TECHNICAL NOTE

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Micromethod for MN Antigen Grouping of Dried Bloodstains

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ABSTRACT: A micromethod based on the absorption elution technique was shown to be applicable to the detection of M and N blood groups of dried bloodstains on cotton cloth. Each antigen M and N was tested using two different types of antisera. Two hundred different bloodstains, stored up to six months, were analyzed. Conclusive results were obtained for M typing on 2.5-mm-long bloodstained threads. For N typing, some cross-reactivity of homozygous M stains with anti-N sera was observed. This may be explained by the structure of the M and N antigens on the red cell membrane.

KEYWORDS: forensic science, serology, genetic typing, antigen systems, absorption elution test, micro method, MN antigens, bloodstains

In the forensic science laboratory, ideally one should be able to detect different blood group antigens by simple methods even on very small size bloodstains.

Recently we have described a new micromethod [*1*] for red cell grouping of dried bloodstains on cotton cloth based on the absorption elution technique. By performing the agglutination on microplates (also used for human lymphocyte antigen [HLA] typing) and observing the different stages of agglutination through an inverted phase-contrast microscope the number of indicator erythrocytes could be reduced to about 3000 cells. This enabled smaller volumes of eluate (2 to 4 μL), and smaller areas of stain to be examined. Using this technique, A or B antigens can be detected on a single bloodstained cotton thread of 1 mm in length, and Rhesus C, c, D, or E antigens on threads of 2.5 mm in length.

The aim of this study was to examine if this method could also be used for M and N grouping of dried bloodstains.

Materials and Methods

Bloodstains

Bloodstains were prepared using 3- to 5-mL samples of fresh blood, taken from the blood bag side tubes at the time of donation, from 200 healthy, randomly selected donors of known

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MN type. Pieces of plain cotton cloth were soaked with blood from these samples and allowed to dry for 48 h at room temperature. These stains were then stored at room temperature for up to 6 months.

Reagents

Five different anti M sera and six different anti N sera (Ortho Diagnostics, Molter, Gödecke, Fresenius, BAG) were tested. The final results were obtained with the anti M sera Ortho MM 501 B and Molter 19385 and the anti N sera Ortho NN 601 A and Molter 21382 (Ortho Diagnostic Systems GmbH, Postfach 104345, D-6900 Heidelberg 1 and Dr. Molter GmbH, Postfach 1320, D-6903 Neckargemünd).

Different potentiators of agglutination were tested: 30% bovine albumin (Gödecke), low ionic strength solution (LISS) (Dade), and AB neutral serum (obtained from a donor of our institute), stored in liquid nitrogen and diluted 1:3 with normal saline before use.

Apparatus

The apparatus used were:

- 1.0- and 0.5-μL Hamilton syringes,
- 1.5-mm-diameter hole punch,
- 0.4-mL Eppendorf tubes,
- vacuum pump with modified canula to suck fluid from the Eppendorf tubes,
- round bottom microplates No. 653180 (Firma Greiner, Postfach 1320, D-7400 Nürtingen), and
- inverted phase-contrast microscope (magnification ×300).

Method

To absorb the antibodies to the antigen, 5 μL of undiluted antiserum were put into an Eppendorf tube along with a piece of the bloodstained cotton cloth (one punch of 1.5-mm diameter = 0.2 mg of dried blood or one thread of 2.5-mm length = 0.075 mg of dried blood). To prevent evaporation, the contents of the tube were covered with a drop of paraffin oil, centrifuged at 2000 × g, and the tube was then incubated at 4°C overnight (12 to 16 h).

For the washing stage the Eppendorf tube was filled with cold physiological saline up to 0.3 mL and the contents were mixed well. The tube was allowed to stand in the refrigerator for 15 min, centrifuged for 30 s at 2000 × g, and the supernatant fluid was discarded. This washing cycle was repeated five times.

For elution the tube was filled with 5 μL of normal saline, covered with a drop of paraffin oil, and incubated in a water bath at 56°C for 10 min. Then 2 to 4 μL of the warm eluate were transferred into the well of a microplate and covered with a drop of paraffin oil.

For the final agglutination reaction, 1.7 mL of venous blood was taken from a homozygous MM or NN donor into a syringe containing 0.3-mL acid citrate dextrose anticoagulant (ACD). This blood was used within 12 h: the red cells were washed twice and suspended in normal saline to a concentration of approximately 3000 cells/μL.

With a Hamilton syringe 1 μL of this suspension was added to the eluate under paraffin oil: MM erythrocytes were added to the anti M eluate and NN erythrocytes were added to the anti N eluate. To each well 1 μL of AB neutral serum, diluted 1:3 in normal saline, was added. After incubation at 4°C for 4 h the degree of agglutination was read with an inverted phase-contrast microscope. The readings were scored from − to +++ as shown in Fig. 1.

