyping of the properdin factor B and reported that Bf^F is not uniform.It can divided into $Bf^{F'}$ and $Bf^{F''}$.

We examined the Bf gene frequencies including $Bf^{F'}$ and $Bf^{F''}$ alleles in Japanese and Italian populations.

Material and Method

Sera of 745 Japanese(living in Tokyo and its suburbs) and 595 Italians (living in Vicenza and its suburbs) were analysed using the PAGIF method as described by Geserick et al.[1] .The Italian sera are separated promptly and transmitted with dry ice by airplane to Japan.

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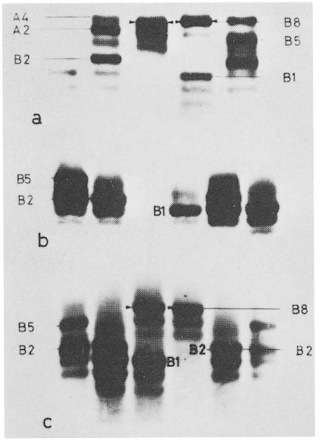
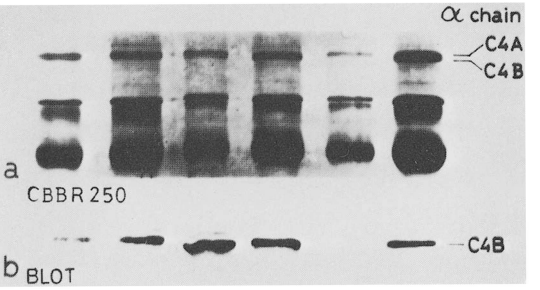
respectively. These five markers except C3 have been employed for parentage testing over 50 cases in our laboratory without any difficulties in typing.



Hemolytically inactive C4B allotype(suspected), which was tentatively named C4B8. a.IAGE pattern b.hemolytic detection c. immunoblotting with a monoclonal antibody specific to C4B epitope

Table 3. Phenotype distribution of C4A and C4B and their gene frequencies.

phenotypes				gene frequencies	
A locus	no.	B locus	no.		
5,4	1	5	29	A^*_*5 = 0.0012	
4	38	5,21	2	A^*_*4 = 0.1580	
4,3	73	5,2	23	A^*_*3 = 0.7127	
4,2	2	5,1	26	A^*_*2 = 0.0727	
3	267	4,1	2	A Qo= 0.0554	
3,2	61	21,1	3		1.0000
Qo	1	2	45	B^*_*5 = 0.0855	
	443	2,1	97	B^*_*2 = 0.2113	
χ^2=50.4(df=4)		12	4	B^*_*1 = 0.5115	
		11	2	B^*_*R = 0.0208	
		1	195	B Qo= 0.1709	
		1,96	1		1.0000
		Qo	11		
			443		
		χ^2=24.1(df=6)			



SDS-gel electrophoresis of α chain of inactive C4B8. From left to right, mother(A4,2B2,0),child (A4,2B8,0),father(A4,3B8,1),controls(C4A null, B null, A4,3B5,2)

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found in a family with insulin-dependant diabetes mellitus and another example which showed mobility close to C4A3 was suspected to be C4B product by immunoblotting with a monoclonal antibody specific to the epitope of C4B protein and α-chain typing. The latter was detected through blood typing for a family in which a child was born with chromosomal abnormality(partial deletion of 3q) and is under further investigation. Gene duplication at both C4 loci is not so rare event. Duplicated genes at C4B locus were found to be transmitted through three generation in one family and through two generation in the other in this study

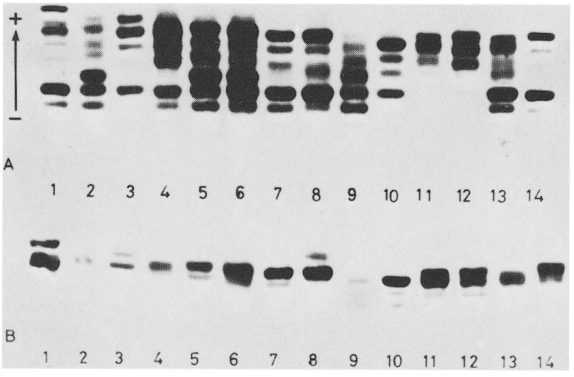

Fig.5.A.Immunofixation patterns of all the C4A allotypes in this study. B.Blot with a monoclonal antibody specific to C4A epitope. 1)A6,3;2)A5,4;3)A5,3;4)A4,3;5)A4;6)A4,3;7)A3;8)A3;9)A3,2;10)A2;11)A3,2;12)A3,13?;13)A3,2;14)A3. 1),9),10)were kind gift from Dr.O'Neill.

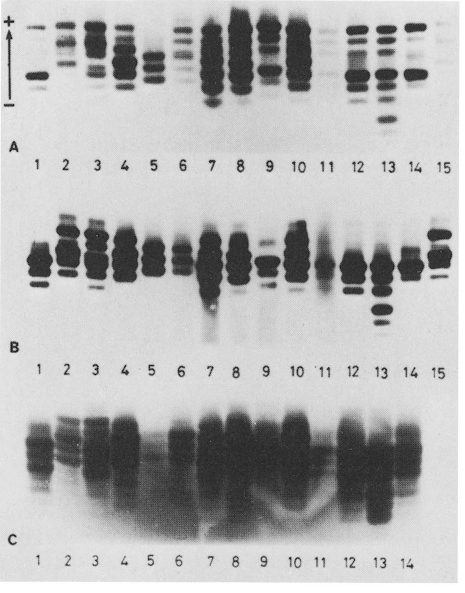

Fig.6.A.Immunofixation patterns of all the C4B allotypes in this study. B. Blot with a monoclonal antibody specific to C4B epitope. C.Hemolytic detection. 1)B1;2)B5,2;3)B5,1;4)B4,21,2;5)B31;6)B29;7)B21,1;8)B2,1;9)B12;10)B21,11;11)B11;12)B1;13)B1,96;14)B1;15)B5,2. 3),5),6) were kind gift from Dr.O'Neill.

Those markers which showed appreciable polymorphism seems to be useful when they are applied to parentage testing. Exclusion ratio is therefore one criterion for selecting effi-ient cient markers. The ratios were calculated as follows, 11.6% for BF, 5.2% for C2, 22.0% for C6, 12.5% for C7, 14.5% for C4A, and 21.6% for C4B,

(Fig.3) and the other for C7 blot(Fig.4). C6 showed appreciable polymorphism in Japanese as well as in other ethnic groups. It was confirmed that C6 polymorphism was controlled by three common alleles, C6*A, C6*B, and C6*B2 in Japanese as previously reported[15)~17)]. Moreover, two rare variants were confirmed, one was identified as M2 by direct comparison and the other was designated B11 on the suggestion by Dr.K.Tokunaga. The genetic polymorphism of C7 was controlled by three common alleles, C7*1, C7*2, and C7*4 in Japanese[18)]. A rare type which seemed to be C7 3 was found in two samples of this study and in one sample of a patient of Buerger's disease. Any significant association between C6 and C7 alleles could not be observed in this study, however, further investigation must be carried out to determine which alleles would be strongly associated each other.

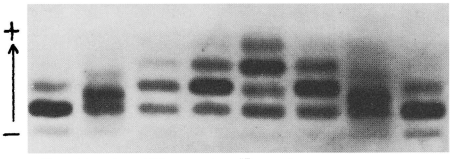

Fig.2. Immunofixation patterns of BF allotypes

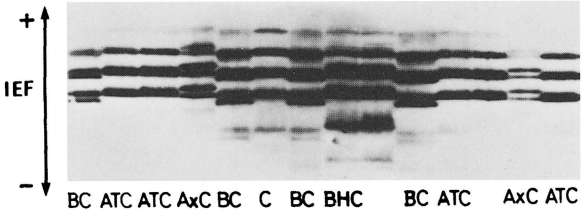

Fig. 1 IEF patterns of C2 revealed by immunoblotting Ax is a new variant and tentatively designated Reference typing was performed by Dr.TOKUNAGA

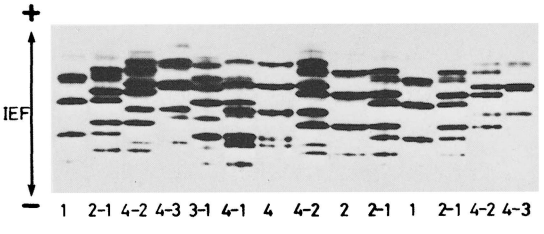

Fig.4 IEF patterns of C7 revealed by immunoblotting

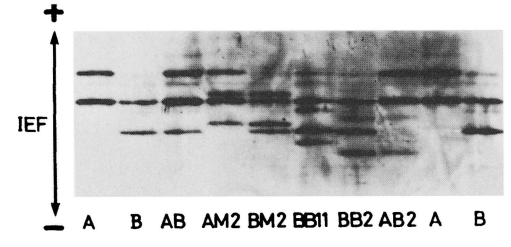

Fig. 3 IEF patterns of C6 revealed by immunoblotting Reference typing of M2 and B11 was performed by Dr.TOKUNAGA

Any other variants except C3S were not observed in this study although s few rare variants, S0.2, S0,25, F0.6, F0.65, and F0.8 were demonstrated among 1692 samples in Japanese[19)].

As for C4(Fig.5,6), some aberrant types were observed in this study. Hemolytically inactive C4B allotype, B4, was

migration than that of C2AT and was tentatively designated C2Ax(Fig.1). The other rare variants which have been found so far only in Japanese[10] and in Korean[11], C2AT and C2BH, were also observed at polymorphic frequencies.

Table 1. Electrophoretic and detecting procedures

C3 :	Agarose gel electrophoresis + protein staining
BF :	Immunofixation agarose gel electrophoresis(IAGE) + protein staining
C4 :	IAGE + protein staining
	AGE + immunoblotting using monoclonal antibody
	AGE + hemolytic detection
	SDS-gel electrophoresis
C2,C6,C7 :	Isoelectric focusing in polyacrylamide gel + immunoblotting

Among the 215 samples of this study, one rare type,BF F0.75, which has been reported so far in Japanese[12) 13)] was detected and another variant was newly observed(Fig.2) in a paternity case and its genetic transmission was confirmed. This new variant was named BFF0.25 considering its relative mobility compared with those of BFF0.75 and BFF[14)]. The significant association of C2*AT with BF*F was estimated using 2 x 2 association analysis by Fisher's exact test(p=0.0021). This association was already described by Tokunaga et.al. with another significant association of C2*BH with BF*F[12)].

C6 and C7 were transferred from the both surfaces of a slab gel to nitrocellulose sheet; one surface for C6 blot

Table 2. Phenotype distribution and gene frequencies of BF, C2, C6 and C7

BF	C2	C6	C7
S = 154	C = 191	A = 43	1 = 164
FS = 53	BC = 13	AB = 97	2-1 = 31
F = 7	ATC = 8	B = 51	4-1 = 15
F0.75S = 1	BHC = 2	AB2 = 11	2 = 2
215	AxC = 1	BB2 = 8	3-1 = 2
	215	AR = 2	4 = 1
		BR = 2	215
		R = 1	
		215	
		R:rare allotypes (M2 , B11)	
BF*S =0.8419	C2*C =0.9442	C6*A =0.4558	C7*1=0.8744
BF*F =0.1558	C2*B =0.0302	C6*B =0.4860	C7*2=0.0814
BF*F0.75=0.0023	C2*AT=0.0186	C6*B2=0.0488	C7*4=0.0395
χ^2=0.829 1d.f.	C2*BH=0.0047	C6*R =0.0094	C7*3=0.0047
0.25<p<0.50	C2*Ax=0.0023	χ^2=0.893 4d.f.	χ^2=0.745 2d.f.
	χ^2=0.753 1d.f.	0.90<p<0.95	0.50<p<0.75
	0.25<p<0.50		

GENETIC POLYMORPHISMS OF HUMAN COMPLEMENT COMPONENTS IN JAPANESE AND THEIR APPLICATION TO PARENTAGE TESTING

K.SUZUKI,H.MATSUMOTO(Dept. Legal Medicine)*,Y.MATSUO,K.SHODA, M.IWATA(Dept. Blood Transfusion, Osaka Medical School),** G.J.O'NEILL(Columbia University)

Genetic polymorphisms of several components of human complement have been successively elucidated by some investigators[1)~8)] and now the existence of genetic polymorphism of the complement receptor as well as of the other components is being demonstrated.

In the field of forensic science, many polymorphic markers in blood have been applied to parentage testings. The typing techniques would be required to be always reproducible and not so difficult to operate when the polymorphic markers are employed for parentage testings. Some components were detected by their function, but this technique required some special and unstable reagents. On the other hand, immnoblotting is easy to perform and detects proteins by their antigenecities. We demonstrate the result of the investigation on complement polymorphisms in Japanese using immunoblotting or other conventional techniques.

MATERIALS AND METHODS

All the samples were prepared as EDTA-plasma by centrifugation. For C4 typing, samples were treated with neuraminidase under continuous dialysis[9)]. Monoclonal antibodies which were kindly provided by Dr.G.J.O'Neill were employed to discriminate the epitopic difference between C4A and C4B proteins. The typing procedures are presenred in Table 1.. Immunoblotting was carried out by simple diffusion from gel to nitrocellulose without any electroblotting apparatus.

RESULTS AND DISCUSSION

Phenotype distribution and gene frequencies observed in this study are shown in Table 2 and 3. Some new variants among Japanese were detected in C2, C6, and C7 and a new BF variant was found in a paternity case.

The band pattern of a new C2 variant showed more anodic

* Prof. Hideo Matsumoto, Osaka Medical School, 2-7 Daigakumachi Takatsuki City, J-569 Osaka, Japan

** 2-7 Daigakumachi, Takatsuki City, J-569 Takatsuki City, Japan

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variation that provides the guide to most studies of C4 molecular genetics.

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but not with the B5 Ch:-1,-2,-3 allotype (Giles, unpublished observations). The immune response of the animal (mouse) in this instance is closer to that of humans, though no details of the immunisation procedure have been given. The haemolytic overlay remains the only method that defines the A1 and B5 allotypes as products of C4A and C4B genes.

Ch determinants on B5 Ch:-1,-2,-3 allotype

The B5 Ch:-1,-2,-3 allotype was termed B5 Rg^+ by Roos and colleagues (1984) but the C4B α chain reacted with a proportion of anti-Ch which could be translated into serological activity. Ch:-1,-2,-3 red cells coated with C4 from the B5 reacted weakly with many anti-Ch but it was evident with inhibition that more than one determinant involved. Recent work (Giles, in preparation) has defined three more Ch determinants expressed at a much lower level. They subdivide existing Ch phenotypes and are inherited as part of the C4/Ch haplotype. The complexity of Ch has therefore been doubled.

Conclusions

The simplistic interpretation for Rg and Ch as single determinants on C4A and C4B no longer holds true; the terms are not synonymous. Both Rg and Ch are multifactorial and are expressed in an increasing number of detectable phenotypes. The association of the Rg/Ch phenotypes with C4 allotypes is strong but they have no direct relationship. The Rg:1,-2 and Ch:1,2,-3 phenotypes subdivide the common A3 and B1 allotypes which are indistinguishable in electrophoretic analysis. When combined with HLA and C4 haplotypes, Rg/Ch haplotypes define the extended haplotype more accurately.

Rg/Ch typing relies on the supply of antisera from transfused patients and the dependence on inhibition, which detects the lack of a determinant, also limits the information obtained.

The aberrant C4 allotypes indicate that the Rg/Ch epitopes are probably independent of the C4A and C4B class differences. Rg/Ch epitopes do not relate to charge differences in the protein and may reflect amino acid differences since the C4d fragment contains only 6% carbohydrate. The precise location of Rg/Ch epitopes on C4 will have to await amino acid or nucleotide sequencing.

DNA studies of C4 will undoubtedly uncover more variation (Palsdottir et al. 1983, Whitehead et al. 1984), but it is the serological and electrophoretic

The C4 A6 allotype found with the B1 that is Ch:-3 has always been Rg1,2 and the B1 in the A*QO,B*1 haplotype has never lacked Ch3.

