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MN Determinations in Bloodstains—Selective Destruction of Cross-Reacting Activity

By definition, serology is the study of antigen-antibody reactions in vitro [*1*]. Forensic serology is, however, an expansion of this study and encompasses not only serology but the essentials of biochemistry, immunohematology, immunology, and immunochemistry. The forensic serologist is faced with the dual responsibility of performing analyses within this framework of disciplines and functioning as a criminalist faced with the science of identifications [*2*]. It is the forensic serologist's ultimate goal to link evidence to a source of origin, if possible.

In terms of potential, blood evidence holds the most promise of being a forensically discriminating tool. This genetic broth requires efficient analysis to yield extensive information as to its source. Currently, serological equipment includes not only the simpler microscopes and centrifuges but also high resolution electrophoresis and the scanning electron microscope.

Periodically, it becomes imperative to pause and reflect on analytical procedures, especially when development is expansive and needs are great. It is necessary to make value judgments on whether tests are appropriate, informative, and ultimately valid. Proficiency testing is an effort to perform such a critique [*3*]. It is sobering when results are less than encouraging. This project is a definitive statement that present laboratory difficulties should be surveyed and corrected.

A subject that warrants elucidation in view of this preface is the MN blood group system. Discovered in 1927 by Landsteiner and Levine [*4*], the MN system, potentially valuable in discriminating between individuals, concerns antigen sites of the same name located on the red cell surface. Landsteiner and Levine gave a frequency of occurrence as 30% M, 20% N, and 50% MN. Combined with the Ss system, it becomes a powerful tool for discrimination [*5*]. For forensic purposes, the detectability of this system in dried blood has been demonstrated [*6*]. The techniques in dried blood are the same as that used for the ABO system: absorption-elution or inhibition. The results of the proficiency testing demonstrate that an error rate as high as 40% is obtained with MN as compared to 1.6% for ABO. These results suggest that the fault is not necessarily in the ability of crime laboratory personnel to use these techniques but quite possibly in the system itself or the application of these particular techniques to the detection of M and N antigens in dried stains.

The MN problem can be considered in its most simplistic terms as being caused by two factors. First, paradoxically, it is possible to detect N antigen on M cells [*7–9*]. This

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cross-reactivity is potentially troublesome because of the possibility of mistyping M stains as MN. Second, the quality of commercially available anti-N sera is not controlled for forensic purposes, resulting in the production of antisera unsuited to bloodstain typing. This report is concerned with the selective destruction of the cross-reactivity associated with the MN system. The results of this work are applied to detection of MN antigens in bloodstains.

Experimental Materials and Methods

Materials

Alpha-Chymotrypsin (thrice crystallized), Type VI, was supplied by Sigma Chemical Co. Amicon Miniconcentrator B15 was produced by Amicon Corp. Ortho Diagnostics supplied the anti-M and anti-N sera (rabbit) (Lots 147 and 148). Acrylamide was produced by Sigma Chemical Co. Cooke microtiter equipment was supplied through Scientific Products.

Direct Agglutination of Known Mx Cells

Whole blood known to be cross-reacting (Mx, M-'N') was washed three times with physiologic saline and made into a 2% saline suspension. This suspension was tested in a Cooke microtiter plate against serially diluted anti-M and anti-N sera. Aliquots (25 ml) of the red cell suspension were added to each test well, the first of which contained neat antisera and the others, sera serially diluted to 1:2048. This test stood at room temperature for 30 min and then was read. The M was reactive to a 1:8 dilution, while the N was reactive only as a neat solution.

Incubation of Whole Red Blood Cells

Experiments were conducted on Mx blood cells to duplicate the results of other investigators. Samples of freshly washed (three times), packed red cells of the same Mx type were treated with one of four concentrations of α-chymotrypsin: 4, 2, 1, and 0.5 mg/ml. Each of these concentrations was tested against a varied timetable for optimum incubation conditions: 120, 60, 40, 20, 10, and 5 min. The test was designed so that one set of 24 tubes represented the anti-M antisera and a second set of 24 the anti-N antisera. The tubes were arranged in four rows; each row was representative of the four α-chymotrypsin concentrations and was six tubes deep to accommodate the different time incubations. Two-millilitre quantities of the α-chymotrypsin were prepared by dissolving the enzyme in 0.01*M* phosphate saline, pH 7.6.