In pilot studies some test parameters were checked and optimized as follows. Titrations using the microplate test were compared with titrations using the conventional macromethods (tile and tube test) and all tests were performed at 4°C. For both macromethods a 0.1%

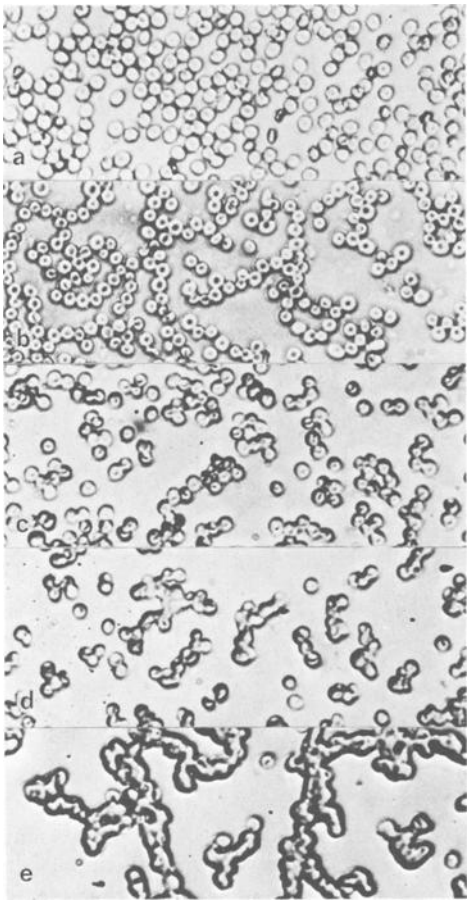

FIG. 1—*Definition of the degree of agglutination on a microplate read through an inverted phase-contrast microscope (×300):* (a) *negative reading,* (b) ± reading, (c) + *reading,* (d) + + *reading, and* (e) + + + *reading.*

suspension of cells was used, otherwise the conditions were the same as for the micromethod. The tubes were centrifuged at 1000 × g before reading. Figure 2 shows the mean endpoint titres using the three different methods. The agglutination occurred more slowly on microplates, but after the appropriate reaction time the titre on the microplate was higher than on the tile, and as high as in the tube.

Eleven different antisera, all used for routine hemagglutination test in our laboratory, were tested for their suitability for the absorption elution test. Only those antisera which gave no false positive or negative results when compared to the donors' known MN types were used in this study.

Fixation of stains with 96% methanol for 15 min at room temperature has been thought to improve results [*2–4*]. However, in the present study, readings obtained with fixed stains were on average one grade less than those with unfixed material.

Maximum absorption was obtained after 12 to 16 h of incubation at 4°C.

Attempts to automate the washing process, as tried by other authors [*4,5*], failed here because of false positive results.

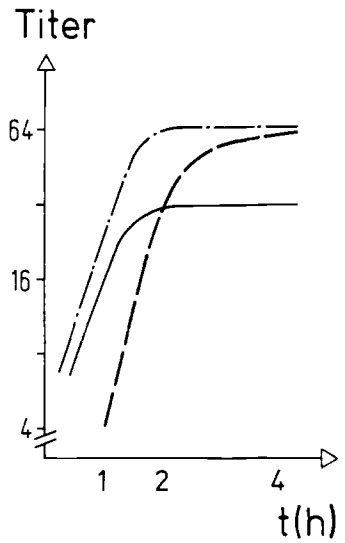

FIG. 2—*Mean endpoint tires of titrations of M and N antisera comparing three different methods:* — · — · — · — · — *tube test (with centrifugation),* _____ *tile test, and* — — — — — — *microplate test.*

For the agglutination reaction, fresh (less than 12 h old), homozygous erythrocytes should be used. As suggested by other workers [*4,6*], the results could be improved by using AB neutral serum as potentiator of agglutination rather than the widely used bovine albumin. Readings with AB neutral serum were 1/2—one grade higher than those with albumin enhancement. The addition of LISS, successfully performed by workers [*6–8*], led in this study only to false positive readings.

The validity of this method was tested by analyzing 200 different bloodstains; 100 blood-stained cotton circles of 1.5-mm diameter (36 MM, 45 MN, and 19 NN punches) and 100 cotton threads of 2.5-mm length (30 MM, 45 MN, and 25 NN threads) were each analyzed using two different anti M and two different anti N sera. The actual blood groups were not known to the analyst beforehand.

Results

For each antigen analyzed, the readings of the degree of agglutination obtained with each of the two antisera were added, so that a final "score" of 0 to 6 was obtained. We define a score of 0 to 1 as a negative result and 2 to 6 as a positive result.

The results of the analysis of 200 different bloodstains are shown in Fig. 3. For larger pieces of cloth (punches), the results were clearer than for smaller stains (threads). For the M antigen, good results were always obtained with punches as well as with threads, whereas for the N antigen, results were not as satisfactory for punches, and there was even overlapping of positive and negative results at score 1 and 2 for threads.

Discussion

As shown in the pilot studies, the microplate test proved to be as sensitive as the conventional macromethods after incubating the plates for at least 4 h. The amount of antiserum used for the micromethod however is less than for the macromethods which enables smaller amounts of stain to be analyzed than in previous studies [*2–5,8–10*].