Table III
Association of Rg/Ch haplotypes with C4 haplotypes.
(Giles et al. 1984)

1,-2		1,-2,3		1,2,-3	
Rg	n	Ch	n	Ch	n
A*3A*2,B*QO	5	A*4,B*2	17	A*6,B*1	11
A*3,B*QO	4	A*QO,B*2	4	A*3,B*1	6
A*2,B*QO	1	A*3,B*2	4		
		A*2,B*2	1	A*6,B*3	1
A*3,B*2	1			A*QO,B*3	1
A*5.1,B*1	1	A*1,B*QO	3	A*3,B*2	1
		A*3,B*6	1	A*QO,B*w2	1
		A*4,B*5.1	1		
Totals	12		31		21

The combination of Rg/Ch typing with C4 allotyping yields more precise data and helps with haplotype interpretation.

Aberrant C4/Rg/Ch allotypes

The first finding of a C4B allotype lacking Ch and a C4A allotype with Ch was made in 1982 (Rittner et al. 1984a). The A*1,B*QO haplotype, when paired either with A*QO or B*QO in the study of family Bk (Rittner et al. 1984b) showed that this A*1 allele lacked Rg1 and Rg2 determinants but had Ch1 and Ch3 (Ch:1,-2,3). The A*4,B*5 haplotype when paired with B*QO was of two types, Ch+ and Ch- (now termed Ch1); analysis of the C4B α chain confirmed this but also demonstrated that Rg1 was present on the B5 that lacked Ch1 (Roos et al. 1984).

The aberrant A1 and B5 allotypes could be hybrid proteins, the products of fusion genes of the Lepore (A/B) and anti-Lepore (B/A) types respectively (Giles et al. 1984); this hypothesis predicted the correct gene order for C4A and C4B but as yet there is no scientific evidence to support it.

Monoclonal anti-C4B

O'Neill (1984) reported a monoclonal anti-C4B which he suggested might replace haemolytic overlay in defining the products of all C4B alleles. It was important to study its reactivity in relation to the aberrant C4 allotypes, A1 and B5. In agglutination tests with red cells of different C4 coats and in inhibition tests, the serological specificity was deduced as anti-Ch1 and not anti-C4B. It reacts with the A1 Ch:1,-2,3 allotype

ficities for Ch2 and Ch3.

Table I Frequency (%) of Rg and Ch phenotypes in the South London donor population (Giles 1984).

Rg phenotype	%	Ch phenotype	%
Rg;1,2	94.5	Ch:1,2,3	87.7
Rg:1,-2	3.2	Ch:1,-2,3	4.9
Rg;-1,-2	2.3	Ch:1,2,-3	3.2
		Ch:-1,-2,-3	4.2

There are three Rg phenotypes and 4 Ch phenotypes; Rg:-1,-2 is usually found with C4A*QO and Ch:-1,-2,-3 is usually with C4B*QO. The Rg:-1,-2 Ch:-1,-2,-3 combined phenotype represents the extremely rare C4 deficient individual not found in random testing. None of the donors (Table I) lacked a determinant for both Rg and Ch, so the frequency of donors lacking one or more determinants was about 18%. There are 12 possible combined Rg/Ch phenotypes of which only two remain undetected (see below).

Two new Ch phenotypes have been observed recently in Rg/Ch typing family material (Giles and colleagues, unpublished observations) which have only one of the three Ch determinants (Table II). These findings all support the concept of multiple determinants and antibody specificities for Rg and Ch.

Table II Eight possible Ch phenotypes.

Ch:1,2,3	Ch:-1,2,3*	Ch:1,-2,-3	Ch:-1,-2,-3
	Ch:1,-2,3	Ch:-1,2,-3	
	Ch:1,2,-3	Ch:-1,-2,3*	

* not detected yet

Association of Rg/Ch phenotypes with C4 allotypes

The Rg:1,-2 and Ch:1,-2,3 phenotypes were strongly associated by Nordhagen and colleagues (1980,1981) with the duplicated C4A haplotype A*3A*2,B*QO (FI) and the C4B haplotype B*2 (M). The Ch:1,2,-3 phenotype (Giles 1984) subdivided the common C4 B*1 allele. Studies of the segregation of rare C4 haplotypes (Rittner et al. 1984b) and the recessive inheritance of partial inhibition (Giles et al. 1984) in families showed that Rg and Ch phenotypes do not have a direct relationship with particular C4 allotypes, but simply that there are strong associations which is inevitable. The absence of two combined Rg/Ch phenotypes, Rg:1,-2 Ch:1,2,-3 and Rg-1,-2 Ch:1,2,-3 can be accounted for in Table III.

haemolytic activity which is useful in classifying the rarer fast C4B products and the slower C4A products whose banding patterns can overlap. There are other differences between C4A and C4B products. The C4A protein is strongly associated with Rg (Rodgers) determinants and C4B with Ch (Chido) determinants (O'Neill et al. 1978a) and an apparent molecular weight difference of 2000 was demonstrated between the heavier C4A and lighter C4B α chains (Roos et al. 1982) which absorb anti-Rg and anti-Ch respectively. A combination of techniques will provide the best definition of the products of the two genes. However the classification into C4A and C4B is becoming less easy with the increase in knowledge of the polymorphism.

Rg and Ch determinants on C4

The Rodgers (Rg) and Chido (Ch) blood groups were described before the association with C4 was known; linkage to HLA had been observed for both and clearly a relationship between them existed (Giles 1977). The frequency of Rg+ and Ch+ did not allow them to be products of alleles at a single locus. This knowledge enabled O'Neill and colleagues to establish Rg and Ch as determinants on C4 and necessitated the proposal that the polymorphism of C4 was controlled by two loci rather than one (O'Neill et al. 1978b).

Patients who lack C4A or C4B and who have received many transfusions of blood may become immunised to determinants on their missing C4 component. The alloantibodies are IgG and do not themselves bind complement in serological tests; they are detected in compatibility tests with normal red cells. Much stronger reactions are obtained with red cells that have been coated with C4. Trypsin treatment of these C4 cells does not affect their reactivity, demonstrating that anti-Rg/Ch are detecting determinants on the C4d fragment (Tilley et al. 1978).

Both anti-Rg and anti-Ch are inhibited by C4 in serum (Middleton & Crookston 1972, Longster & Giles 1976) but two types of inhibition, complete and partial, were described for anti-Rg. Partial inhibition of anti-Ch was reported later (Nordhagen et al. 1980). It was not clear whether partial inhibition represented a quantitative, as the name implied, or qualitative difference, but it was inherited recessively and linked to HLA (Giles 1977). Recent studies (Giles 1985) have shown that all anti-Rg/Ch capable of detecting partial inhibition are polyspecific and that there are separable antibodies which detect two Rg determinants (Rg1,Rg2) and three Ch determinants (Ch1,Ch2,Ch3). All anti-Rg contain anti-Rg1+2 whereas most anti-Ch contain only anti-Ch1 but a few antisera have additional speci-

POLYMORPHISM OF C4 WITH SPECIAL REFERENCE TO THE SEROLOGICAL DETERMINANTS, RODGERS AND CHIDO.

Carolyn M. Giles. Department of Immunology, Royal Postgraduate Medical School, Hammersmith Hospital, London.

Introduction

The fourth component of complement (C4) in man has an important central role in the classical pathway of complement activation. It interacts with other complement components (C1, C2, C3) and binds covalently to antibody or antigen on cell surfaces. The highly complex polymorphism is thought to relate to its diversity of function (Porter 1983) and is limited, with one reported exception (Mauff et al. 1983a), to the α chain of the molecule or more precisely to the C4d (α_2) fragment (Tilley et al. 1978, Mevag et al. 1981). Since the C4d fragment contains structures involved in the activation, inactivation and covalent binding of C4 and also 13 of the 15 reported nucleotide sequence differences (Belt et al. 1985), Porter suggests that the α chain is probably on the exterior of the C4 molecule.

There are two closely linked genes, C4A and C4B, which probably arose by duplication and, with genes for two other complement components C2 and Bf, comprise the class III genes of the Major Histocompatibility Complex (MHC) on chromosome 6 in man. The gene order is C2, Bf, C4A, C4B (Carroll et al. 1984 a,b) though the orientation in relation to HLA-B and -DR loci is not known. The term complotype was proposed (Alper et al. 1983) for the class III complement proteins as no cross-over has been detected between them and the extended haplotypes including HLA-B and -DR products show marked linkage disequilibrium (Awdeh et al. 1983). It is clear that the complex polymorphism of C4 has a role to play as a genetic marker particularly in the studies of disease associations and susceptibility (Fielder et al. 1983, Dawkins et al. 1983), but many of the interrelationships of demonstrable polymorphism are not completely resolved so the value in forensic investigations must be rather limited at present.

Each C4 gene, C4A and C4B, has a series of alleles and relatively frequent silent alleles termed A*QO and B*QO. Generally the C4 allotypes have been detected by immunofixation electrophoresis of fresh EDTA plasma after neuraminidase treatment and the recommended nomenclature (Mauff et al. 1983b) is based on this method. Distinction between the C4A and the C4B products can be made by their marked difference in

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In the determination of phosphoglucomutase it was found that particular phenotypes may be demonstrated in the inner ear lymph and that they agree with those obtained in control blood samples. The PGM_1 phenotypes was not determined in only 5 samples of inner ear lymph and in blood of the same cadavers with signs of autolysis.

Acid phosphatase /AcP/ and esterase D /EsD/

The identification of AcP and EsD isoenzymes in inner ear lymph was not possible.

Conclusion

The results obtained by determination of PGM_1 in inner ear fluid appear very encouraging and should be useful in medico-legal practice.

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Results and discussion

Phosphoglucomutase /PGM_1/

The results obtained are shown in the table and figure.

Table 1

The presence of PGM_1 phenotypes in inner ear fluid and autolytic changes of examined cadavers

Cadavers	No	PGM_1 phenotypes			
		1-1	2-1	2-2	none
without autolysis	49	22	20	7	
putrefactive changes	7		3		4
small autolysis	6	4	1		1
total	62	26	24	7	5

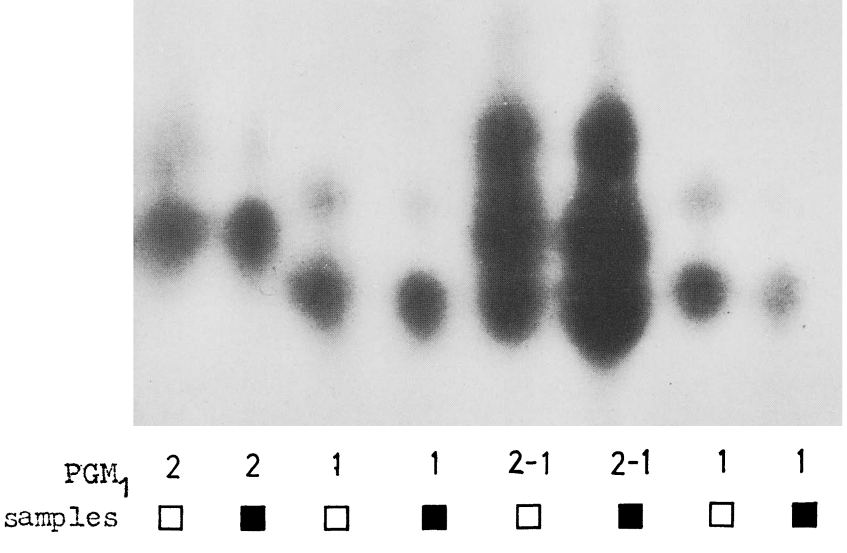

Fig. 1. The photograph of PGM_1 pattern on starch gel. Comparison of the phenotypes of isoenzymes origin from inner ear lymph □ and from blood ■ of the same cadaver.

THE ISOENZYMES AcP, EsD AND PGM_1 IN HUMAN INNER EAR FLUID

Bożena Turowska and Franciszek Trela

Sero-Haematology Unit, Institute of Forensic Medicine, Copernicus Academy of Medicine, Cracow, Poland

Introduction

Searching for genetically conditioned characters not only in the red cells but also in other tissues and body fluids is quite comprehensible in forensic medicine. New discovery in this sphere increases the changes of individual identification of human cadavers in cases in which cannot be made in the red blood cells.

Positive results of determinations of group substances in the ABO system, Gm/1/, Gm/2/ and Inv/1/ factors in endolymph taken from human cadavers /1,2,3/ encouraged us to attempt to show the presence of the isoenzymes AcP, EsD and PGM_1.

Material and methods

Samples of human blood and inner ear fluid taken at postmortem examination of 62 human cadavers were used for this study. Endolymph was obtained in the manner described by Trela et al. / 4 / from both ears. The time elapsed from death to autopsy ranged from 2 - 60 days. The 7 bodies revealed marked putrefactive changes: greenish discoloration of skin, swelling of the face and liquefaction of organs. The 6 bodies were autolytically changed at the beginning.

Starch gel electrophoresis and visualization of the iso enzyme pattern of PGM_1 was carried out as described by Spencer et al. / 5 /. AcP and EsD isoenzymes were investigated according to the technique of Karp and Sutton / 6 /.

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limits between 61% and 93%).

The results allow the following conclusions concerning the best storage conditions for a successful redetermination of PGP and Bf types in blood samples, hemolysates and sera:

1. PGP groups can be determined exactly for at least two years with hemolysates that are prepared immediately after the blood is drawn or the sample is sent and stored at -40°C.

Differing storage conditions shorten the redetermination interval in direct relation to the storage temperature and the lapse of time until preparation of the hemolysates.

2. Bf groups can be determined exactly for at least ten months in sera that are decanted right after the drawing of the blood or the receipt of the samples and stored at -25°C.

Other storage conditions shorten the time when an exact determination is possible.

3. Surprisingly in sera of hemolytic or otherwise altered blood samples an exact determination of Bf groups is obviously possible in many cases when the blood samples are stored as soon as possible after the drawing (1-5 days) at 4°C for a longer time.

As the determination of blood samples for alcohol tests that were 2 - 13 months old showed the rate of success, 81,5%,was unexpectedly high.

Summary

Phosphoglycolate phosphatase (PGP) and properdin factor B (Bf) groups can be determined or redetermined with success in stored blood samples, hemolysates and sera when optimal storage conditions exist. In freshly prepared hemolysates stored at -40°C the PGP groups can be determined for at least 2 years. In freshly separated sera stored at -25°C the Bf groups are determinable for at least 10 months. The more unfavourable the storage conditions - storage at 4°C or at room temperature and storage of blood samples instead of hemolysates or sera - the shorter the period of determinability. Surprisingly in blood samples drawn for alcohol tests and stored at 4°C for 2 - 13 months the Bf groups were determinable in about 80%.

The Bf groups were determined by immunofixation after electrophoresis on cellogel foils according to the method of MARTIN and ZIEGLER (1981). All Bf types we found were included in this investigation.
In addition the Bf groups from blood samples drawn for alcohol tests were investigated after storage at 4°C for 2-13 months and compared to the Bf types determined in freshly drawn blood samples of the same persons.
The following results were obtained:

1. In those hemolysates that were prepared at once or after a 3-days storage at 4°C the PGP patterns could be determined without any alteration for at least two months. During the third month this was not possible any more since the heterocygous types showed vapid and strongly weakened spots.
2. With the blood samples stored at room temperature and later at 4°C safe PGP determination was possible up to the eleventh day.Afterwards the PGP types could not be determined with the heterocygous types any more in spite of storage of the hemolysates at -18°C. With homocygous types correct determination was possible in some cases. The results agree in principle with those described by BRINK et al.(1981).
3. With the hemolysates prepared at once and stored at -40°C determination was unaltered after two years and the PGP typing was as correct as right after the samples were drawn.
4. The Bf types of serum samples stored at room temperature were determinable exactly only for two days. During the next three days some patterns could still be identified, others could not. Bf FS for instance showed only a washed-out area. After the fifth day a correct determination was not possible any more.
5. With the sera stored at 4°C an exact determination of Bf of all types was possible up to the tenth day, but from then on during the next two days the determinability decreased rapidly, so that from the thirteenth day no safe results could be obtained any more.
6. With the sera stored at -25°C completely exact determination of Bf groups could be obtained for at least ten months.
7. Amazingly the Bf typing of 27 partly very old, hemolytic and by no means well stored blood samples from alcohol tests showed results that were identical with those obtained right after the blood was drawn with 22 out of 27. This means a rate of success of 81,5% (with confidence

DETERMINATION OF RED CELL ISOENZYME GROUPS OF THE PHOSPHOGLYCOLATE PHOSPHATASE (PGP) AND THE PROPERDIN GROUPS (Bf) IN STORED SAMPLES OF BLOOD, HEMOLYSATE AND SERUM UNDER VARIOUS CONDITIONS.

