APPLICATION OF IMMUNOGLOBULIN ALLOTYPING IN FORENSIC STAIN ANALYSIS; RELIABILITY AND SENSITIVITY OF Gm AND Km TYPING. "The demonstration of the factors Gm (z, a, x, f, n, g and b^O) and the factors Km (1 and 3) in relation to the Immunoglobulin G concentration in the stain extract".

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Abstract

This paper reports the detection of a wide scope of immunoglobulin alloantigens in bloodstains. Commercially available antisera and their corresponding anti-D reagents were evaluated in agglutination-inhibition tests for bloodstain grouping in forensic serology. The determination of a maximum of different allotypes increased the value of immuno-globulin allotyping in forensic bloodstain analysis. Particular reference is made to the application of a sensitive semi-quantitative IgG estimation in relation to the detectability of the respective allotypes. The relative performance of the individual antigens in serum and extracts of fresh and aged bloodstains were obtained. On the basis of these results it was often possible to interpret negative reactions of the bloodstain extract in the hemagglutination-inhibition method with confidence. There is no evidence for the preferential loss of any of the alloantigens after drying and after aging of the blood.

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INTRODUCTION

In forensic medicine the determination of immunoglobulin allotypes is valuable for the identification of bloodstains. A recurrent problem in Gm typing is the interpretation when no inhibition of the anti-allotype antiserum occurs (Khalap, 1979 and Schmitter, 1980). This result can represent the true absence of the Gm factor or the concentration of the marker in the extract was too low for detection. In this paper we report the application of a sensitive semi-quantitative IgG estimation in relation to the detectability of the Gm factors (z, a, x, f, n, g and b^0) and the Km factors (1 and 3). The relative performance of the individual antigens in serum and stain extracts is also studied.

MATERIALS

Anti-allotype antisera, their corresponding anti D-coats and controlsera both positive and negative for the relevant allotype were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) in Amsterdam. A representative set of allotyping reagents is given in table 1. Foetal Calf serum (heat inactivated) was from Flow Laboratories. V-bottom microtiter plates were purchased from Greiner. Coombs Control cells and anti-human IgG (H-chain specific) were from Ortho Diagnostics Systems. Standardserum (HOO-03) for the quantitative determination of serumproteins was obtained from the CLB.

METHODS

Extraction for bloodstain typing

0,5 cm^2 Of bloodstained fabric was extracted in 1,5 ml of saline at roomtemperature for 1 hour. The mixture was heat treated at 65°C for 10 minutes, frozen at -20° C, thawed and the supernatent was centrifugated until clear.

Agglutination-inhibition method for determination of allotypes

Allotyping of sera was done in at least three dilutions of the serum (1 :15, 1 : 45 and 1 : 135). Serum dilutions were heat treated for 10 minutes at 65°C, frozen at -20°C and centrifugated until clear. Samples of stain extracts were usually tested in doubling dilutions starting undiluted. In microtiterplates 1 drop (25 ul) of serum dilution or stain extract, 1 drop of optimally diluted antiserum and 1 drop of allotype-coated 0,1% red cells in 5% FCS were added successively. Plates were mixed and left overnight at 4°C. Agglutination reactions were read macroscopically with the help of a light box after being placed for 10 minutes at an angle of 60°. The highest dilution of stain extract giving complete inhibition of agglutination is read. Controls included always: saline controls to show sufficiently strong agglutination of the coated red cells with the anti-allotype antiserum, positive serum controls, negative serum controls and a control for the serum or extract with the coated red cells only, which must not show agglutination.

Agglutination-inhibition method for semiquantitative IgG estimation

Doubling dilutions of stain extract were made in microtiter plates. To 25 ul of stain extract dilution 25 ul optimally diluted IgG antiserum (usually 1 : 300 in saline) and 0,1% Coombs control cells in 5% FCS were added successively. Incubation and reading of the plates were analogous to the procedures for determination of allotypes.

