Jerome S. Levkov,[1] *Ph.D.*

A Method for the Determination of MN Antigens in Dried Blood

ABSTRACT: MN phenotypes of experimentally prepared dried blood samples, some as old as six months, were obtained using sodium dodecyl sulfate polyacrylamide gels, electroblotting, and monoclonal antibodies.

Human blood contains hundreds of genetically determined group specific antigens, enzymes, and other proteins that provide the potential for uniquely characterizing a sample of blood. However, at the present time, only about 14 genetic marker systems have been sufficiently evaluated to be used for forensic science purposes. Typing is usually accomplished using electrophoretic or serological methods or both [1–3]. The specific markers that will be determined in a particular case depend on the skill and training of laboratory personnel, availability of equipment, potential value of a marker in effecting a discrimination of the blood, cost of the analysis, as well as the age, quantity, and condition of the bloodstain. Typically, not more than 6 to 8 markers are determined in a given case.

In 1927, Landsteiner and Levine described the existence of the MN blood group in humans, and classified blood according to its MM, NN, or MN phenotype [4]. This activity in chimpanzee blood was subsequently demonstrated [5]. Among blood group marker systems, MN possesses one of the better population discrimination indices. For example, in the United States white population, the occurrence of MM, NN, and MN antigens is 28, 22, and 50%, respectively [1]. A number of studies have shown that the antigenic activity is carried by a transmembrane protein, α glycophorin,[2] which comprises about 1.6% of the erythrocyte membrane proteins. This protein has a molecular weight of 31 000 daltons and is composed of 131 amino acids with the amino terminus external to the cell and the carboxy terminus in the cell cytoplasm. Fifteen oligosaccharides, all external to the cell, make up some 60% of the protein mass. The difference between M α glycophorin and N α glycophorin resides in two amino acid substitutions at Positions 1 and 5 of the polypeptide. M glycophorin has serine and glycine and N glycophorin has leucine and glutamic acid at these

positions, respectively. MN individuals have both molecules present in their red blood cell membranes [6–8]. In addition, several minor glycophorin-like proteins are present, including δ glycophorin that carries the antigenic determinants for Ss blood groups [9].

Difficulties have been encountered in working with this blood group system in forensic science studies. For example, in a laboratory proficiency study, the frequency of error in the determination of the MN phenotype was much larger than with the ABO system, 20 and 1.6%, respectively [10]. Furthermore, 123 laboratories participating in the study reported results for the ABO system, but only 30 analyzed for the MN phenotype. Shaler et al. have attributed at least part of the error observed in MN serological typing of dried blood to the presence of N antigen on M cells, and they present a technique for selectively inactivating this antigen [11]. As discussed below, recent studies indicate the minor glycoprotein, δ glycophorin, has N antigenic activity and is the source of the cross-reaction of N antisera with Type M blood [8]. The second contributing factor is the lack of control of the quality of commercial MN antisera.

This paper presents preliminary results for a procedure that provides a means of determining MN phenotypes in bloodstains representing less than 1 μL of blood. We show that these phenotypes can be distinguished in blood from stains as old as six months. The procedure involves separation of the blood cell antigens using electrophoresis of sodium dodecyl sulfate polyacrylamide gels (SDS/PAGE), transfer by electrophoretic blotting onto nitrocellulose membranes, formation of an antigen-monoclonal antibody complex, and visualization with horseradish peroxidase (HRP) labeled antibody.

Experimental Procedure

Materials

Antibodies—Monoclonal antisera to M α glycophorin provided by Margaret Nichols, The New York Blood Center, 310 E. 67th St., New York, NY 10021.[3]

Monoclonal antisera to N α glycophorins provided by William L. Bigbee, Ph.D., Senior Research Scientist, Lawrence Livermore Laboratory, Biomedical and Environmental Sciences Division, University of California, P.O. Box 5507, Livermore, CA 94550 [12].

Polyclonal antiserum to α glycophorin prepared by injection of rabbits with MN α glycophorin antigen [13].

Glycophorins—M α glycophorin and N α glycophorin: prepared by the method of Blumenfeld and Adamany [7].

Rabbit Anti-Mouse Immunoglobulin G (IgG)—Manufactured by Cappel Laboratories, Cochranville, PA.

SDS/PAGE—Materials were prepared as described by Laemmli [14].

BIO-RAD Immun-Blot Solutions—Solutions were prepared as described by the manufacturer (BIO-RAD Laboratories, P.O. Box 708, 220 Maple Ave., Rockville Center, NY 11571).

Analytical Procedures

Blood Samples

Blood was obtained from laboratory personnel and typed by hemagglutination according to standard procedures using commercial anti-M and anti-N rabbit sera (Ortho Diagnostic Systems, Inc.). To assess the effect of aging and drying in bloodstains, as may be encountered in forensic science analysis, the following protocol was used: blood was drawn by

fingerprick and 5-μL samples were placed on 1.5-cm Whatman No. 1 qualitative filter papers. The blood-stained filter papers were stored at ambient temperature in glass petri dishes with the lids ajar.

To prepare samples for analysis, the portion of the filter paper containing blood was cut into small pieces (approximately 2 mm^2) and placed in 15-mL narrow tipped glass centrifuge tubes. Sample buffer, 75 μL, were added. The test tube was vortexed vigorously for 1 min and then placed in boiling water for 90 s immediately before loading onto the gel. Of this solution, 10 to 30 μL were loaded per lane. It is estimated that this was equivalent to about 0.7 and 2 μL, respectively, of whole blood.