Ilse Adamzik,G.-R.Christiansen,V.Sachs and Renate Dörner,
(Dept.of Immunohematol.and Blood Transf.,Univ.of Kiel,D-2300 Kiel

It is desirable to know under which storage conditions later investigations of blood samples will be sucessful,e.g. for a control investigation of known samples or to disprove a presumed mix-up of samples drawn for a blood alcohol test. These questions can also become important for those cases where blood samples have to be investigated that were stored or transported for some time under unfavourable circumstances.

In order to obtain informations concerning these problems for the red cell isoenzyme of the phosphoglycolate phosphatase (PGP) and the properdin factor B (Bf) we determined the groups of these two systems in samples which were stored under various conditions.

For the determinations the following storage conditions were chosen:

1. Preparation of the hemolysates, immediately after the blood was drawn or after a one-night storage at $4^{o}C$ and storage afterwards at $-18^{o}C$.
2. Preparation of the hemolysates after keeping the blood samples at $4^{o}C$ for 3 days and storage afterwards at $-18^{o}C$.
3. Preparation of the hemolysates after first keeping the blood samples at room temperature for 3 days then at $4^{o}C$ for 8 days and finally storage at $-18^{o}C$.
4. Preparation of the hemolysates immediately after the blood samples were drawn or sent in and storage afterwards at $-40^{o}C$.

The PGP groups were determined in the hemolysate by starch gel electrophoresis following the method of BARKER and HOPKINSON (1979) modified by MARTIN et al. (1981). With the exception of PGP 3 all types were included in the investigation.

For the Bf determination the sera were separated immediately after the blood samples were drawn or received and stored as follows:

1. at room temperature.
2. at $4^{o}C$.
3. at $-25^{o}C$.

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rabbit anti human orosomucoid followed by swine anti rabbit antibody linked to alkaline phosphatase. The fluorescent band pattern of isofocused, neuraminidase treated orosomucoid is seen in figure 4. Normal Hp-subtyping could subsequently be performed on the same blot.

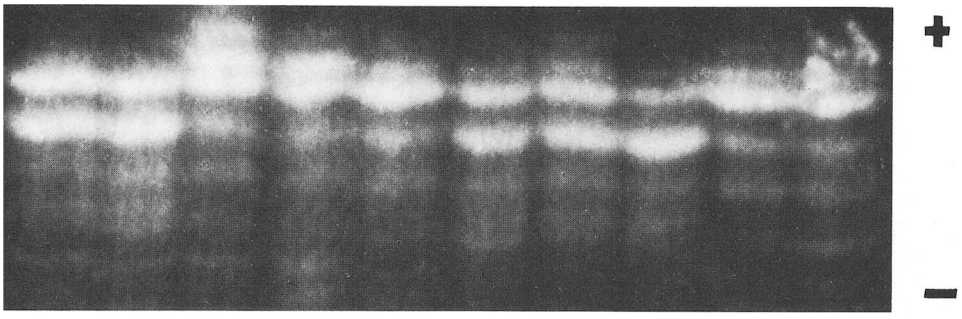

Figure 4. Neuraminidase treated orosomucoid, isofocused and immunoblotted. Staining: Alkaline phosphatase/4-methylumbelliferyl phosphate.

Conclusion

Detection of specific proteins on nitrocellulose blots is easily performed using secondary antibodies linked to alkaline phosphatase and 4-methylumbelliferyl phosphate as substrate. The method is sensitive. The fluorescent band pattern thus obtained does not interefere with subsequent colour detection of other proteins on the same blot.

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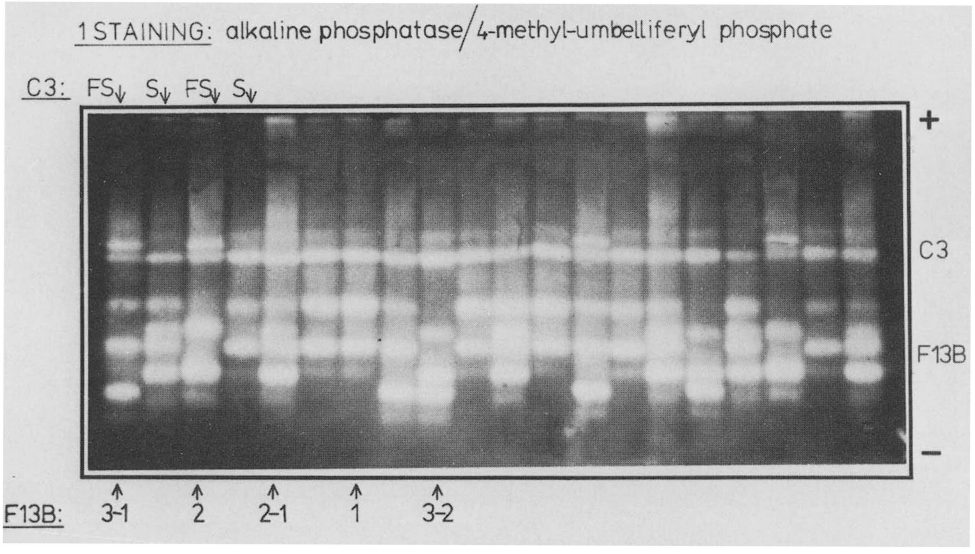

Figure 2. The electrophoretic band patterns of coagulation factor 13B and complement factor C3. The method is described in the text.

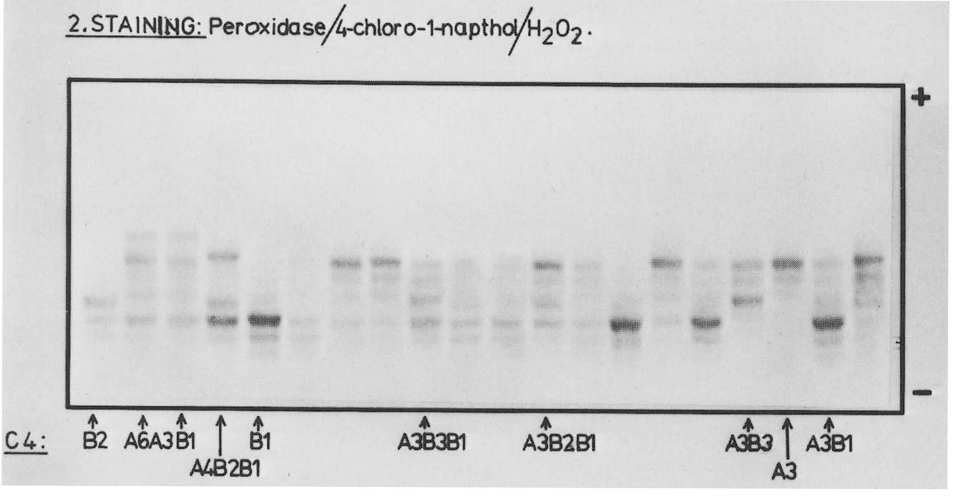

Figure 3. The electrophoretic band patterns of complement factor C4. The staining is performed on the same blot as seen in fig. 2. The method is described in the text.

Immunoprint procedure

Step	Antibody directed against	Antibody raised in	Conjugated enzyme	Enzyme substrate	Detection
1.	Human 13B and C3	Rabbit			
2.	Rabbit	Swine	Alkaline-phosphatase		
3.				4-methylumbelliferyl dihydrogen phosphate	Fluorescent bands of factor 13B and C3
4.	Human C4	Goat			
5.	Goat	Rabbit	Peroxidase		
6.				4-chloro-1-naphtol/H_2O_2	Blue bands of C4

The following antibody dilutions were used: anti C3 1:2000, anti factor 13B 1:500, anti C4 1:1000, swine anti rabbit antibody linked to alkaline phosphatase and rabbit anti goat antibody linked to peroxidase 1:1000. The washing procedure was as described for Hp-subtyping (4). Fluorescent bands were obtained by immersing the blot for approximately 1 minute in 50 ml diemal buffer, pH 8.6, containing 20 mg 4-methylumbelliferyl dihydrogen phosphate. The blot was then dried between two sheets of filterpaper and photographed in UV-light.

In figure 2 is seen the fluorescent band patterns of factor 13B and complement factor C3 photographed in UV-light. In figure 3 is seen the same blot after peroxidase/ 4-chloro-1-naphtol/H_2O_2 staining of complement factor C4.

The fluorescent detection technique was further tested on a nitrocellulose blot of an isofocused polyacrylamide gel intended for Hp-subtyping (4,5). Before Hp-detection, the blot was subjected to

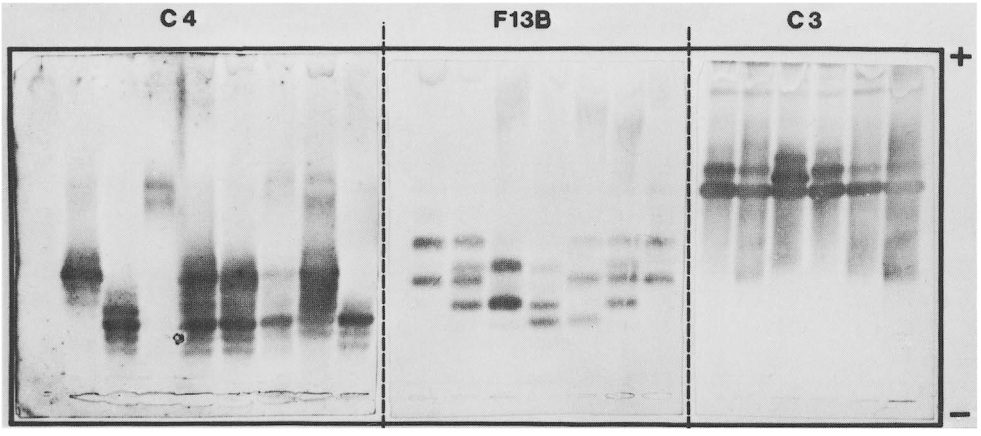

Figure 1. Complement factor C4, coagulation factor 13B and complement factor C3. Neuraminidase treated serum samples are separated on agarose gel and transferred to nitrocellulose paper. One third of the blot is subjected to anti human C4, one third to anti human F13B, and one third to anti human C3. Protein detection: Peroxidase conjugated secondary antibody/4-chloro-1-naphtol/H_2O_2.

Coagulation factor 13B and complement factor C3 are well separated and may be stained on the same blot without interference. Complement factor C4 and factor 13B have similar band positions and cannot be typed simultaneously.

As a test system for protein detection with soluble and "washable" staining was chosen swine anti rabbit antibody linked to alkaline phosphatase combined with 4-methylumbelliferyl dihydrogen phosphate as substrate. The reaction between phosphatases and 4-methylumbelliferyl phosphate produces the highly fluorescent substance 4-methylumbelliferone. This reaction is well known in the detection of acid phosphatase (2) and is also used in ELISA techniques (3).

Our access to suitable combinations of antibodies was limited, and the experiments were designed to avoid crossreactions. The following detection procedure was used on the blots obtained after agarose separation of neuraminidase treated serum samples:

Fluorescent stains in protein detection on immunoblots

Brita Teige, Bjørnar Olaisen, Reidun Jonassen
Institute of Forensic Medicine, University of Oslo,
Rikshospitalet, 0027 Oslo 1, Norway

Transfer of proteins to nitrocellulose paper and subsequent staining with an immunoprint technique, is an efficient method for identification of specific proteins as well as for detection of genetic variations. Proteins may be transferred to nitrocellulose paper from different kinds of gels used for separation, and in our experience proteins are readily transferred by passive blotting both from agarose gels and from polyacrylamide gels. The immunoprint technique implies a series of antigen-antibody reactions forming immunocomplexes on the nitrocellulose surface. The first antibody is directed against the protein in question, and protein staining is usually achieved by an enzyme-linked second antibody combined with a suitable substrate. Horseradish peroxidase and alkaline phosphatase are the two most widely used enzymes in commercially available enzyme-linked antibodies. The reaction between enzyme and substrate usually involves the formation of insoluble, coloured products that indicate protein band position on the blot. This staining method is suitable when only one protein on each blot is examined, or when stained proteins are so well separated that their band patterns do not interfere.

Most techniques for electrophoretic separation and staining of serum proteins allow only one or two genetic marker systems to be studied. Several proteins may, however, be detected on one blot if an immunoblot staining method were available in which the stain could (i.e. after photographic documentation) be washed off leaving the blot "clean" for another protein to be detected. Such a method would require a selection of suitable combinations of primary and enzyme-linked secondary antibodies.

The test system we selected in order to establish a method for successive staining of proteins on one blot was neuraminidase treated serum proteins separated on agarose gels, and transferred to nitrocellulose paper by passive blotting. The electrophoretic procedure employed was a slight modification of the method described by O'Neill (1) for separation of complement factor C4. With these experimental conditions, satisfactory separation of coagulation factor 13B phenotypes as well as separation of complement factor C3 phenotypes were also achieved. In figur 1 is seen the electrophoretic band pattern of these three serum proteins detected on nitrocellulose blots with specific antibodies and peroxidase/4-chloro-1-naphtol/H_2O_2 staining.

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their Handbook of 1976-78 (see Beaudet 1985 for Bibliography of cloned human DNAs). Such probes make it possible to study in blood samples those loci where the enzyme is normally only expressed in less easily available tissue such as liver, gut and muscle. Also, using appropriate restriction enzymes it is often possible to reveal RFLPs at loci which are relatively invariant at the protein level.

Conclusions

1. The basic ideas promulgated more than 10 years ago about the incidence of human enzyme polymorphisms and their role in forensic science remain intact.

2. New enzyme polymorphisms can be identified by the application of the original methods; progress depends on the ingenuity of the investigator and the availability of substrates and other reagents necessary to generate specific staining methods.

3. The use of specific antibodies to detect genetic variation in human enzymes and other proteins in immunoblots prepared from IEF or conventional electrophoresis gels offers a powerful new approach for the forensic scientist. These procedures should be of general application but may be especially valuable for the detection and individualisation of trace amounts of human material on stains and other scene of crime objects.

4. The immunoblot approach will never be as powerful as DNA analysis for the identification of individuals in cases such as paternity disputes where conventional fresh blood samples are available, but it may have some advantages for the assessment of forensic material, especially that which has had to endure unfavourable conditions and where there has been some deterioration of the protein and DNA markers.

stain (e.g. Whitehouse & Putt, 1983; Blake et al. 1984).

The nitrocellulose filter provides a replica of the pattern which would be obtained by direct isozyme staining but the immunoblotting technique is usually considerably more sensitive than direct staining (Towbin & Gordon, 1984). The sensitivity depends largely on the quality of the first antibody and it is often possible to detect as little as a few nanograms of enzyme protein with a good antiserum. Even greater sensitivity may be achieved by the use of amplifying techniques such as "immunogold" (Moermans et al. 1984). So far the blot procedure has been used to analyse several human serum protein polymorphisms (e.g. Whitehouse & Putt, 1983; Whitehouse et al. 1985; Teige et al. 1985) but preliminary experiments with human enzymes (unpublished data) such as adenosine deaminase (ADA) and carbonic anhydrase (CA) gave convincing resolution of variant phenotypes in 2-5 µl aliquots of blood samples diluted down to 1 in 50. Thus the procedure may be expected to have a wide application to the study of human enzyme polymorphisms in forensic material.

Cloned human enzyme loci: The rapid progress which has been made in the cloning of human DNA sequences and their use as probes in clinical genetics and in linkage analysis has been referred to earlier and the specific applications of DNA probes in forensic science and paternity testing will be the subject of other presentations at this Conference. However in the context of this brief review of current work on human enzyme polymorphisms it is relevant to note that cDNA probes have now been generated for about half of the polymorphic enzyme loci listed by Harris & Hopkinson in

required for the attachment of asparagine-linked oligosaccharide side chains. Another mutant enzyme protein identified by SDS gel electrophoresis is a rare variant of hypoxanthine phosphoribosyl transferase with a neutral amino acid substitution which appears to have led to a change in the SDS-binding properties of the HPRT molecule (Wilson et al. 1983).

Enzyme detection methods: Many ingenious and specialised stains for enzymes have been devised and the principles underlying such methods which depend on the catalytic activity of the enzymes are given in Harris & Hopkinson (1976) and by Naylor (1980). The most important new and general alternative method to emerge in the past few years takes a different approach, and depends on the use of antibodies to detect the specific immunological determinants on the enzyme protein molecules. The enzyme under analysis is subjected to electrophoretic separation in agarose or polyacrylamide gels and the isozymes are then transferred electrophoretically from the gel onto a nitrocellulose filter. This electroblotting method, which was originally described by Towbin et al. (1979) for SDS gels also works well for non-denaturing polyacrylamide or isoelectric focussing gels. Passive transfer also may allow adequate retrieval of the enzyme protein (Gershoni & Palade, 1983).