RESULTS

Serological properties of allotyping reagents

The optimal sensitizing anti-D dilutions along with the titers of the anti-allotype antisera were determined in one operation using two-dimensional titration schemes. Subsequently, the optimum anti-allotype antisera dilutions (working dilutions) were obtained from two-dimensional titration schemes with the anti-allotype antisera titrated against dilutions of control-sera both positive and negative for the relevant factor using optimally coated red cells. The optimum dilutions of the allotyping reagents which are listed in table 1 are given in table 2. The addition of Foetal Calf Serum to the agglutination-reaction prevented adherence of red cells to the plates. In many different batches of FCS unspecific reactions due to its addition were never encountered.

Detectability of Gm and Km antigens in relation to IgG concentration in limited diluted bloodstain extracts

To determine the sensitivity of Ig allotyping in bloodstain analysis a large series of extracts of control bloodstains with several different Gm/Km antigen compositions were tested. Used bloodstains (n = 77) were not older than 6 weeks in this experiment. The heat treatment of the bloodstain extract effectively eliminated occasionally occurring unspecific agglutinations of the extract with the coated red cells only, but had no effect on the titer of the extract in het HAI reactions for IgG estimation or Ig allotyping. Limiting dilution experiments were conducted to determine the maximal dilution of the bloodstain extract at which the respective allo-types present in the bloodstain could be detected. From this the minimal IgG concentration required for the detection of the respective alloantigens present in the bloodstain extract was established (table 6). The relative performance of the different alloantigens can also be substracted from this table. For example: Less than 0,6 ug/ml IgG in the stain extract is required for Km(3) detection while at least fortyfold more IgG in the extract is required for Km(1) detection. The amount of IgG which is required for the detection of the respective Gm antigens lies between 4,6 and 17 ug/ml IgG. As the sensitivity of the HAI test can be affected by the quality and the freshness of the coated red blood cells, it is essential to determine the sensitivity of the test for any alloantigen in each new experiment.

Ig allotyping of bloodstains in casework

Tests for Ig allotyping were made on a large number and variety of actual case material in conjunction with semiquantitative IgG estimation. In most circumstances it was possible to interpret negative inhibition results of the bloodstain extract by this results of the IgG determination. A representative example is

given in table 7. The table summarizes the results of the allotypic marker testing conducted on reference blood samples from victim and accused. The evidence consisted of two blood stains (stain x and y).

DISCUSSION

This study was undertaken by our laboratory in order to get the availability of a reliable and feasible immunoglobulin allotyping method. Allotyping was performed by a haemagglutination-inhibition test. For forensic purposes the test must be as sensitive as possible. The inhibition method can be optimized by using optimally diluted antisera. Firstly we selected appropriate allotyping reagents. The working dilutions of anti-allotype antisera and corresponding anti-D coats were obtained. The features and serological properties of a representative set of allotyping reagents are given in table 1 and 2. Optimally diluted allotyping reagents were firstly extensively tested with sera and bloodstain extracts both negative and positive for the relevant factor before the reagents were released for staintyping in actual casework. The limits of sensitivity in terms of the amount of bloodstain extract which is needed for the positive identification of alloantigens was thoroughly investigated. Our approach to this problem was the measurement of the concentration of IgG, the protein on which the allotypes are actually located. IgG was measured by an agglutination-inhibition method. This method was sufficiently sensitive and accurate for our purposes. The quantification of IgG appeared highly useful in bloodstain allotyping. Knowing the actual IgG concentration in the extract, negative inhibition results of the extract in the inhibition test for allotyping could be interpret by using the figures of table 6 where the detectability of Gm and Km antigens in relation to the IgG concentration in the bloodstain extract are given. The relative performance of the alloantigens in the HAI test was also used for the interpretation of negative reactions. The presence of a certain positive marker can rule out any possibility of an immunoglobulin-subclass deficiency. A representative example of an actual case is given in table 7. The IgG concentration in the stainextract from stain x allows positive identificattion of all allotypes when present. From this it was substracted that the phenotype of the blood on stain x was Gm (fnb), Km (1-, 3+), the same type as the blood from the victim. The low IgG concentration in the bloodstain extract of stain y did not allow to report the true absence of the factors Gm (a, x and g) and Km(1).