One Pasteur pipet drop of an appropriate α-chymotrypsin concentration was added to each of the four tubes in the row representing a 120-min incubation. Two Pasteur pipet drops of a 2% cell suspension of the previously prepared cells were added to each of the four tubes. The tubes were finger-tapped and placed in a 37°C incubator for 60 min. At that time the row representing 60 min was prepared and both sets were incubated for an additional 20 min. The rows continued to be prepared in this manner until all were completed and 120 min total time had elapsed. At this point, the tubes were centrifuged (3400 rpm) in a clinical centrifuge for 15 s. The supernatant enzyme was removed and the cells were washed three times with physiologic saline, the last wash being decanted.

One drop of anti-M serum, 1:8, was added to each tube in the first set of 24 and one drop of neat anti-N serum to each tube in the second set. All tubes were finger-tapped and stood at room temperature for up to 30 min. Readings were taken at 30 min or as soon as visible agglutination occurred prior to that time. After being read, all the tubes

were centrifuged for 30 s. The supernatants were tested against indicator cells of the corresponding type, that is, M cells against the M supernatants.

Incubation of Bloodstains

The next series of experiments was run with an absorption-elution technique on Mx (M-'N') bloodstained fibers.

Absorption-elution plates were prepared and run, initially without α-chymotrypsin treatment and later with the four concentrations of the varied time study. It was noted with the first plates that commercial-strength anti-N sera were ineffective in demonstrating the N antigen even when new lots were substituted.

Disk Electrophoresis of Membrane Components

Membranes were isolated and their components were separated by detergent polyacrylamide gel electrophroesis according to the method of Fairbanks et al [*10*].

Results and Discussion

It is generally believed that cross-reactivity is due to the presence of N antigen on M cells as a result of the former being a precursor molecule. [*8,9*]. Thus a variety of M blood cell samples would exhibit various degrees of N reactivity dependent on the amount of N to M conversion that had taken place.

The ability to detect N antigen is directly proportional to the strength of the anti-N sera.

To clarify the experiments performed in this report some background serology and biochemistry on MN blood group factors must be presented.

In dried blood analyses the absorption-elution technique, as described by Howard and Martin [*11*] or as modified by Kind [*12*], is extremely sensitive: only a minute quantity of antigen need be present to be detected. Thus, if a bloodstain (tube-typed with antisera against whole cells) from a type-M demonstrates cross-reacting N activity, it may be typed by the absorption-elution technique as MN. Also, historically, it has been known that cross-reactivity is related to S activity [*5*].

In 1960 Allen [*13*] described an individual on whose red cells N antigen could not be detected. This was the first demonstration that all M cells do not contain N antigen. The blood type MS-s-U that Allen described is rare and was considered an exception to the rule. For nearly twelve years after that announcement, research continued concerning the biochemical nature of these antigens and their structures, and their biosynthesis was moderately well established [*9,14-22*]. During the course of these investigations, knowledge concerning the structure of red cell membranes has increased and has been correlated with the different serological antigens [*23-25*].

Several laboratories have demonstrated the protein and glycoprotein constituents in red blood cells by detergent disk polyacrylamide gel electrophoresis. Figure 1 shows a diagrammatic representation of the results obtained in the forensic science laboratory of the Aerospace Corp. It can be noted in Fig. 1*B* that several different proteins are present and in Fig. 1*A* that only a few glycoproteins are present, notably PAS 1, 2, and 3, where PAS indicates the periodic acid-Schiff reagent used to stain for carbohydrates. In an attempt to illustrate the rationale behind experiments in this report, more biochemical evidence concerning the nature of M, N, 'N', and S antigenic activities must be explained.