The nitrocellulose blot is incubated with a specific antiserum raised against the purified enzyme protein (for example in a rabbit) and the enzyme-antibody complexes are detected with a second antibody (such as a goat anti-rabbit IgG) which carries some type of conjugated signal protein such as peroxidase or alkaline phosphatase which can be visualised by a conventional histochemical type

yet directly applicable in forensic science has already made important advances possible in the field of clinical medicine for the early and accurate diagnosis of specific genetic defects.

Enzyme separation techniques: The most powerful technique currently available for uncovering enzyme polymorphisms due to charge change substitutions is flat bed polyacrylamide gel isoelectric focussing. There are now many examples of subtypes, attributable to allelic variation, which are recognised by this method but are not visible by conventional methods such as starch gel electrophoresis. The IEF technique is capable of considerable experimental variation, using "spacer" molecules for example, to extend the range of separation and this procedure has infinite possibilities. The very exciting new method which depends on immobilised pH gradient is capable of even better resolution (Gianazza et al. 1983; Righetti et al. 1983), but has not yet been widely adopted for routine use.

Another high resolution technique is SDS - polyacrylamide gel electrophoresis and this procedure, which gives separations on the basis of molecular size is especially valuable for monitoring the phases of protein purification. Due to the denaturation of enzyme activity which occurs with the SDS method, it is not generally suitable for the detection of enzyme polymorphisms which depend on activity staining but this may be overcome by radio-labelling and immunological techniques. For example Waheed et al. (1983) recently identified a polymorphism of human arylsulphatase by SDS electrophoresis which appears to be due to an alteration in the number of carbohydrate side chains on the enzyme molecule. The probable site of the mutation is in the Asn-X-Thr(Ser) sequence

frequencies are low there are examples of very common "null" alleles at enzyme loci; for example the mitochondrial aldehyde dehydrogenase (ALDH2) polymorphism in Oriental populations (Goedde, Harada & Agarwal, 1979) and the recently identified glutathione-S-transferase (GST1) polymorphism in the European population (Board, 1981; Strange et al. 1984).

Recent developments

Enzyme purification: Protein purification has been revolutionised by the advent of affinity methods of separation and by the introduction of reverse phase high pressure liquid chromatography for the comparative analysis of amino acid sequences (see Scopes, 1982; Harris, 1985 for review). Immunoadsorption chromatography using polyclonal and monoclonal antibodies and affinity ligands based on substrates and inhibitors have been particuarly important. For example the synthesis of a specific affinity matrix for the purification of alcohol dehydrogenase (ADH) has recently led to the derivation of the entire amino acid sequence of different forms of ADH (Bühler et al. 1984) and the elucidation of the molecular basis of the human ADH2 polymorphism (Jornvall et al. 1984). Coincidentally, rapid progress has also been made in gene cloning experiments on human ADH (Duester et al. 1984; Ikuta et al. 1985) and it should now be possible to study the ADH polymorphisms using DNA prepared from white cells or any other nucleated cell rather than liver specimens, which were needed hitherto to examine the expressed gene product. This type of dual approach, which combines protein and DNA analysis, to investigate structure-function relationships in human enzymes is extremely powerful and while not

Secondary isozymes: In addition to the complexities of enzymes due to allelic variation and mutliple loci there is also the phenomenon of secondary modification leading to the occurrence of multiple isozymes. This is a universal phenomenon and may arise due to a very great variety of causes. It can have a great nuisance value in forensic studies since secondary modification of enzyme proteins may mimic genuine genetic polymorphism and lead to misidentification. However some secondary changes of enzyme proteins are tissue specific and may therefore be useful in pointing to the origin of suspect human material in certain cases or they may provide clues as to the ways in which the material has been stored or handled prior to analysis.

Quantitative variation; "null alleles": A number of enzyme polymorphisms have been identified by quantitative assay techniques and in several cases these polymorphisms first came to light as a result of idiosyncratic responses to drug therapy. Their applications in the field of forensic science are limited however due to the difficulties of accurate identification of the variant phenotypes in the kinds of material usually available for analysis.

A more important category of quantitative variation is the so called "null" allele which is characterised by no discernable enzyme product. When such an allele occurs in a polymorphic enzyme system, such as PGM1, ACP1, ESD, difficulties may arise in paternity testing and of course individuals homozygous for such "null" alleles may present with specific clinical disorders, e.g. ADA, PEPD, GALT, αFUC. "Null" alleles have now been identified at almost every polymorphic enzyme locus and although in most cases their population

electrophoresis so that on average electrophoretic, i.e. charge-change, mutants occur with a frequency of about 1 in 3,600bp. Allowing for the fact that electrophoretic methods detect only about 30% of all the possible isozyme variants with single amino acid substitutions, the overall incidence of such polymorphisms in the DNA of enzyme polypeptide coding sequence must be in the region of about 1 in 1,000bp. This is an order of magnitude lower than the estimated incidence of variation in non coding DNA sequence (Jeffreys, 1979) demonstrable by direct analysis of restriction fragment length polymorphism (RFLP) using molecular probes.

Multiple loci: The occurrence of multiple gene loci encoding enzymes is a common phenomenon. Estimates based on the study of more than 100 human enzymes indicate that at least a quarter are encoded by more than one structural locus. These multiple loci, coding for similar though structurally distinct polypeptides have mostly arisen as a result of gene duplication during the course of evolution and the duplicate genes have subsequently diverged in structure as a result of point mutation. In many cases the polypeptides coded by each of the loci are synthesised together in the same cell but there are often marked disparities in the rates of synthesis in different tissues and at different stages of development. Such differences are important in forensic analysis since they may be used as indicators of the species and tissue of origin of an unknown biological stain or other types of sample and in some cases may also give an indication as to whether the specimen was derived from an adult or an infant.

Current research on enzyme polymorphism

D.A. Hopkinson, MRC Human Biochemical Genetics Unit, London.

Introduction

Overall incidence of variation: Enzyme polymorphism is a well documented phenomenon in human populations. Estimates derived from electrophoretic studies indicate that approximately one third of all human enzymes exhibit genetic polymorphism where "polymorphism" is defined as the occurrence of heterozygotes with a frequency greater than 2%. Taken as a whole the data indicate that the average heterozygosity per locus is about 0.06 and this implies that any single individual in any human population is likely to be heterozygous at about 6% of the loci encoding enzyme proteins, for alleles which give rise to electrophoretically distinct isozyme forms (Harris & Hopkinson, 1976). The complexity of the isozyme patterns varies according to the subunit structure of the enzyme proteins and on average monomeric enzymes exhibit a higher incidence of genetic polymorphism than multimeric enzymes (Harris, Hopkinson & Edwards, 1977).

The data derived from electrophoretic studies of enzyme proteins can be used to obtain crude estimates of the incidence of mutation in the coding regions of the DNA of the structural genes for comparisons with the estimates derived from the direct molecular analysis of non coding (viz. the flanking and intervening) DNA sequences. Since the average enzyme polypeptide size is about 45,000 Daltons, corresponding to about 400 amino acid residues, it must be encoded by about 1,200 base pairs (bp) of DNA. About a third of all human enzymes exhibit polymorphism when examined by

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CREDO

1. Polymorphism of introns is extensive and many new genetic system of restriction fragments await their detection.

2. α1-antitrypsin and its variants give a model system. Our aim should be to get comparable insight in other genetic systems, their importance in physiology and pathology.

3. The genetic control of recognition molecules of the immune system shows special features such as allelic exclusion and extensive crossreactivity between idiotypes of anti-allotypic antibodies.

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are not significantly differing from those in normals, neither in African nor in Caucasian populations (R. Grubb et al 1985).

Observations chiefly concerning rabbit Ig allotypes have indicated that rabbits may express not only their nominal allotypes but also allotypes characteristic of other strains of rabbits. These "latent" allotypes appear irregularly and usually at a level of 1 per cent or less of the nominal allotypes. These observations have led to the concept that genes for a majority or all Ig allotypes of a species is normally present in the IgG genome of a single individual (see Kindt et Yarmush 1981). The question is now whether this concept does or does not apply to the human Ig allotypes. Most of the human Ig allotypes are markers of the constant part of the IgG molecules and none are characteristics of the hypervariable regions. They are thus generally determined by IgG H C genes. Direct studies of the human IgG H C genome shows that there are not more than 2 IgG H C genes within the haplotype of one individual (see Honjo 1983).It is diffucult to reconcile this evidence with an idea of IgH C control of possible latent allotypes in Man. An alternative explanation is therefore required. I believe that at least some of the observations may be explained within the framework of idiotypes, anti-idiotypes and internal images. Several investigators have established that in rabbits and mice there is extensive crossreactivity between the idiotypes of anti-allotypic antibodies of a particular allotypic specificity. This is true even for anti-allotypic antibodies raised in different species. It has also been shown that anti-idiotypic antibodies to antiallotypes not infrequently are similar to the allotypes themselves in inhibition and binding experiments (see Jerne et al 1982, Kazdin et Horng 1983, Cazenave et Roland 1984). The anti-idiotypies of anti-allotypes may thus mimic allotypes. These observations were made in animals but findings concerning anti-allotypic specificities in rheumatoid arthritis patients are compatible with this type of explantion (see R Grubb 1970, 1985).

sequence data banks. In 1968 a strong inhibitor of papain was isolated from hen eggwhite (Fossum et Whitaker) and the sequence of this inhibitor of a potent plant cysteine proteinase was worked out in 1984 (Brzin et al). It immediately became apparent that there is extensive homology between human γ-trace and the papain inhibitor of hen eggwhite. It was soon established that the function of γ-trace is to inhibit the human cathepsins B, H and L which are similar to papain (Barrett et al 1984) and γ-trace was accordingly renamed cystatin C.

From a medical point of view it is of interest to note that the levels of cystatin C is markedly reduced in the cerebrospinal fluid in individuals struck by hereditary cerebral haemorrhage with amyloidosis in young years. This disastrous disease is particularly common in Iceland (see A. Grubb et al 1984 and A. Grubb et Löfberg 1984). In these individuals γ-trace accumulates in the cerebral vessels.

If the function of a protein is found it is probable that important pathology will also be encountered.

Some special features of immunoglobulin (Ig) control

A special feature of Ig genetic control is allelic exclusion. The phenomenon was found already in 1961 by Mårtensson in distinguishing those human Ig allotypes which we today call G1m (f) and G3m (b). Allelic exclusion, or allelic preference if you prefer, has so far been shown to hold only for immunoglobulins although very recent data indicate that it is valid also for T cell receptor molecules (see Epstein et al 1985). An important corollary of this type of control is that Ig molecules are symmetrical. Köhler and others (see Köhler 1985) are now engaged in elucidating the control mechanism of allelic exclusion and a stochastic model now appears less probable. Immunoglobulin gene loci and the c-myc oncogene locus are without exception involved in the chromosomal translocations which regularly take place in Burkitt lymphoma (see Klein 1983). In more than 80 % of Burkitt cases the c-myc gene is translocated to the IgC heavy chain locus. The remaining cases engage c-myc and IgC light chain loci. The c-myc gene is thus invariably brought to immediate vicinity of IgC loci. As mentioned allelic exclusion is a very special feature of these loci. It is tempting to speculate that allelic exclusion might be a prerequisite for the emergency of Burkitt lymphoma. Speculation apart, I am able to inform you that Gm type frequencies in Burkitt lymphoma cases

	Abnormality	Substitution
S gene	Deficiency	264 Glu - Val
Z gene	Severe deficiency	342 Glu - Lys
Pittsburgh gene	Antitrombin	358 Met - Arg

The Pittsburgh mutation strikes exactly at the crucial Met site at position 358 and gives a complete change of the function of the molecule rendering highly active antitrombin.

The amount of antitrypsin in ZZ people is about 15 per cent of the normal level. As is well-known such persons, which are 1 in a 1000 Europeans, have an increased risk of becoming ill with pulmonary emphysema particularly if they are smokers. Straight - forward relations between structure and function are thus known to apply to α_1 antitrypsin and its variants. Normal α_1 antitrypsin is of potential therapeutic interest in the deficiency state in ZZ individuals and antitrombins are also of medical interest in common clinical syndroms associated with thrombosis (for recent reviews see Carrel et al. 1982, Jeppsson et Franzén 1982, Carrel et Travis 1985). Rosenberg et al (1984) and Courtney and his group in Strasbourg (1984) have succeded in isolating human α_1-antitrypsin cDNA clones. These clones and modifications thereof can be made to express themselves in yeast or E. coli and produce substantial amount of the desired proteins.

The methionine present in normal α_1-antitrypsin at the crucial position 358 is readily oxidizable and therefore less stable. Replacement of the reactive centre methionine by valine gives stability to oxidation and retains the elastase inhibition. This replacement and thus improval of the natural product has been achieved by Rosenberg and coworkers and by Courtney and his group. If the clinical trials will verify the expectations, this achievement may be classed as an example of instant evolution.

The function of γ-trace (=cystatin C) is found by sequencing and data bank searching

An recent example of how the function of a protein may unexpectedly be resolved is given by γ-trace, also called post-γ-globulin. This protein which is a normal constituent of body fluids was detected in 1961 by Clausen. Its amino acid sequence was elucidated in 1982 (A. Grubb et Löfberg) and was incorporated in several

RECENT RESEARCH ON GENETIC REGULATION AND FUNCTION OF SOME SERUM PROTEINS.

Rune Grubb, (Department of Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 LUND Sweden)

Intron variation

With the advent of recombinant DNA techniques it soon became clear that eukaryotic genes usually are organized in exons and introns. The polymorphic variants of serum proteins and of other immunogenetic systems which we hitherto study are, of course, ascribable to nucleotide variation in the corresponding exons. The introns are not expressed, but are cut out in messenger RNA production. Hybridization between messenger RNA and single-stranded DNA demonstrates that very substantial parts of DNA are not translated (see Chambon 1981). For a majority of genes the base pairs of the introns is twice or more the base number of the exons (see for example Efstratiadis et al 1980). In addition, the base pair composition of the introns commonly vary more than those of the exons (see Milner-White 1984). DNA hybridization techniques thus give access to a new and abundantly rich source of polymorphisms: Study the variation of allelic restriction fragments of numerous introns! As an example Krontiris and coworkers (1984) studied the allelic restriction fragments of the oncogene Harvey-ras. As is wellknown we all possess this oncogene and its DNA can easily be extracted from leukocytes. The Southern blot pattern of restriction fragments from different individuals could be systematically ordered. It is of more than passing interest that the Ha-ras alleles met with in cancer patients frequently differed from those found in normals.

The Pi system as a model system of serum protein polymorphism

As is wellknown the genetic variants of human α_1-antitrypsins within the Pi system are quite useful in forensic medicine. The protein moiety of the molecule consists of 394 amino acid residues in a known sequence. The reactive site of this inhibitor is crucially dependent upon a methionin residue at position 358, 37 residues from the C terminus. The main function of α_1-antitrypsin is not really to be an antitrypsin but to be an inhibitor for elastase from leucocytes. The S, Z and Pittsburgh variants are important from a clinical point of view. Fortunately, the Pittsburgh variant, which gives rise to intractable bleeding, is rare indeed. These variants are caused by single amino acid substitutions as shown below.

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II. Proteins and Enzymes

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(homosexuals, i.v.-drug addicts and hemophiliacs). The negative outcome of subsequent confirmatory tests again may not be conclusive in cases with low antibody concentrations.

In conclusion, we have shown HLA-DR4 antibodies as one possible cause for false-positive HTLV-III antibody ELISA restuls, since the probability, that all nine different donors of the DR4 antisera were HTLV-III infected, is very small.

The sensitivity and specificity of the commercially available tests (Abbott, ENI, Organon) is satisfactory in general, as we may judge from our routine blood donor screening (n = 20.000, Hessen, FRG).

Of course the detection of DR4 antibodies in individuals belonging to the known risk groups does not exclude a true HTLV-III infection. Absorption experiments with DR4-positive cells led to a variable decrease of the absorption values measured in the HTLV-III antibody ELISAs in homosexuals and hemophiliacs. These preliminary data may indicate, that both HTLV-III and DR4 antibodies occur in homosexuals HLA ABC DR-immunized by sperms and white blood cells as well as in polytransfused patients.