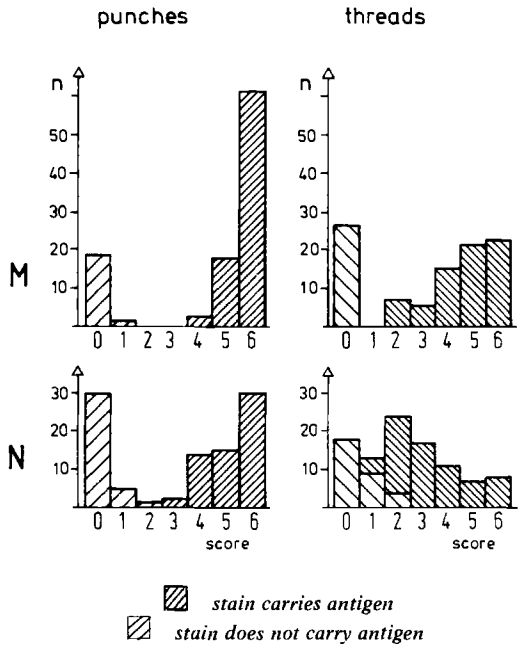

FIG. 3—*Results of the absorption elution microtest grouping 200 different bloodstains (punches of 0.5-mm diameter and threads of 2.5-mm length). The readings with two different antisera for each antigen were added to the "score."*

Not all antisera used in standard hemagglutination tests are equally applicable to the absorption elution test. Antisera for routine grouping of dried bloodstains therefore have to be carefully selected. MN antisera show more variability than ABH reagents [*11*]. Reliability of the results can be improved by testing each antigen with two different antisera.

The results of the final analysis of 200 bloodstains in the microplate test were dependent on several factors such as the number of antigenic receptors (approximately the same for the M and N antigen [*12*]) and the size of the bloodstains (punches or threads). M typing was straightforward, but for N typing, false negative and false positive results were obtained.

The weak agglutinations (scored 1) for N antigen positive threads (treated as false negative results) were not reproducible in this study. On repeat testing clear-cut positive results were always obtained. Apparently those false negative results can be avoided by appropriate test conditions.

False positive results, however, obtained by incorrect typing of some homozygous M stains with anti N sera, also called cross-reactivity, could even under optimal test conditions not be avoided. These false positive results seem most likely to be due to the structure of the M and N antigens on the erythrocyte membrane.

Most workers generally agree that M antigen typing is satisfactory whereas the detection of the N antigen is more problematic. Difficulties with N grouping were described in early reports [*13–15*]. Schwerd [*2*] observed false negative results, whereas Pereira [*9*] described false positive reactions of M stains and attributed this to the presence of some N-type substance in the M-type stains.

This has been confirmed by the investigation of the biochemistry of the M and N antigens [*16–19*]. In the human erythrocyte membrane important transmembranous proteins include the sialoglycoproteins, for example, glycophorin A and B. On the outside of the red cell membrane glycophorin A carries the polypeptide chain which determines the M and N antigen (see also Fig. 4). The smaller glycophorin B has been associated with the Ss activity. The

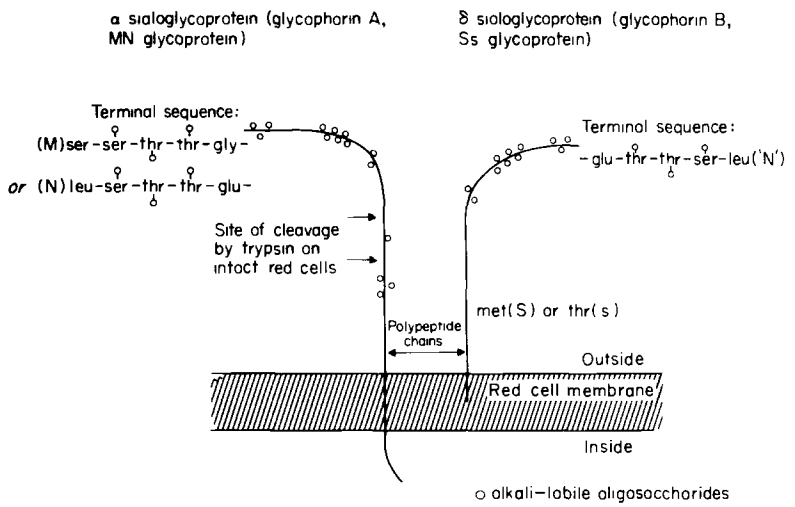

FIG. 4—*Diagrammatic representation of the MN antigens according to Mollison* [21].

first 26 amino acid residues and tetrasaccharide side chain attachments of glycophorin B are identical to those found in glycophorin A determining the N antigen, and therefore termed "N" antigen, for example, all cells, if not S and s negative, carry the "N" antigen on glycophorin B. To this "N" antigen N antibodies may be bound in some homozygous M stains.

The "N" antigen might be hidden in native M erythrocytes, but as erythrocytes undergo changes in conformation on drying, this could account for some false positive reactions [*20*].

The described micromethod proved to be applicable to M and N typing of small dried bloodstains. For M typing the method can be recommended as reliable. For N typing, however, the method has no advantages over the conventional methods. The problem how to avoid the cross-reactivity of some M stains with anti N sera remains to be solved.

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