TABLE 1. REAGENTS FOR Gm AND Km TYPING

anti-allotype	manufacturer	batchnr.	anti-D-batchnr.
anti z	CLB	3272	3471
anti a	CLB	3294	3471
anti x	CLB	2984	3545
anti f	CLB	2871	3480
anti n	CLB	R120	n-protein 1)
anti g	CLB	4040	3285
anti b^o	CLB	3955	2127
anti Km(1)	CLB	3951	2447
anti Km(3)	CLB	3674	3597

1) The IgG2 (n+) myeloma protein (code Jas) was a gift from Dr. G. de Lange from the CLB.

TABLE 2. SEROLOGICAL PROPERTIES OF REPRESENTATIVE ALLOTYPING REAGENTS

Anti-Allotype Antiserum				O R_2R_2 Sensitization	
allotype	antiserum	titer(3)	working dilution(4)	reagent	optimal concentration
z	anti z	160	60	anti D (za)	1:1
a	anti a	160	40	anti D (za)	1:1
x	anti x	80	20	anti D (x)	3:1
f	anti f	160	60	anti D (f)	1:1
n	anti n	1600	400	n-protein (2)	0,08 mg/ml
g	anti g	160	60	anti D (g)	3:1
b^o	anti b^o	160	40	anti D (b)	1:1
Km(1)	anti Km(1)	140	80	anti Km(1)	3:1
Km(3)	anti Km(3)	160	60	anti Km(3)	1:1

1. The optimal anti-D concentration is the optimal sensitizing volumeratio of anti-D to saline using a cell concentration of 10% group O R_2R_2 cells in the reaction mixture.
2. The optimal sensitizing amount of n-protein is the concentration of n-protein in saline with 10% group O cells and 0,02% $CrCl_3$ in the reaction mixture.
3. The titer of the anti-allotype antisera is expressed as reciprocal dilution giving the last 1+ agglutination reaction with optimally sensitized cells.
4. The optimum anti-allotype antiserum dilution (working dilution) was obtained from a two dimensional titration scheme with the anti-allotype antiserum titrated against dilutions of Gm (+) and Gm (-) control sera.

TABLE 6

DETECTABILITY OF Gm AND Km ANTIGENS IN RELATION TO IgG CONCENTRATION IN LIMITED DILUTED BLOODSTAIN EXTRACTS

Minimal IgG concentration required for the detection of allotypes present in limited diluted stain extracts.

allotype	phenotype	minimal IgG concentration number tested	range ug/ml	mean ug/ml
z	zaxg	1		9,2
z	zafngb	18	3,4 - 12,0	7,6
a	zaxg	1		9,2
a	zafngb	18	3,6 - 31	14,0
x	zaxg	1		4,6
x	zaxf(n)gb	18	2,2 - 18,6	7,4
f	fnb	35	4,6 - 18,6	11,6
f	zafngb	18	9,2 - 31	17
n	fnb	35	3,4 - 26,4	11,4
n	zafngb	18	4,6 - 18,4	9,4
g	zaxg	1		13,2
g	zafngb	18	9,2 - 19,6	14
b^0	fnb	35	2,4 - 18,4	7,0
b^0	zafngb	18	3,2 - 9,8	6,6
Km(1)	Km1+3+	12	6,6 - 37,2	23,4
Km(3)	Km1-3+	65		<0,6
Km(3)	Km1+3+	12		<0,6

The minimal IgG concentration required for the detection of Gm and Km antigens in diluted extracts of control bloodstains is obtained from the IgG concentration in the extract and the maximum dilution at which the allotype present in the extract could be detected.

TABLE 7

FORENSIC STAIN ANALYSIS

Semi-quantitative IgG estimation and Ig allotyping

Case

Victim : V

Accused : A

Pieces of evidence : bloodstain x
bloodstain y

Titer in HAI

	z	a	x	f	n	g	b°	Km1	Km3	IgG	IgG (ug/ml)
Serum V	+	+	-	+	+	+	+	-	+		
Serum A	-	-	-	+	+	-	+	+	+		
Control bloodstain V	4	4½	0	3	5½	2	2	0	>6	6½	28
Control bloodstain A	0	0	0	3½	>6	0	3	2½	>6	7	37
Stain x	4	4	0	3½	5½	3	3	0	>6	7	37
Stain y	1	0	0	1	3	0	1	0	4	4	4