In forensic serology, the question of an incidental self-inoculation occuring in the laboratory or at postmortem work with possibly infective materials is of some importance.

It should be mentioned, that hitherto no AIDS cases have been reported after simple incidental needlestick injuries of laboratory personal. One case of a "microinjection" of freshly drawn venous blood in a British nurse may rather be looked at as a "transfusion-associated" (TA) case of AIDS, a category which accounts for approximately one percent of all AIDS cases.

The extreme polymorphism of the HTLV-III envelope-proteins (env-gene) may thus reflect a response to the extreme human polymorphism of the MHC and also of a possible genetically determined T4 heterogeneity, which influences the clinical course of an individual after HTLV-III-infection.

The possible implications of false-positive HTLV-III antibody results caused by anti-DR4 are summarized in table 5:

POSSIBLE IMPLICATIONS OF FALSE POSITIVE HTLV-III ANTIBODY RESULTS CAUSED BY ANTI DR4:

1. Homosexuals immunized by sperm and WBC via rectal mucosa
2. I.v. drug addicts sharing contaminated needles
3. Hemophiliacs under FVIII concentrate substitution therapy
4. Hemodialysis patients (often polytransfused) in view of scheduled transplantations and immunosuppressive therapy
5. Women after pregnancies with development of HLA antibodies, which may be rejected as blood donors. Confirmatory tests like Western blot, immunofluorescence assay or radio-immunoprecipitation may be inconclusive at low HTLV-III antibody levels observed in early stages of HTLV-III infection

It is obvious, that lacking specificity of HTLV-III assays may cause severe problems in the interpretation of the restuls. In particular, HTLV-III positive hemodialysis patients may be excluded from transplantation and the immunosuppressive therapy requested thereafter. Confirmatory tests (WB, IFA, RIPA) cannot resolve this problem, since they are based on antibody detection as well and low antibody levels can be expected in immunocompromised hemodialysis patients (e.g. also after HBsAg vaccination).

In women, the development of HLA DR antibodies may lead to their exclusion as blood donors after pregnancies with HLA DR antibodies. It is also conceivable, that positive test results obtained with this first generation of HTLV-III antibody ELISAs are observed in individuals belonging to the known AIDS risk groups

expresses the "B-cell-specific" DR molecules. A depression of an operator gene by conversion of a "normal" into a leukemic T-cell may be one of the mechanisms, which is responsible for the presence of DR on a T-cell. The fact, that only 9 of 27 (= 33 %) DR antisera tested, reacted with the ELISA antigen preparation might be explained by

a) a partial destruction of the DR4 epitope on the native DR4β-chain by ultrasonication and detergent treatment of HTLV-III infected cell culture supernatants
b) the expression of HLA-like structures on human tumor cells, as this has been reported for the prostate adenocarcinoma PC93 (Claas and Steenbrugge, 1982).

The question, why there is no obvious reaction with HLA class I antigens (ABC) remains unsolved. The complete HLA pattern of the H9 cell line has not yet determined by standard microlymphocytotoxicity assays to our knowledge. Monoclonal antibodies, directed against DR4 (also together with other HLA specificities) gave no reactions in the HTLV-III ELISAs. They are not likely to react with the goat AHG (Coombs serum) used as a conjugate in the ELISA, since these antibodies were produced in mice.

In this context it is remarkable, that the main target of the HTLV-III attack, the T4 (helper/inducer) lymphocytes recognize their antigen MHC-class II-(= DR,DQ,DP)-restricted, whereas T8 (suppressor/killer) cells cooperate in connection with MHC-class I (HLA ABC) molecules as restriction elements. An interaction of the T4 antigen of T-cells, and the class II MHC antigen of the antigen-presenting cell (APC = macrophage) appears possible; this interaction would mean another positive signal besides the antigenic stimulation of the T-cell receptor. We may speculate if the T4 antigen, which is apparently the "crucial" anchorage for later HTLV-III incorporation in the cell, and HLA DR molecules are also involved in the "budding" of the virus from the cell surface.

dures (0 to 2.91 % in different German regions) and 0.07 to 0.40 % in the confirmatory tests: Western blot (WB), immunofluorescence assay (IFA) or radio-immunoprecipitation assay (RIPA).

We tested 248 HLA ABC-antisera directed against all common HLA-specificities as shown from our experience with local serum sets, Eurotransplant (ET) and Collaborative Transplant Study (CTS) trays.

In addition, 336 HLA DR-antisera (ET DR'85, CTS, local FM DR'85 set and 9. IHWS DR antisera set including MCA) were tested by three different HTLV-III antibody ELISA's (Abbott, batch # 74089 HR, 75207 HR; ENI (Viramed) # 2362 and Organon # 850307). The results are shown at table 4:

RESULTS

POSITIVE HTLV-III ANTIBODY EIA RESULTS WERE OBTAINED WITH

0/248 HLA ABC ANTISERA

9/336 HLA DR ANTISERA; SPECIFICITY *

ANTI-DR4	(7)
ANTI-DR4+7	(1)
ANTI-DR4+7+9+ DQW3	(1)
ANTI-DR7	(1) **

* 9/27 tested DR4 antisera gave positive results

** weak positive

Apparently the DR4 specificity is present on the HTLV-III infected H9 cell line in homozygous or heterozygous state. Alternatively, a loss of the second DR antigen on the leukemic H9 cells may be discussed. We assume, that HTLV-III incorporates the DR4 molecules from the cell surface during the process of "budding". DR4 may than be "harvested" together with the virus from the cell culture and be present as a contaminant in the HTLV-III antigen preparation used for the ELISA test (both Abbott and ENI). It remains unclear, why the T-cell-derived-leukemic H9 cell

PRINCIPLES FOR PREVENTING AIDS TRANSMISSION IN HEALTH-CARE WORKERS AND ALLIED PROFESSIONALS

Persons performing necropsies or providing morticians services:

1. As part of immediate post-mortem care, deceased persons should be identified as belonging to one of the above three groups (AIDS, ARC, individuals with epidemiologic risk) and identification should remain with the body
2. The procedures followed before, during and after the postmortem examination are similar to those for hepatitis B. Personnel involved in performing an autopsy should wear double gloves, masks, protective eyewear, gowns, waterproof aprons and shoe coverings. Contaminated instruments and surfaces should be handled as infective
3. Appropriate precautions to prevent parenteral or mucous-membrane exposure of personnel to body fluids should be evaluated

The biosafety guidelines for use of HTLV-III and related viruses mention the accidental parenteral self-inoculation, droplet exposure to mucous membranes by splashing or spraying of infectious materials (possibly also aerosols), contact exposure of broken skin as well as pricking, puncturing, cutting of skin with scalpels or other sharp objects like broken glassware. In view of the epidemiological situation arising in Europe and the quality of the different commercial ELISA-techniques, the question of sensitivity and specificity of those tests for the reliable identification of infectuous materials is of importance from different forensic-serological aspects.

After completion of a HTLV-III antibody screening pilot study in German blood donors (Seidl and Kühnl, 1985), we were interested in possible causes for false-positive reactions observed in HTLV-III antibody ELISA-tests. In particular the question, if HLA antibodies might cause such reactions was investigated. A survey of HTLV-III antibody tests on 33.603 German blood donors correlated to confirmatory tests (Kühnl et al., 1985) has shown a marked discrepancy between the primary ELISA screening proce-

As of December 1984, a number of 135 AIDS cases has been officially registered in the FRG; a number which has now increased to 180.

HTLV-III virus isolation studies have shown the infective agent in blood, semen and saliva of HTLV-III antibody positive (symptomatic and asymptomatic) individuals, exceptionally also from sero negative individuals. The modes of transmission are shown in table 2:

HTLV-III VIRUS ISOLATION STUDIES

HTLV-III has been isolated from : blood
semen
saliva
of HTLV-III antibody positive (symptomatic and asymptomatic) individuals, exceptionally also from seronegative individuals

MODES OF TRANSMISSION

1. Intimate sexual contact
2. Sharing of contaminated needles, razors, toothbrushes
3. Transfusion of whole blood
   blood cell components
   plasma
   clotting factor concentrate (not heat-treated)
   (immunoglobulin, albumin, plasma protein fraction and hepatitis B vaccine are not involved so far
4. Transplantation of body organs
5. Sperm (cryo-preserved)
6. Mother to child at birth

Besides these routes of transmission, the question of a new, possible occupational hazard for health care workers (clinical, laboratory and allied professionals) has been discussed in the USA (MMWR 32, 450-452, 1983). The principles for preventing AIDS transmission in persons performing necropsies or providing mortician's services are shown in table 3:

THE INFLUENCE OF HLA CLASS II ANTIBODIES ON HTLV-III ("AIDS") ANTIBODY ELISA RESULTS

P. Kühnl, S. Seidl, G. Holzberger

Institute of Immunohematology, University Frankfurt, Sandhofstrasse 1, D-6000 Frankfurt/Main, FRG

Epidemiological data suggest, that the human T-cell-lymphotropic virus (HTLV-III) ist the causative agent of the acquired immune deficiency syndrome (AIDS). Based on virus antigen preparations of the infected leukemic H9 cell line, commercial HTLV-III ELISA tests have become available a few months ago. Screening for HTLV-III antibodies at every blood donation will probably become mandatory in the Federal Republic of Germany (FRG) after October 1, 1985 and is already now performed routinely in some blood banks. The number of reported AIDS cases is rapidly increasing in many European countries; with a rate of 6.6 per million population, Denmark and Belgium are the two countries with the highest rates (Table 1, MMWR 34/11, 147-150, 1985, CDC, Atlanta, USA).

TABLE 1. Reported acquired immunodeficiency syndrome cases and estimated rates per million population — 17 European countries

Country	Oct. 1983	July 1984	Oct. 1984	Dec. 1984	Rates
Austria	7	0	0	13	1.7
Belgium	38	0	0	65	6.6
Czechoslovakia	0	0	0	0	0.0
Denmark	13	28	31	34	6.6
Finland	0	0	4	5	1.0
France	94	180	221	260	4.8
Federal Republic of Germany	42	79	110	135	2.2
Greece	0	2	2	6	0 6
Iceland	0	0	0	0	0.0
Italy	3	8	10	14	0.3
Netherlands	12	21	26	42	2.9
Norway	0	0	4	5	1.2
Poland	0	0	0	0	0.0
Spain	6	14	18	18	0.5
Sweden	4	7	12	16	1.9
Switzerland	17	28	33	41	6.3
United Kingdom	24	54	88	108	1.9
Total	**260**	**421**	**559**	**762**	**2.0**

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Fig. 2. Examples of prenatal HLA determination.

a) Cultured cells from amniotic fluid, sampled at the 16th week of gestation.

Mother : HLA-A3,A11;B15,B40;Cw2
Father : HLA-A2,A11;B21,B44;Cw-
Fetus : HLA-A11 ;B21,B40;Cw2
Cord blood, at delivery : HLA-A11 ;B21,B40;Cw2

Index = 310.0

b) Cultured cells from villous biopsies taken in or before the 11th week of gestation have given negative reactions in the cytotoxic technique.

Cultured cells from a villous biopsy taken at the 15th week of gestation.

Mother : HLA-A1,A11;B7,B40;Cw3
Father : HLA-A1,A31;B7,B40;Cw3
Culture: HLA-A1,A31;B7,B40(Cw3 not tested)

Index : 60.2

c) Aborted fetus, 16th week of gestation.
Microabsorption with fetal liver tissue: HLA-A3,A28;B16,B35 Cw4: only one serum available, weak absorption.
Cytotoxic test on cultured fetal fibroblasts: HLA-A3,A28;B35,B16;Cw4.(Cw4: only one serum available).

Combined results of case:

Mother: HLA-A3, ;B7,B35;Cw4
Man : HLA-A2,A28;B5,B39
Fetus : HLA-A3,A28;B35,B16(B38/B39);(Cw4)

Index = 188.3

Fig. 1. Complement dependent cytotoxic microtechnique for prenatal HLA-determination.

Microchambers with 60 wells à 10 µl.

A modified NIH-technique:

No medium in the wells.
2 µl of Hanks' solution+20% FCS/well.
Wells covered by paraffine oil.
+ **2 µl** HLA antiserum/well.

30 minutes at room temperature.

+ 4 µl **rabbit** complement/well.

60 minutes at room temperature.

+ 2 µl trypanblue solution (0.1%)/well.
20 minutes at room temperature.

A modified KN-technique:

No medium in the wells.
6 µl of Hanks' solution+20% FCS/well.
Hanks' solution **replaced** by **4 µl** HLA antiserum/well.
Wells covered by paraffine oil.

At once:

2 µl **human** + 2 µl **rabbit** complement/well.

30 minutes at 37°C.

+ 2 µl trypanblue solution(0.1%)/well.
20-30 minutes at room temperature.

READING.

Number of dead, stained cells in each well counted by means of an inverted microscope according to the scale:
0-5% : -, 50% : ++, 75% : +++, 100% : ++++

toxic test using lymphocytes from appropriate test donors. The case is described as case c in fig. 2. In this case it was possible to distinguish, by absorption, the crossreacting antigens A2 and A28, because the serumbattery comprised two antisera reacting with A2 and not with A28, and three sera reacting with A2-A28, and the antibody activity was not reduced in the two reagents reacting only with A2. In the same way two reagents containing anti-B5+B53 showed no reduction in antibody activity after absorption, while the antibody was removed in an anti-B35+B5.

Conclusion: A prenatal HLA-determination is possible after the 11th week of gestation with these methods. Investigation of the HLA type of a fetus may be indicated for medical reasons for example for diagnosis of the HLA linked trait, congenital adrenal hyperplasia. In forensic medicine an HLA-determination of a fetus during pregnancy could be indicated for example in cases of rape, in which doubt exists about the paternity of the fetus, as well as in case of abortion following rape or sexual abuse.

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Vejerslev, L.O., H.E. Hansen, J.G. Westergaard, & F. Søndergaard: Amniotic fluid cell and chorionic villi culturing for prenatal HLA-determination. This issue.

all six test cell suspensions, are tested for complement activity by means of HLA antisera with a well known antibody titer. Rabbits with non-toxic serum often have a poor complement activity. As a result 0-2 rabbits out of 10 may pass the test. The complement used is a pool of serum from 4-6 animals. The rabbits are bled for 40 mls of blood about once a month. The serum is separated within two hours, pooled and stored in liquid nitrogen. Once or twice each year one animal in the group is replaced by a new one.

The cytotoxic microtechnique: The method is essentially the same as described for HLA typing of cells from hydatidiform moles (Hansen & Vejerslev, J. Immunol. Meth., in press), and is used when the cells are grown in monolayer cultures for HLA determinations in situ. For the prenatal determinations two variants of the cytotoxic technique have been used: a modification of the two stage NIH-technique and a modification of the Kissmeyer-Nielsen (KN) method (fig. 1). So far 10 cultures of amniotic fluid cells, 22 cases of hydatidiform mole, and 3 cultures from normal villous biopsies have been investigated, furthermore in three cases cultured fetal fibroblasts from an aborted fetus have been typed.

Amniotic fluid cells could be typed readily, though with the best results in the NIH-modification, since they often gave weak results in the KN. Cells cultured from villous biopsies could not survive at room temperature, and the best results were thus obtained in the modified KN-technique. Cultured fetal fibroblasts from the fetuses could be typed in both cytotoxic techniques.

In one case microabsorption of specific HLA antisera with a crude suspension of fetal liver tissue from an aborted fetus was performed. The microabsorption method has been described in details elsewhere (Hansen & Gürtler, 1981 & 1983). In this case 30 µl samples of centrifuged fetal liver was mixed with 50 µl of selected, specific antisera, and incubated for 1 hour at 37°C before the serum was recovered. The ability of the fetal liver to reduce specifically the antibody titer of the HLA antisera was then measured in the routine cyto-

PRENATAL HLA-DETERMINATION.

Hanna E. Hansen, Lars O. Vejerslev, Jes G. Westergaard & Finn Søndergaard.

University Institute of Forensic Genetics, Copenhagen.
Department of Medical Genetics, the J.F. Kennedy Institute, Glostrup, Copenhagen.
Department of Obstetrics and Gynecology, Odense University Hospital, Odense, Denmark.
Department of Obstetrics and Gynecology, Copenhagen Municipal Hospital in Hvidovre, Copenhagen, Denmark.

The present paper comprises the methods used for prenatal HLA-determination in a number of cases. When live cells are available, through a villous biopsy, amniocenthesis, or directly from an aborted fetus, a cytotoxic microtechnique is applied on cultured cells (cell culture: L.O. Vejerslev et al., this issue). After spontaneous or induced abortion a microabsorption method with the fetal tissue may be used.

