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

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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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RESULTS 2.-

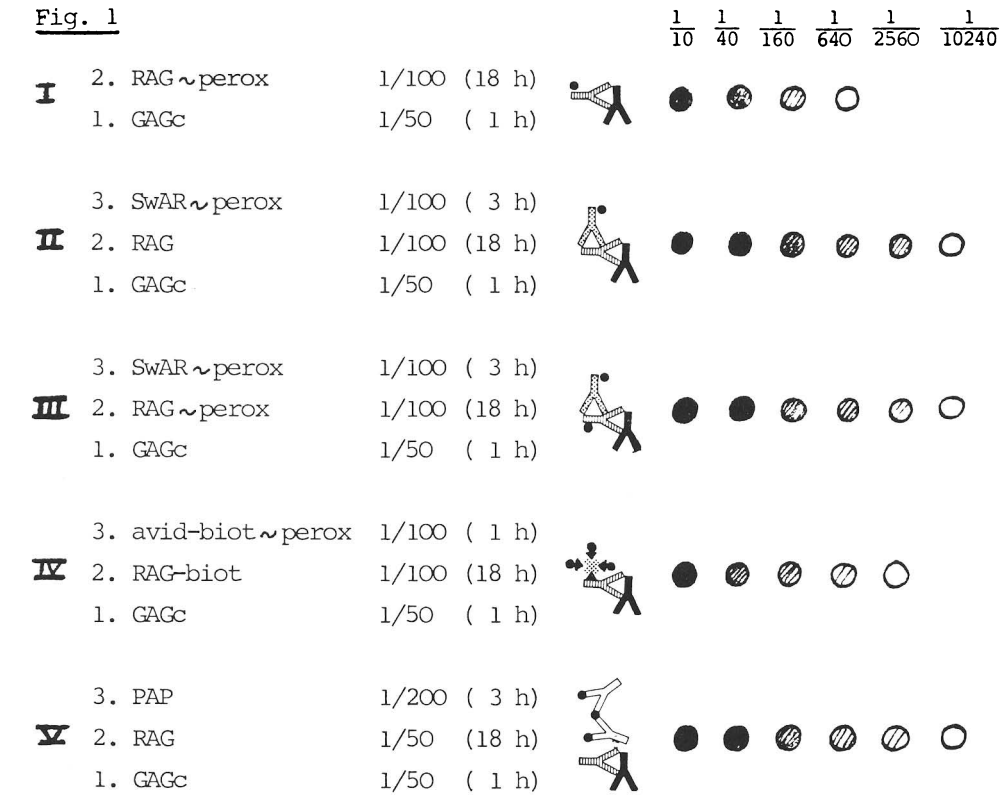
COMPARISON OF VARIOUS ENZYME-IMMUNO-ASSAYS BY DOT BLOTTING

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)

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).

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.

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