The M, N, 'N', and S antigens reside on glycoprotein molecules within the red cell membrane (PAS Bands 1, 2, and 3) [*26-28*]. These glycoprotein molecules can be separated by electrophoresis in detergent acrylamide gels (Fig. 1). Two of these glycoproteins,

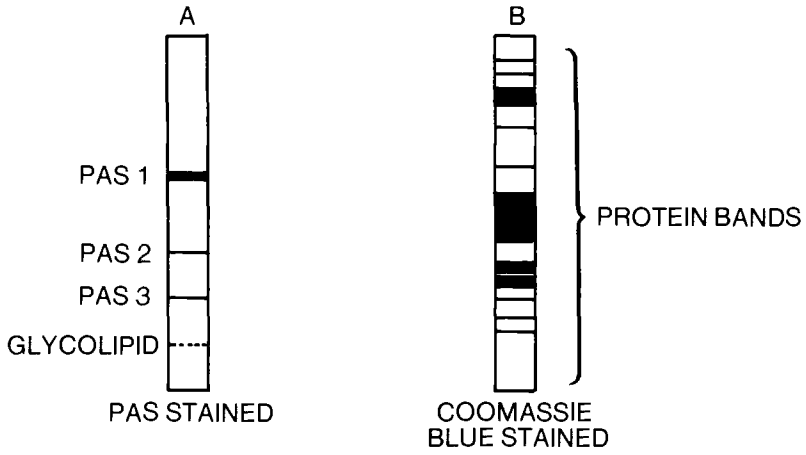

FIG. 1—(A) *Continuous 10% polyacrylamide disk gel stained with periodic acid-Schiff and* (B) *identically run gel stained with brilliant Coomassie blue.*

PAS-1 and PAS-2, contain M and N antigenic activity while the third, PAS-3, contains cross-reacting N ('N') and S antigenic activity [*26,27*].

Although Springer [*9*] has shown that M and N specificities are related to the number of neuraminic acids on the molecules, a more penetrating analysis [*27*] of these structures demonstrated that, possibly to a greater extent, the antigenic specificities are dependent on the amino acid environment, that is, the stereochemical environment of the neuraminic acid residues. It is known that treatment of M cells with neuramindase to remove sialic acid molecules (sialic acid and neuraminic acid are treated as interchangeable terms) will result in the production of nonspecific N activity. Also, treatment of M cells with proteolytic enzymes such as trypsin or pronase will achieve the same result.

An important extension of these studies has shown that treatment of M or N cells with the proteolytic enzyme α-chymotrypsin has no effect on M or N antigenic activities. However, α-chymotrypsin will selectively destroy 'N' and S antigenic activity, that is, in essence, the PAS-3 band [*14,27,28*]. Table 1 illustrates the results obtained with the incubation

TABLE 1—*Effects of α-chymotrypsin on M and 'N' in intact red blood cells. Freshly washed Mx (M-'N') red blood cells were treated with various concentrations of α-chymotrypsin for different time periods. The presence of agglutination* (+) *indicates the detectability of antigenic activity while no agglutination* (−) *indicates the inability to detect activity.*

Incubation Time, min	Anti-M (1:8) α-Chymotrypsin Concentration in mg/ml				Anti-N (Neat) α-Chymotrypsin Concentration in mg/ml			
	4	2	1	0.5	4	2	1	0.5
No enzyme	+	+	+	+	+	+	+	+
5	+	+	+	+	−	−	−	−
10	+	+	+	+	−	−	−	−
20	+	+	+	+	−	−	−	−
40	+	+	+	+	−	−	−	−
60	+	+	+	+	−	−	−	−
120	+	+	+	+	−	−	−	−

of whole M-'N' blood cells with α-chymotrypsin. These results show that reduction of 'N' activity without concomitant destruction of M antigenicity is possible.

Throughout the experiment only concentrated anti-N sera were used. This was accomplished by concentrating a 5-ml bottle of anti-N serum, Lot 148 and later Lot 147, in a miniconcentrator to the 5X mark. This worked very well and as the test proceeded was found to be even a bit strong. The strength was eventually adjusted to an optimum 2.5X. On all plates, the anti-M sera were used neat. (The diluted 1:8 anti-M sera were of insufficient strength for absorption-elution testing.)

The actual preparation of the plates consisted of affixing pairs of threads with nail enamel to the individual wells of ceramic ring slides. The prepared plates were permitted to dry overnight. The threads selected for the tests were these:

(1) control threads cut from new clean cotton sheeting,
(2) cross-reacting M threads previously determined,
(3) M threads from a stain made with type M blood, and
(4) N threads from a stain made with type N blood.