For the cytotoxic microtechnique special reagents and selected complement are needed.

HLA antisera: Reagents are obtained through the laboratory screening programme, and tested on B- and T-lymphocytes at room temperature and at 37°C (Hansen & Gundolf, 1981). After throughout testing on peripheral lymphocytes, the sera are tested for possible use on cultured cells. Sera which act operationally monospecific in routine HLA-typing very often show unexpected positive reactions with cultured cells. The cultured cells seem to be more vulnerable, and thus more susceptible to weak antibodies and/or crossreactions which would not effect normal peripheral lymphocyte suspensions. Some reagents operationally monospecific in routine typing give strong unspecific positive reactions with all cultured cell populations. So far this phenomenon remains unexplained.

Complement: Most normal rabbits have antibodies toxic to human cells. To assure non-toxicity of the rabbit complement it is necessary to test the toxicity of the serum of each rabbit separately. Both B- and T-lymphocytes, freshly prepared as well as frozen and thawed, are used in three non-toxic media. Rabbit sera, which are absolutely non-toxic to

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the background of dead cells observed immediately after trypsinization. The culture of villus stromal cells provides a prenatal HLA-determination about 4 weeks earlier than it is possible to obtain by amniocentesis.
It is now possible to obtain the proper material for HLA-determination, whether it is performed on amniotic fluid or villus cells and whether the purpose is based on Forensic or Medical Genetics.

Acknowledgement.
The investigation of hydatidiform mole is supported by the Danish Medical Research Council, j.nr. 12-4122, 12-4514 and 12-5580.

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Vejerslev L.O., N. Tommerup & A. Hallberg. Hydatidiform moles: Methods for culture and cytogenetic analyses. Cancer Genet. Cytogenet. In press.

are replaced by stromal cells. When trophoblast cells are removed before the villi are set up in culture the time for HLA-determination is reduced considerably. By centrifugation the villus stems are concentrated in the pellet and set up for culture.

Primary cultures are established in Chang medium in T 25 flasks. Chang medium, which is supplemented with several growth factors and hormones, was originally designed for improved growth of amniotic fluid cells, but applied to chorionic villi the time in culture is reduced too. Outgrowth of fibroblastlike, stromal cells is observed around the tissue explant within 1-3 days. On the 3rd day the medium is replaced by DMEM with 20% fetal calf serum and 10% pooled human serum. Secondary cultures are usually established on day 4-5 and confluent on day 8-10. HLA-determination on cells in suspension immediately after harvest with trypsin revealed a very high percentage of dead cells - often about 40%. This was reduced to less than 10% by final culture in microchambers used for HLA-determination.

After harvest 200.000 cells are suspended in 1.5 ml medium and 15 mikroliters of this suspension are placed in each well of 2-4 microchambers used for HLA-determination. When the cells are attached to the bottom of the wells 5 ml of medium are added and the microchambers incubated for 24-48 hours to obtain a sufficient number of cells. The incubation period reduced the background of dead cells due to the preceeding trypsinization and increased the number of cells expressing HLA.

Conclusion.

Isolation and culture of villus stromal cells by the present techniques has improved the growth rate of cells expressing the fetal HLA-haplotypes. The final culture of amniotic fluid cells and villus stromal cells in the microchambers used directly for HLA-determination reduced

ally after fetal death placental cells may show the best growth potential.

Material and methods.

Cell cultures were established on from 3 chorionic villus biopsies, 10 samples of amniotic fluid and 22 samples of villi from hydatidiform moles. The molar pregnancy is in most cases characterized by absence of a fetus, cystic swelling of the villi and 46 chromosomes originating from a duplication of the 23 paternal chromosomes. As the maternal chromosomes are missing only paternal antigenes are expressed. Finally 3 cultures of fetal fibroblasts were processed.

After amniocentesis the culture of amniotic fluid cells followed the routine procedure at the lab. Briefly, the cells in 15-20 ml amniotic fluid are spun down and incubated in medium 199 or McCoy's medium supplemented with 10% fetal calf serum and 10% pooled human serum. Usually the cells are subcultured on day 10-14 and the secondary cultures confluent after 1-3 days. When a sufficient number of cells was obtained in the secondary cultures the cells were trypsinized and transferred for final growth into microchambers used for typing as described for villus cells below.

Placental parts of fetal origin are obtained prenatally by villus biopsy or after abortion by dissection of the placenta under the microscope. In both situations the initial important step is isolation of fetal villi and removal of blood clots or tissue of maternal origin. The following enzymatic disaggregation of the tissue by collagenase removes the trophoblast layer on the surface of the villi and exposes the stromal cells. Villus trophoblast cells do not express HLA-antigens and it is thus intended to obtain cultures exclusively of stromal cells. Furthermore untreated villi in culture show primary outgrowth of slowly dividing trophoblast cells that later on

Amniotic fluid cell and chorionic villi culture for prenatal HLA-determination.

Lars O. Vejerslev, Hanna E. Hansen, Jes G. Westergaard & Finn Søndergaard.
Department of Medical Genetics, The J.F.Kennedy Institute, Glostrup, Copenhagen.
University Institute of Forensic Genetics, Copenhagen.
Department of Obstetrics and Gynecology, Odense University Hospital, Odense, Denmark.
Department of Obstetrics and Gynecology, Copenhagen Municipal Hospital in Hvidovre, Copenhagen, Denmark.

HLA-determination on placental or fetal tissue are of interest in Forensic Medicin as well as in Medical Genetics.I Forensic Medicine prenatal HLA-determination of the fetus supports a paternity diagnosis. In Medical Genetics one important aspect is prenatal diagnosis of congenital adrenal hyperplasia, as there is a close linkage between the HLA-B locus and a deficiency in 21-hydroxylase. Furthermore prenatal HLA-determination is of interest in relation to transplantation immunology.

We have elaborated methods for culture of amniotic fluid cells and villus cells for HLA-determination. HLA-determination on cultured amniotic fluid cells was previously not performed in Denmark. The recent introduction of chorionic villus biopsies for prenatal diagnosis made an investigation about 4-6 weeks earlier than amniocentesis possible. As the time for a villus biopsy may have passed when the investigation is wanted it will be necessary to handle both techniques.

The material for a fetal diagnosis can be obtained during pregnancy as a villus biopsy in the 8-12 week or as fetal cells isolated from amniotic fluid after amniocentesis in the 16th week of gestation. Abortion material, whether spontaneous or induced, consists of the fetal parts of the placenta, the membranes or the fetus proper. Especi-

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Waltz H, Prokop O, Mayr WR: HLA-DR in der Paternitätsbegutachtung. Referate 10. int. Tagung Ges. f. forens. Blutgruppenkunde, München, 1983, S. 41

In spite of this excellent applicability of HLA-A,B,C (and in some cases also HLA-DR), there are several pitfalls which can occur during the serological determination of these alloantigens. The problems arise in connection with the various reagents used in the lymphocytotoxic assay: lymphocytes (media for resuspension, viability, contamination with granulocytes or platelets, storage), antisera (cross-reactivity, gene dosage effect, synergism, non-HLA antibodies), rabbit complement, other reagents (mineral oil, formaldehyde) or atypical HLA alloantigens (variants, changes by diseases or drugs, blocking antibodies); for details, see Mayr, 1977. The knowledge of all these factors and the use of well-defined antisera which have been tested against a large cell panel, however, ensure correct and reproducible typing results.

For the forensic practice, the genetic systems investigated in affiliation cases should be tested in the following order: ABO, MNSs, Rh, HLA, and thereafter all the other markers. This modus procedendi which takes into account the high chance of paternity exclusion of HLA and the short life span of the lymphocytes used for the definition of the HLA alloantigens avoids multiple bleedings of the individuals and provides all the informations for the evaluation of a case in only one series of tests.

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Taking into account the HLA-DR alleles in addition to HLA-A,B,C, the number of HLA phenotypes and genotypes rises to 4×10^8 and 4×10^9, respectively. The chance of exclusion of HLA-A,B,C,DR amounts to 98%; the cumulative chance of exclusion with the non-HLA systems routinely used reaches 99.97%.

The determination of the HLA-DR alloantigens in paternity testing (see e.g. Waltz et al., 1983) should be carried out only by specialised laboratories, as specific antisera in sufficient quantities are not available and some factors cannot easily be defined. In our opinion, the two-colour-fluorescence (TCF) method is preferable to the use of separated B lymphocytes due to the fact that the former technique can be performed with rather small volumes of blood (3-4 ml) which can be easily obtained from small children, while the amount of blood necessary for the preparation of B cells is significantly higher.

The results obtained by using the HLA system in affiliation cases are excellent (see e.g. Mayr et al., 1981), especially in problematic ones (cases with 2 men not excluded in non-HLA systems, cases without mother or cases in which only relatives of the putative father could be tested). The analysis of 1130 affiliation lawsuits from Austria and the German Democratic Republic (Mayr et al., 1981) for instance showed that the inclusion of HLA-A,B,C could reduce in cases with 2 accused men the number of problematic situations from 66% to 9%.
In this material, the frequency of non-HLA exclusions without HLA exclusion is 4%; this figure corresponds very well to the calculated chance of paternity exclusion of HLA-A,B,C (96%).
The inclusion of HLA-A,B,C also has a strong effect on the biostatistical computations: in the above-mentioned material, the plausibility of paternity according to Essen-Möller reached the W value of 99.8% without HLA only in 72 out of 832 cases (8.7%), while with HLA, $W > 99.8\%$ was observed in 464 out of 832 cases (55.8%). A similar increase of the chance of paternity exclusion in the single cases could be found.

X genes are not yet detectable, they formally follow a recessive mode of inheritance.
There exists a strong linkage disequilibrium between several alleles of the 3 loci, that means that in general the frequencies of the HLA-A,B,C haplotypes are not in accordance with the products of the corresponding gene frequencies.
Due to the large number of alleles in the 3 loci, there is a prodigious polymorphism in HLA-A,B,C: approximately 5×10^6 phenotypes and 23×10^6 genotypes. The most "common" phenotypes in Caucasoids are HLA-A1,3;B7,8;Cw7 and HLA-A1,2;B8,44;Cw7 with frequencies of less than 1%.

The tremendous HLA-A,B,C polymorphism and the low frequencies of the phenotypes are the reasons for the extreme usefulness of this system in solving problems of parentage: the investigation of the HLA-A,B,C gene products gives a chance of exclusion in false accusations of paternity of 96%. Together with the routinely used non-HLA systems ABO, MNSs, P, Rh, K, Fy, Jk, Lu, Xg, Se, acP_1, AK_1, ADA, PGM_1, GPT, EsD, GLO, Hp, Gm, Km, Gc, C3, Bf, Tf and Pi a cumulative chance of exclusion of 99.94% can be reached.

The alloantigens of the HLA-D region which are present on B lymphocytes, monocytes, some epithelial and endothelial cells consist of one α and one β chain being non-covalently bound. With serological techniques, it is possible to define the polymorphism of the HLA-DR and the HLA-DQ gene products. Up to now, 12 DR alleles coding for serologically detectable specificities and one X allele (DRX) are known; a linkage disequilibrium exists between some HLA-DR and HLA-B alleles. The polymorphism of DQ encompasses only 4 alleles (DQw1, DQw2, DQw3 and DQX). Due to the extremely strong linkage disequilibrium between DR and DQ, the DQ polymorphism adds no further information to the DR typing results for paternity testing. The same applies for DRw52 and DRw53 which are products of the DR subregion and show an extreme linkage disequilibrium with the other DR alleles.

THE HLA SYSTEM IN FORENSIC PRACTICE

W.R. Mayr (Institut für Blutgruppenserologie der Universität Wien,
Spitalgasse 4, A-1090 Wien / Österreich)

The HLA system which represents the major histocompatibility complex of man encompasses approximately one thousandth of the human genome. It is governed by a gene complex situated on the short arm of chromosome 6 (between 6p21.1 and 6p21.3) and contains a series of closely linked loci (for review, see Histocompatibility Testing 1984):
- HLA-A, B and C coding glycosilated polypeptides with a molecular weight of 44000 daltons (on the cell surface, these chains are non-covalently bound to β_2-microglobulin, a polypeptide governed by chromosome 15),
- the HLA-D region with loci for α and β chains (glycosilated polypeptides with 34000 and 29000 daltons, respectively),
- at least 4 loci for proteins of the complement system (C2, C4A, C4B and Bf),
- loci for the 21-hydroxylase.

Out of the cell-bound HLA gene products, the alloantigens coded for by HLA-A, B and C present on all nucleated cells of the organism fulfil without doubts all the criteria which are demanded for the use of genetically defined markers in cases of disputed paternity: the mode of inheritance is known with certainty, the techniques of determination are reliable and simple, the phenotype reflects the genotype only, and the characteristics are developed at birth.
The loci HLA-A, B and C show a remarkable multiple allelism: the gene products of 19 HLA-A, 37 HLA-B and 8 HLA-C alleles can be defined for the moment. The analysis of family and population data demonstrates that not all specificities of these loci are yet serologically detectable. These "unknown" alloantigens are governed by alleles designated as AX, BX and CX, respectively. The alleles of HLA-A, B and C except the X genes are inherited in a codominant way; as the products of the

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The HLA System: Biological Function and Association with Disease.

A.Svejgaard, Tissue Typing Laboratory of the
Department of Clinical Immunology,
University Hospital (Rigshospitalet) of
Copenhagen, Denmark.

The HLA system is the major histocompatibility complex (MHC) of man and controls transplantation antigens, various immune responses, certain complement components, and the susceptibility to a variety of diseases. More specifically, the system codes for three sets of characters: the class I-III molecules. The Class I molecules are cell surface molecules carrying the HLA-A, B, and C antigens and present on all nucleated cells and on platelets. The Class II molecules are also cell surface molecules; they carry the HLA-DR, DQ, and DP antigens but are only present on some cell types, macrophages and B-lymphocytes in particular. The Class II molecules are properdin factor Bf of the alternative and factor 2 (C2) and 4 (C4) of the classical complement activation pathway. All Class I, II and III molecules are genetically highly polymorphic: there is a large number of different HLA types in the population, but all HLA factors show pronounced linkage disequilibrium with at least one other factor controlled by genes at a nearby HLA locus.

The Class I and II molecules are intimately involved in the specific thymus-dependent immune response. Thus, Class II molecules are required when macrophages present antigen to T-helper lymphocyted and when B-lymphocytes receive help from these. In analogy, Class I molecules are involved when virus-infected target cells present viral antigens to cytotoxic T-killer lymphocytes. Different Class I and Class II molecules present antigen with different efficiency and thus, they serve as immune response (Ir) determinants.

This biological function of the Class I and II determinants is probably the reason behind the associations between certain HLA factors and various "autoimmune" diseases. For example, insulin-dependent diseases occurs almost exclusively in DR3- and/or 4 positive individuals, rheumatoid arthritis mainly in DR4--positives, and multiple sclerosis in DR2-positives. Most recently, an absolute association between narcolepsy and DR2 have been found and suggest that this disorder may be due to autoimmunity against an as yet unknown receptor in the brain. However, not all HLA associated diseases are characterized by autoimmunity (e.g. idiopathic haemachromatosis and congenital adrenal hyperplasia) demonstrating that the HLA complex also control certain non-immunological functions.

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When different subclones of hybridoma II V 10 with anti-N specificity are tested against red cells treated with various enzymes, their reactivities are reduced differently, suggesting that each recognized a distinct and different epitope (table 4). Hemagglutinating reactivity for all subclones are readily destroyed by papain, whilst the reactive sites for subclones E 11 and H 10 are resistant to chymotrypsin and trypsin. The reactive sites of MN (heterozygous) red cells demonstrated greater sensitivity to neuraminidase treatment than those of NN (homozygous) red cells, possibly due to dosage effect. There may be distinct epitopes on the N antigen: The sensitivity of subclones D 1 and D 2 epitopes to neuraminidase and trypsin treatment is in contrast to the resistance of sublones E 11 and H 10. Similar results regarding the M antigen were demonstrated by Nichols et al. (6), who postulated two blood group M epitopes applying anti-M monoclonal antibodies.

In conclusion, the microtiter hemagglutination method provides considerable advantages. In our experience, based upon more than 40.000 reactions, it es a simple, rapid and economic method. If large numbers of samples are examined (as in the case of production of monoclonal antibodies with blood group specificity)economy of equipment, reagents, and especially time, is achieved in comparison to standard techniques carried out in tubes. Furthermore, the microtiter hemagglutination method gives important information about comparative strength of reactions. For reading reactions, agitation of the plates gives more uniform cell dispersal as by shaking individual tubes. However, the most important advantage is the obvious rapidity. One technologist can perform as many as 600 reactions per hour including reading and manual recording. Last, but not least, the method allows the automatic evalution of agglutination reaction patterns by a photometer combined with a computer, programmed to produce print outs.

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Table 2: Specificity of 118 antigen-positive hybridoma cells (VCN=Vibrio cholerae neuraminidase, PAP=Papain)

Out of 118 antigen-positive hybridomas 64 (54 %) showed defined blood group specificity, whilst 54 (46 %) reacted with all native testcells. However, if red cells were treated with neuraminidase or papain, different agglutination reaction patterns could be observed.

Subclone	cell growth	reactivity	yield	reactivity
I J 12-10	61	0		
-25	85	0		
-50	86	0		
I J 13- 5	17	15 (88%)	15	15/15 (100%)
-25	51	47 (92%)		
-50	72	70 (97%)		
I K 4-10	23	0		
-25	54	2 (4%)	1	1/2
-50	79	3 (4%)	2	1/3
I T 12- 5	18	5 (28%)	3	3/5 (60%)
-25	76	15 (20%)	14	13/15(87%)
-50	92	90 (98%)		

Table 3: cell growth and hemagglutinating activity of some selected subclones with Anti-B specificity.

24 selected hybridoma cells were finally cloned by limiting dilution (9 anti-B, 4 anti-AB, 2 anti-N and 9 panagglutinins). Hybridomas may stop synthesizing antibodies despite good cell growth (see table 3). Therefore the supernatants have to be tested frequently. In subclone I J 12 the antibody producing cell line is lost. In subclone I K 4 only 4 % of growing cells show hemagglutinating activity. The many "non-producers" may overgrow the "producers". To avoid the loss of these few antibody producing cells, the hybridomas have to be recloned. Other subclones like I J 13 and I T 12 are good antibody-producers and stable cell lines.

Subclone		Erythrocytes								
II V 10	ONN	VCN	TRY	CHY	PAP	BMN	VCN	TRY	CHY	PAP
D 1	+++	+	0	+++	0	++	0	0	0	0
D 2	+++	+	0	+++	0	++	0	0	0	0
D 5	+++	++	++	+++	0	+++	0	++	+	0
E 10	+++	++	++	+++	0	+++	0	++	+	0
E 11	+++	+++	+++	+++	0	+++	0	+++	+++	0
H 10	+++	+++	+++	+++	0	+++	0	+++	+++	0

Table 4: effect of enzyme treatment of tested erythrocytes on the reactivity of different subclones with anti-N specificity (VCN = Vibrio cholerae neuraminidase, TRY = Trypsin, CHY = Chymotrypsin, PAP = Papain).

RESULTS AND DISCUSSION:

In contrast to polyclonal antisera containing many unrelated immunoglobulins beside the specific antibody, in hybridoma supernatants the total amount of immunoglobulin is a specific antibody and too small to inhibite the antiglobulin serum. Therefore, during the screening and identification of blood group specific monoclonal antibodies, the AGT can be performed WITHOUT WASHING. Thus, the microtiter method including AGT is extremely rapid without decreased hemagglutination sensitivity.

	Microplates	Cluster trays	Petri dishes
supernatant (Vol.)	50-100 µl	1-2 ml	10 ml
growing hybridoma	721	624/721 (87%)	152/721 (21%)
antigen positive	500/721 (69%)	363/721 (50%)	118/721 (16%)
		363/500 (73%)	118/500 (24%)
		363/624 (58%)	118/152 (78%)
			118/363 (32%)

Table 1: cell growth and agglutinating activity of hybridomas during expanding after cell hybridization (B-28-1-85).

Supernatants of growing cells in Greiner fusion plates, microplates, cluster trays and Petri dishes are tested systematically for hemagglutinating activity using the microtiter hemagglutination method. As shown in talbe 1, 500 out of 721 cells growing in microplates were reactive with the red cells used for immunization, whilst in cluster trays the results were 363. In Petri dishes only 118 supernatants were angigen positive. There is a remarkable loss of antibody production during the first days after hybridization: 31 % after 10 days (microplates), 42 % after 15 days (cluster trays), whilst 4 weeks later (Petri dishes) the clones are more stable (only 22 % loss of antibody production). 118 out of 500 (24 %) primary antigen-positive clones could be preserved and stored in liquid nitrogen.

64 (54%) with defined blood group specificity:

- 34 Anti-B
- 13 Anti-AB
- 14 Anti-N
- 3 Anti-N/Anti-B

54 (46%) with undefined specificity:

- 27 Panagglutinins (saline): 10 VCN -/- PAP; 12 VCN +/- PAP; 5 VCN +/+ PAP
- 27 IgG-Panagglutinins (AGT): 6 VCN -/- PAP; 5 VCN +/- PAP; 16 VCN +/+ PAP

well indicates an antigen-positive clone or at least highly enriched clone. The antigen-positive hybridomas are transferred into cluster trays and expanded in Petri dishes until enough cells are available for storage in liquid nitrogen and for final cloning. Thus a particular antigen-positive clone can be isolated under optimal conditons after 2-3 tests. Antigen-positive hybridomas are isolated by final cloning by limiting dilution in microtiter plates (4) and evaluated according to Fazekas de St. Groth (3). Since hybridomas may stop synthesizing antibodies, the supernatants are tested against red cells from time to time, and only positive clones are expanded and aliquots of the cells are frozen and stored in liquid nitrogen.

Microtiter hemagglutination method:

Rigid plastic microtiter plates containing 96 U bottom wells (Greiner) are used for all tests. 25 µl of hybridoma supernatant are distributed by a repetitive dispenser (Multipette Eppendorf) and mixed with 25 µl of a 2 % red cell suspension in PBS (red cells used for immunization, and appropriately selected cell sets for antibody screening and identification). Plates are covered to prevent evaporation and are incubated 10 - 30 min at room temperature. After centrifugation 30 - 60 sec at 100 x g, using a centrifuge adapted for plates (Hettich), the plates are agitated by a shaker (Titertek, Flow) for 10 - 30 sec, to resuspend the cells completely. The agglutination reactions are read from the bottom of the plate by a reading device with an illuminated and magnifying mirror (Biotest).

INDIRECT ANTIGLOBULIN TECHNIQUE (AGT):

Applying monoclonal antibodies the AGT can be performed WITHOUT WASHING because the hybridoma supernatant contains only specific antibodies, and the total amount of immunoglobulin is too small to inhibit the antiglobulin serum. Microplates are centrifuged 30 - 60 sec at 700 x g and the supernatant fluid is removed by flicking the plate over a sink. 50 µl of appropriately diluted anti-mouse-immunoglobulin (IgG + IgM, Jackson) are added by a multi-microdiluter (8 oder 12-channel graduated pipette Multistepper, Titertek, Flow). Plates are centrifuged again for 30 - 60 sec at 100 x g, agitated and read. Coombs control cells may be added to each negative AGT to confirm the antiserum's reactivity.

INTERPRETATION:

Positive reactions are to be interpreted based on the normal appearance of agglutination reactions. A negative reaction will appear as a smooth cell suspension in the bottom of the well. Verification of weak reactions may be accomplished by interpreting the settled red blood cell pattern after 20 - 30 min. The settled pattern ist then read by observing the underside of the well through the magnifying mirror. Negative reactions appear as smooth round cell buttons, whereas strong positive reactions will appear as jagged clumps of cells. Weak positive reactions will appear as a cell button with a "halo" surrounding it.

A RAPID SCREENING ASSAY FOR BLOOD GROUP SPECIFICITY OF MONOCLONAL ANTIBODIES USING THE MICROTITER PLATE SYSTEM:

Alois Poschmann, Volker Müller, Hans H. Hoppe and Konrad Fischer

Abteilung für Klinische Immunpathologie der Universitäts-Kinderklinik and Zentralinstitut für Transfusionsmedizin, Hamburg, Germany.

When producing monoclonal antibodies of blood group specificity it is important to screen as early as possible and rapidly hundreds of hybridoma supernatants on antibody activity and specificity. The standard techniques for erythrocyte antibody screening and identification are time consuming and expensive. Therefore, we tested the microtiter plate system which was adapted to blood grouping firstly by Wegmann and Smithies in 1966 (10), using V-bottom wells. In 1970 Crawford et al. (2) used U-bottom wells and modified the microtiter plate system for antibody screening and identification, titration and cell typing. Since then, this microtiter hemagglutination method has achieved increasing importance(1,7,9). The method is simple, rapid, economic and makes it possible to read, interprete and record automatically the agglutination reactions. The advantages of the microtiter hemagglutination method are demonstrated by rapid detection and isolation of antigen-positive hybridomas after fusion and final cloning. Our experience with this method is based upon more than 40.000 reactions.

MATERIALS AND METHODS:

Immunization and somatic cell hybridization (4,5,8):

8-week-old female BALB/c mice were immunized according to the schedule of Stähli et al. (8) with washed erythrocytes of a healthy donor of the following type: B MN ss P Le(a-b+) Rh pos CcD.ee K neg Fy(a+b+) Jk(a+b+). Spleen cells were obtained 1 day after the final boost and were fused in the ratio 5:1 x 10^7 with BALB/c, P3-X63-Ag8-U1 (11) in the presence of 0,5 ml of 40 % polyethylene glycol 4000 (Merck) and 15 % dimethylsulfoxide.

Selection and growth of hybridomas (4,5):

RPMI 1640 culture medium containing 20 % fetal calf serum and supplemented with glutamine, penicillin and streptomycin plus hypoxanthine, aminopterin and thymidine (complete HAT medium) was used for the selection and primary culture of growing colonies(4). The fusion mixture was suspended in 50 ml of complete HAT medium and distributed into 48 wells of 2 Greiner fusion plates each containing normal spleen cells (1-2 x 10^5/well). They were used as feeder cells prepared 1 day prior to fusion. 10 days after fusion cellular growth was observed macroscopically in all primary culture wells. Culture supernatant from each well was tested for hemagglutinating activity. With the small wells at the bottom of a big well the Greiner fusion plate provides higher resolving power than any other multi-well tissue culture plate (early cloning). Several cell clones in a big well are not mixed with each other. All cell clusters of the antigen-positive wells are transferred using 50 or 100 µl Eppendorf pipettes into microtiter plates. After several days of proliferation the supernatant of each well is tested again for antigen reactivity: each positive

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pronounced effect on binding of anti-N to MM than to NN red cells. Furthermore, modification of the amino groups such as acetylation (18-21) abolishes antibody binding.
These findings also indicate that the antibody recognizes an epitope at the amino-terminal end of the polypeptide chain (anti-N_{leu}) since free amino groups do not occur at other sites of the chain.
The anti-N_{leu} type of the antibody is also corroborated by lack of reactivity with trypsinized NN,He+ red cells since trypsin selectively destroys glycophorin A on the red cell surface (22,23).
In contrast to 35/5 F and to most polyclonal anti-M and anti-N reagents of human or animal origin, antibody 425/2 B agglutinates desialylated red cells almost as well as untreated cells.
The epitope recognized by the antibody 425/2 B seems to be located at an interior part of the polypeptide chain, i e the antibody seems to be of the rare anti-M_{gly} type. The agglutination obtained with two NN,He+ samples supports this assumption. Further proof would be non-reactivity with rare red cells of the McMc type since such cells have amino acid Ser at position 1 as normal M but Glu at position 5 as normal N (10,11). However, no such cells were available.
The binding and inhibition experiments also point to an epitope located at an inner portion of the polypeptide chain. The antibody bound strongly to M glycoprotein and its acetylated derivative, and the binding to the untreated glycoprotein was strongly inhibited by the acetylated derivative.
The non-specific binding to N glycoprotein and the ability of this protein and its derivatives to inhibit antibody binding points to an internal epitope which includes amino acid(s) beyond the fifth position in the polypeptide chain since amino acids 6-131 are identical in M and N glycoprotein (6,14). Conformational change supposedly imposed at a higher pH might make the part of the epitope that is common to M and N less available to antibody binding. Such conformational change could also explain the pH dependence of the antibody since blocking of free amino groups by other means such as acetylation does not abolish the antibody activity. Studies are in progress to elucidate these points.

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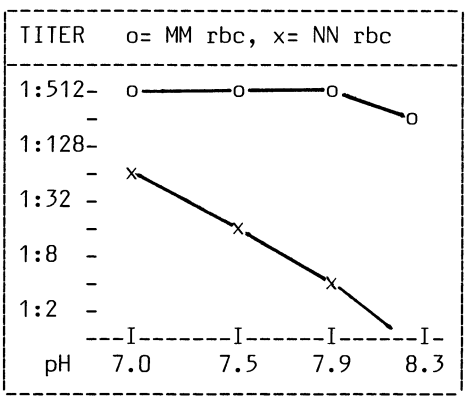
Antibody 425/2 B (anti-M):
Native tissue culture supernatant (pH 7.2) strongly agglutinated MM and NN red cells. Upon increasing the pH the titer with NN cells decreased and at pH 8.3 no agglutination was observed. With M red cells a slight decrease in titer was observed at pH 8.3 (fig 4). Trypsin treatment almost completely abolished the reactions with MM and NN cells at pH 8.3. Chymotrypsin treatment decreased the titer with MM red cells at pH 8.3, but with NN red cells no reactions were observed at this pH.

FIG 4



After desialylation with neuraminidase, reactions with MM cells at pH 8.3 decreased with increasing neuraminidase concentration. The degree of desialylation was monitored by using anti-T (peanut lectin,Gamma Biologicals). Even at an enzyme concentration of 0.21 mg/ml the antibody gave strong reactions (titer 1:8) with MM red cells.
Two samples of NN,He+ red cells were agglutinated to an intermediate degree (2+) by the antibody. Neither sample was agglutinated by polyclonal anti-M raised in rabbits.
The agglutination of MM cells at pH 8.3 was strongly inhibited by untreated and N-acetylated M glycoprotein but only slightly by the desialylated derivative, and even less with N glycoprotein (fig 5)
In ELISA at pH 8.3 a considerable binding to M glycoprotein was seen whereas only negligible binding to N glycoprotein occured.

FIG 5

pH	RED CELL	INHIBITOR	CONCENTR (µG/ML)
8.3	MM	Glycoprotein M:	
		Untreated	40
		Acetylated	<40
		Desialylated	312
		Glycoprotein N	625

The binding to M glycoprotein could be inhibited by M glycoprotein and its N-acetylated or desialylated derivatives. N glycoprotein or its derivatives did not inhibit the binding to M glycoprotein at pH 8.3.

DISCUSSION
Both antibodies were of the IgM type and gave completely specific reactions in hemagglutination when used at an appropriate pH.
The antibody 35/5 F did not agglutinate desialylated NN red cells and therefore seems to be highly dependent upon the presence of intact sialic acid residues. This was confirmed by the results of the desialylation and periodate/borohydride experiments since such procedures either remove or partially degrade (15-17) the NeuAc residues.
The pH dependency of the antibody indicates that it requires free amino groups for reactivity (20). The amino groups are not ionized (20) at a high pH. Since NN red cells contain a large number of N antigenic sites, carried on both glycoprotein MN and Ss, a slight increase in pH will affect antibody binding only to a small degree. MM red cells, on the other hand only have a small amount of N antigen carried exclusively on Ss glycoprotein. Therefore, an alkaline pH will have a much more

The immunization protocol, procedures for cell fusion, cloning and cultivation of growing hybridomas have been described earlier (13).Tissue culture supernatants were tested for hemagglutinating activity against MM and NN red cells in microtiter plates. Hybridomas giving strong and specific reactions were expanded in tissue culture and the culture supernatants were used in further studies.
M,N and Ss glycoproteins and desialylated or N-acetylated derivatives were prepared as described earlier (14).
For hemagglutination studies a panel of red cells of different phenotypes was used. The red cells were used native or after treatment with TPCK-trypsin (Merck), trypsin (Merck) or neuraminidase (Sigma), and suspended in isotonic saline. Tests were performed in tubes or on tiles and read macro-and microscopically.
ELISA was performed in microtiter plates coated with purified M or N glycoproteins or their derivatives as described earlier (14). The same substances were also used for inhibition of antibody binding in ELISA and/or hemagglutination.