The α-chymotrypsin was incubated on the threads at 37°C in a moisture chamber at the four concentrations and for times of 120, 60, 40, 20, 10, and 5 min. The plates were rinsed in ice-cold physiologic saline and placed in beakers of fresh cold saline for three periods of 15 min each in the freezer compartment of a refrigerator. The plates were blotted dry with paper towels and incubated with antisera for 1 h at 4°C. The washing procedure was repeated as before, including the blotting dry at the end. Room-temperature saline, one drop per well, was placed on each pair of threads, and the plates were incubated at 56°C for 15 s in a moisture chamber. At this point, red cells were added to the test in the form of a 1 to 2% suspension, one drop per well, from a Pasteur pipette, and the plates were placed on a rotator for 5 min. Readings were taken at this time and the plates replaced on the rotator. These readings continued every 5 min until maximum agglutination occurred or until 1 h had passed.

The extension of these results to bloodstain analysis with the absorption-elution technique poses problems. The concentration of antisera to be used is different. Higher concentrations of antisera, especially to identify N antigen, are required, and the incubation times with α-chymotrypsin are also different. The ability to extend wet blood biochemistry to dried blood analyses is possible [*6,29,30*]. These extensions are not always a direct one-to-one adaptation, however, and the procedures require significant modification before workable systems are produced.

The selective destruction of 'N' activity with α-chymotrypsin has shown promising results for the identification of the true M and N antigens in bloodstains. Table 2 illus-

TABLE 2—*The effect of α-chymotrypsin on M, N, and 'N' activity in bloodstains. Incubation of bloodstained fibers was for 2 h at 37°C with α-chymotrypsin (4 mg/ml). The absorption-elution conditions are described in the text. The anti-N serum was commercially prepared Lot 148 from Ortho Diagnostics, concentrated 2.5 times.* (S = strong reaction and W = weak reaction.)

Bloodstained Threads	Incubation with 4 mg/ml α-Chymotrypsin	Incubation After Elution, min						
		5	10	15	20	22	32	37
M	–	+1	+4	+4	+4	+4	+4	+4
N	–	+2	+4	+4	+4	+4	+4	+4
'N'	–	+2	+4	+4	+4	+4	+4	+4
M	+	+2	+3	$+3^{s}$	+4	+4	+4	+4
N	+	+2	$+3^{s}$	$+3^{s}$	+4	+4	+4	+4
'N'	+	–	–	–	+1	+2	$+2^{s}$	$+3^{w}$

trates the effect of α-chymotrypsin on M, N, and 'N' antigens in bloodstains. With the 4 mg/ml α-chymotrypsin solution, after elution and after rotation with indicator cells for 20 to 25 min, all M positive controls will read +4 while 'N' will show only a +1 to +2. At 35 to 40 min 'N' will produce only a weak +3. Early in the testing it became obvious that the lower concentrations of enzyme and shorter incubation time were ineffective in removing 'N' activity. The 4 mg/ml concentration of α-chymotrypsin incubated for 2 h produced the most dramatic results. Stronger concentrations of 8 and 13 mg/ml incubated for 1 h showed little influence in inhibiting 'N' activity.

To illustrate the speed with which the reaction is visible, note that at 5 min controls are +2 and the test threads still negative. It appears that prolonged incubation with α-chymotrypsin has a detrimental effect on the ability to determine any of the desired antigenic activities. Thus, conditions were adjusted to accomplish this desired end without either a reduction in sensitivity or the production of nonspecific activity.

The results of this preliminary work are not intended to be a new procedural inclusion into crime laboratory routine. Instead, these data are presented to illustrate to forensic serologists the complex nature of serological systems. The interpretation of serological results must be done with a great deal of caution, especially until all the facts, serological, immunological, and biochemical, are known. When there are obvious inconsistencies in results and errors are being made during blood grouping, as there are in the MN system, then there is wisdom in a cautious and skeptical approach before an attempt is made to use that system in practice.

Acknowledgments

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