RESULTS
Antibody 35/5 F (anti-N):
Native tissue culture supernatant (pH 7.2) gave an immediate and strong agglutination reaction with all NN and MN red cells, and a weaker or no reaction with MM red cells. Stronger reactions were observed with MM cells that also carried the S antigen. The titer with MMS cells from different individuals after serial dilution of the supernatant with PBS at pH 7.2 varied between 1:8 - 1:64. At the same pH the titer with NN red cells from different individuals varied between 1:500 and 1:1000. When the titration was performed with sodium phosphate buffer at pH 6.0 the reactions with MM red cells increased. At pH 7.8, no agglutination was obtained even with MMSS red cells.
Desialylation with neuraminidase completely abolished the reactions with all cells (fig 2). Weak agglutination was obtained with trypsin or chymotrypsin treated NN cells. Chymotrypsin abolished the agglutination reactions with MMS and MMss cells but after trypsinization stronger reactions than in saline were obtained (fig 2).

FIG 2

RED CELL PHENOTYPE	UNTREATED RED CELLS	TITER WITH RED CELLS TREATED WITH TRYPSIN	CHYMOTRYPSIN	NEURAMINIDASE
NNss	1:500	1:8	1:2	neg
MMS	1:8	1:64	neg	neg
MMss	neg	1:4	neg	neg

The antibody strongly agglutinated two NN,He+ red cell samples but the reactions were practically abolished after trypsinization of these cells.
The agglutination of N cells could be inhibited (fig 3) by untreated glycoprotein N and Ss but neither by M glycoprotein nor by desialylated, N acetylated or periodate/borohydride treated N glycoprotein.

FIG 3

INHIBITOR	CONC µ G/L
Glycoprotein M	>5 000
Glycoprotein N:	
Untreated	40
Desialylated	>5 000
N-acetylated	5 000
Periodate/borohydride	>5 000
Glycoprotein Ss	40

MONOCLONAL ANTIBODIES AGAINST M AND N ANTIGENS

Lisbeth Messeter, Elwira Lisowska and Arne Lundblad
Blood Bank and Dpt of Clinical Chemistry, University hospital,Lund, Sweden, and Dpt of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

INTRODUCTION

The MN antigenic determinants reside in the amino-terminal part of glycophorin A (MN glycoprotein) of the human erythrocyte membrane (1-6). The amino-terminal part of glycophorin B (Ss glycoprotein) is structurally identical with that of glycophorin A of NN individuals (2,3,6) and therefore also carries N determinants. The N activity of MM cells is low but still large enough to cause weak reactions with some polyclonal anti-M sera particularly with red cells that also carry the S antigen since they contain more glycophorin B than ss cells (7,8). The MN blood group antigenic determinants consist of a sequence of 5 amino acids (fig 1), and acids 2-4 are substituted with identical tetrasaccharide structures (5,6). Amino acids 1 and 5 are Ser and Gly in M, and Leu and Glu in N antigen, whereas acids 2-4 are identical in both (fig 1).The sugar moiety consists of an internal Gal-GalNAc sequence with two molecules NeuAc attached (fig 1). Most polyclonal reagents recognize the amino-terminal end of the glycoprotein molecule including NeuAc. For this reason the majority of these sera do not react with cells that have been desialylated. Variant M and N antigens exist and the structures of some of them have been elucidated eg M^c and He (fig 1). The variant structures contain amino acid substitutions at positions 1-5 in the polypeptide chain. M^c for instance contains Ser at position 1 as normal M and Glu at position 5 as normal N. This will give rise to atypical reactions with some anti-M and anti-N reagents. It is of great importance particularly in paternity testing to be able to disclose such anomalies and therefore a precise characterization of the epitope recognized by any particular reagent is of interest. In the present study one monoclonal antibody of each specificity were characterized.

FIG 1

BLOOD GROUP	ON GLYCOPHORIN	AMINO-TERMINAL STRUCTURE	REF
M	A	O* O O | | | Ser-Ser-Thr-Thr-Gly-	1-4, 6
N	A and B	O O O | | | Leu-Ser-Thr-Thr-Glu-	1-4, 6
M^c	A	O O O | | | Ser-Ser-Thr-Thr-Glu-	10, 11
He	A	O O O | | | Try-Ser-Thr-Thr-Gly-	10, 11

*O = NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-

MATERIAL AND METHODS

Balb/cABom female mice were injected either with native human A_2, MNSs red cells or with purified glycoprotein prepared from red cell membranes of MM donors.

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Fig. 1: Amino-acid sequences (one-letter-code) and glycosylation sites (+) of MN SGP, Ss SGP (partial) and hybrid SGP from Dantu+ RBC.

MN SGP:

1 S/L-S-T-T-G/E-V-A-M-H-T-S-T-S-S-S-V-T-K-S-Y-I-S-S-Q-T-N-D-T-H 29

30 K-R-D-T-Y-A-A-T-P-R-A-H-E-V-S-E-I-S-V-R-T-V-Y-P-P-E-E-E-T-G-E-R-V-Q 63

64 L-A-H-H-F-S-E-P-E-I-T-L-I-I-F-G-V-M-A-G-V-I-G-T-I-L-L-I-S-Y-G-I-R 96

97 R-L-I-K-K-S-P-S-D-V-K-P-L-P-S-P-D-T-D-V-P-L-S-S-V-E-I-E-N-P-E-T-S 129

130 D-Q 131

Ss SGP:

1 L-S-T-T-E-V-A-M-H-T-S-T-S-S-S-V-T-K-S-Y-I-S-S-Q-T-N-G-E-M/T 29

30 G-Q-L-V-H-R-F-T-V-P-A-P-V-V-I-I-L-I-I-L-F-V-M-A-G-I-I-G-T-I-L-L-I-S 63

64 Y-X-I-R-R-L-I-K-K 72

Hybrid SGP:

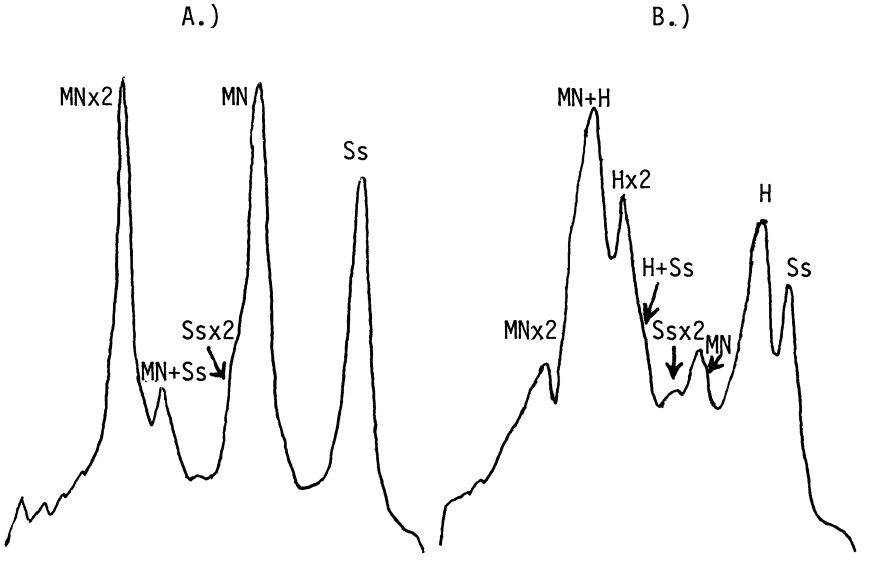
1 L-S-T-T-E-V-A-M-H-T-S-T-S-S-S-V-T-K-S-Y-I-S-S-Q-T-N-G-E-T 29

30 G-Q-L-V-H-R-F-T-V-P-E-I-T-L-I-I-F-G-V-M-A-G-V-I-G-T-I-L-L-I-S-Y-G-I 63

64 R-R-L-I-K-K-S-P-S-D-V-K-P-L-P-S-P-D-T-D-V-P-L-S-S-V-E-I-E-N-P-E-T-S 97

98 D-Q 99

Fig. 2: Densitometric scans of autoradiographs obtained after SDS-PAGE of membranes and incubation of the gels with radio-iodinated Vicia graminea lectin. A.) M-N+ control, B.) M+N+S-s+U+Dantu+ sample exhibiting N activity on the MN, Ss and hybrid (H) SGPs. MN, Ss and H = apparent monomer of the respective SGP; MNx2 = dimer of MN SGP; MN+H = heterodimer involving the monomers of the MN and hybrid SGPs etc.



al. (12), the Dantu phenotype appears to the rather rare in Caucasians. However, recently (16) we have detected a Dantu+ RBC sample from a Caucasian. The ratio of the MN and hybrid SGPs in the membranes from that donor is similar to that in Ph ghosts (13). However, the Dantu gene complex of this individual appears to produce an Ss SGP, as judged from the amount of this molecule in the membranes, in contrast to the Dantu alleles of the N.E. and Ph varieties. We had initially (11) assumed that the Dantu allele in N.E. had been generated by an unequal crossing over between a u and an En allele. On the basis of the data described above it is likely that the Dantu alleles in N.E. and Ph were generated by a misalignment between a u and a normal gene complex. The reason for the different amounts of MN and hybrid SGPs in N.E. and Ph RBC remains to be elucidated.

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Author's address: Prof. Dr. W. Dahr, Abteilung für Transfusionswesen, Universitätskliniken Köln, Stelzmann Str. 9, D-5000 Köln 41, FRG.

Contreras et al. (12) have suggested that N.E., Sh. and Vi. RBC exhibit weak U antigens. According to our previous (11) and further data, this receptor is absent from RBC of all individuals assumed to possess the genotype Dantu/u. SDS-PAGE analyses of membranes from all Dantu+U+ individuals revealed an Ss SGP content of about 50%, consistent with the genotype Dantu/U.

In our initial studies (11) N.E. was found to react weakly with all anti-s sera and with most of our anti-S reagents. Subsequent studies by Contreras et al. (12) revealed that the agglutination of Dantu/u and Dantu/sU RBC by anti-S sera is attributable to a separable anti-Dantu which occurs frequently in sera containing antibodies against low incidence antigens. Our further data confirm this conclusion. Conversely, the weak reactions of anti-s sera with Dantu/u and Dantu/SU RBC can be attributed to a weak s antigen produced by the Dantu allele. We have detected one potent anti-s that fails to react with such RBC, suggesting that this s receptor is altered in a qualitative manner. Just like the N antigen (11,12), the weak s and Dantu receptors are not destroyed by protease treatment of intact Dantu+ RBC. This suggests that they are located on the hybrid SGP which is not degraded by incubation of RBC with various proteinases (trypsin, chymotrypsin, ficin, papain, pronase, V8 proteinase, thermolysin), as revealed by SDS-PAGE. Anti-Dantu could be inhibited by Triton X-100 (0.5%) extracts from Dantu+ membranes which contain the hybrid SGP, but not by SGPs prepared by the phenol and butanol methods. This suggests that the Dantu antigen represents a labile receptor which is located in proximity to the lipid bi-layer of the membrane.

Since the hybrid SGP, in contrast to the MN and Ss SGPs, is not degraded by proteinase treatment of intact RBC, we have used trypsin (and chymotrypsin) treatment of cells, followed by membrane isolation, phenol extraction and gel filtration in the presence of Ammonyx-LO, in order to isolate the hybrid SGP from N.E. and a pool of RBC from Dantu+ individuals who are not related to N.E. The complete structure of the hybrid SGP from N.E. was deduced from manual DABITC/PITC and carboxypeptidase Y sequencing of the intact molecule and various peptides prepared by trypsin, chymotrypsin, V8 proteinase and cyanogen bromide treatment as well as a comparison of the amino-acid composition of peptide C2 (res. 67-86) with the res. 115-118 of the MN SGP (5). We have only sequenced the N-terminal 16 res. of fragment C2 (res. 67-86) and not established an overlap between peptide C2 and the adjacent fragment C3 (res. 87-99). 10 Edman-degradation cycles on the intramembraneous chymotryptic peptide (res. 35/37-66) of the hybrid SGP isolated from the pool of Dantu+ RBC served to establish that the relevant part of the sequence is identical with that of the hybrid molecule from N.E. (Fig. 1).

As shown in Fig. 1, the Dantu-specific hybrid molecule exhibits the res. 1-38 (or 39) of a blood group s-specific Ss SGP and the res. 71 (or 72)-131 of a MN SGP. As judged from its lability, the Dantu antigen appears to be located within the res. approx. 30-40 of the molecule. The proposed structure also explains the absence of the U antigen from u/Dantu RBC, since this receptor is located C-terminal of position 32 or 34 of the Ss SGP (3).

The above-described phenomenon that the Dantu allele expresses M and N antigens of roughly normal strength is of forensic significance. Similarly, the presence of a weak s antigen in Dantu+ RBC and the frequent occurrence of anti-Dantu in anti-S sera represent sources of error in forensic blood typing of Blacks. As judged from the data of Contreras et

bation of $NaJO_4/NaBH_4^3$ labelled Ox. II-3 membranes with anti-CB2 serum (5) and subsequent SDS-PAGE analysis of the antigen-antibody complexes that bound to protein A-Sepharose, after solubilization with Triton.

The data of Contreras et al. (12) had provided evidence that the Dantu gene complex expresses N (protease-resistant and located on the hybrid SGP) and M activity, although the MN SGP content of membranes from Dantu heterozygotes is only approx. 50% of normal. Further evidence for the assumption that the Dantu allele encodes an MN SGP possessing M activity was obtained from the following results: 1) All Dantu+ RBC samples that we have studied (n = 24) were found to exhibit the phenotype M+N+. 2) Studies on one pedigree (H.P.; 14) in which a mother had made anti-Dantu during pregnancy yielded the phenotypes M-N+S-s+Dantu- (mother) and M+N+S-s+Dantu+ (father and child), respectively. 3) Separation of membranes by SDS-PAGE and subsequent incubation with radio-iodinated anti-N lectin from Vicia graminea, in order to visualize the SGPs carrying N activity (15), revealed that 3 (H.P., Sh., Vi.) of 12 Dantu+ RBC exhibit N activity on the MN SGP. Typical patterns obtained by this method are shown in ref. (15) and Fig. 2. The presence of M activity on the MN SGP in all Dantu+ samples is suggested by the destruction of this receptor by trypsin treatment of RBCas well as amino-acid composition and sequence analysis of a peptide (C1, res. 1-64), isolated from the MN SGP of N.E. In order to account for the data described above, it has to be assumed that the hybrid SGP, produced in large amounts, suppresses the incorporation of the MN SGP, encoded by the Dantu allele (M active) and its normal counterpart, into the RBC membranes of Dantu heterozygotes.

In our previous report (11) we had described that the major membrane protein (coomassie band 3) in N.E. ghosts exhibits a decreased molecular weight. This phenomenon was attributed to a shortening of the carbohydrate chains on band 3, due to sterical hindrance of glycosyl-transferase(s) resulting from the formation of complexes with the MN SGP and the hybrid molecule. Further evidence for this hypothesis was obtained by galactose oxidase/$NaBH_4^3$ labelling of N.E. ghosts which revealed a strong (about 80%) decrease of label in the gel region corresponding to band 3. This alteration of band 3 could be detected for all (n = 18) Dantu+ samples that were investigated by SDS-PAGE and coomassie staining.

In our initial SDS-PAGE experiments (11), weak bands (10-20% of normal) at the positions of the mono- and dimer of the Ss SGP were detected for N.E. and her baby (D.E.) that had suffered from hemolytic disease of the newborn, due to anti-U and anti-D. In subsequent experiments we found that this minor component in N.E. and other Dantu+ membranes exhibits a slightly higher mobility than the monomer of the Ss SGP. Due to some variability of the SDS-PAGE separation, it is not resolved from the Ss SGP in some electrophoretic experiments. The significance of this minor componentwhich was detected for all Dantu+ samples is not yet clear - it might represent a degradation product of the hybrid SGP. It is also detectable in Dantu+ membranes of the Ph variety (13). When the SDS-PAGE analyses on D.E. were repeated about one year after delivery, the Ss SGP content of membranes from D.E. and an additional child (K.E.) of N.E. was found to be approx. 50% of normal, suggesting that both are heterozygous for the allele u. It has to be concluded that coating of the cord RBC sample from D.E. with anti-U or -D had caused a selective degradation of the Ss SGP. The data described above suggest that N.E. is heterozygous for the genes Dantu and u. As judged from our results, 4 other individuals (Sh., Vi., Bl., H.B.) also exhibit the genotype Dantu/u.