Washed erythrocytes obtained from fresh blood samples were used as controls. Of sample buffer, 150 μL were added to 5 μL of cells; after placing in boiling water for 90 s, 10 μL of the solution were used for loading. The α glycophorin standards were prepared by adding 75 μL of sample buffer to 5 μL of standard and boiling for 90 s. The smallest amount of standard loaded onto the gels was about 0.1 μg.

SDS/PAGE

Ten percent gels were run by the method of Laemmli [14]. Electrophoresis proceeded at about 125 V for 2 to 3 h.

Electrophoretic Blotting

Electrophoretic transfer to nitrocellulose paper was accomplished using the method of Towbin et al. [15]. A BIO-RAD Trans-Blot electrophoretic cell was used for this purpose. Transfer times were typically 16 h.

Enzyme Immunoassay Detection of Glycophorins

Transferred glycophorin was detected using a BIO-RAD Immuno-Blot assay kit. The method involves making a sandwich between the protein, bound to nitrocellulose paper, and HRP labeled antibody, and then reacting the peroxidase with a color reagent.

The procedure suggested by the manufacturer was followed.

Results and Discussion

Figure 1 presents the glycophorin bands made visible by the application of polyclonal antiserum (to α glycophorin) and HRP labeled antibodies, to immunoblots of gels prepared by SDS/PAGE of MN red blood cells. (Periodic acid Schiff staining, specific for sialic acid rich glycoproteins, reveals the same bands.) The positions of α glycophorin dimer and monomer are marked by Arrows 1 and 4, respectively. Arrow 3 indicates the position of δ glycophorin dimer. (The nature of the glycophorin indicated by Arrow 2 is less certain.) δ glycophorin is present in much smaller quantities than α glycophorin. This minor glycoprotein is present in red blood cells of all Ss individuals regardless of their MN phenotype.[2] However, note that α glycophorin monomer and dimer bands, the two that determine the M or N phenotype of the cells, are sufficiently well resolved so that interference from δ glycophorin and other minor glycophorin bands does not occur.

The results of the analysis of dried bloodstains with the SDS/PAGE immunoblot technique using monoclonal antibodies are shown in Figs. 2 and 3. It is apparent from the reaction with standard α glycophorins that the M and N monoclonal antibodies are specific and react with the monomer and dimer bands of α glycophorins isolated from M and N homozygous individuals. Thus, the M monoclonal antibody reacts exclusively with α M glycophorin (Fig. 2, Lanes A and B), and the N monoclonal antibody reacts only with N α glycophorin
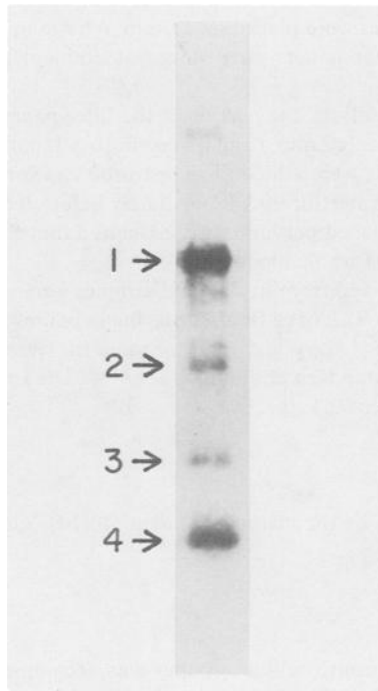
FIG. 1—*SDS/PAGE electrophoretic immunoblots of M,N red blood cells reacted with polyclonal antiserum to α glycophorin. Bands 1 and 4 contain M α or N α glycophorin dimer and monomer, respectively. Band 3 contains δ glycophorin dimer.*

(Fig. 3, Lanes A and B). In the dried blood samples, the α glycophorin Bands 1 and 4 are readily identified by comparison with the standards and the reactivity with the monoclonal antibodies confirmed the blood group phenotype obtained by conventional hemagglutination typing. In each case, the MM erythrocytes reacted with the M monoclonal antibody (Fig. 2, Lanes C and E), but did not react with the N monoclonal (Fig. 3, Lanes C and E); whereas the NN erythrocytes reacted with the N monoclonal antibody (Fig. 3, Lanes D and F), but not with the M monoclonal antibody (Fig. 2, Lanes D and F). The MN erythrocytes reacted with both monoclonal antibodies (Fig. 2 and 3, Lane G). These results are typical of the six experiments performed, in which twenty dried blood samples were tested.

As expected, δ glycophorin monomer and dimer were displayed with the N monoclonal antibodies in the dried blood samples of all phenotypes (Fig. 3, Arrows 5 and 5′, respectively). As also noted by others, the position of migration of the δ glycophorin did not interfere with evaluation of dimer and monomer bands (Arrows 1 and 4) of α glycophorins.

It is significant that the integrity and electrophoretic mobility of α glycophorin was preserved even in blood dried on filter paper for a period of about five months (Fig. 2 and 3, Lanes E, F, and G). (Samples six months old were also tested successfully.) However, it became evident that, as the dry blood aged, the amount of α glycophorin monomer and dimer decreased and a band appeared at the head of the gels. This became noticeable after approximately six weeks of drying and may be due to oligomer formation.
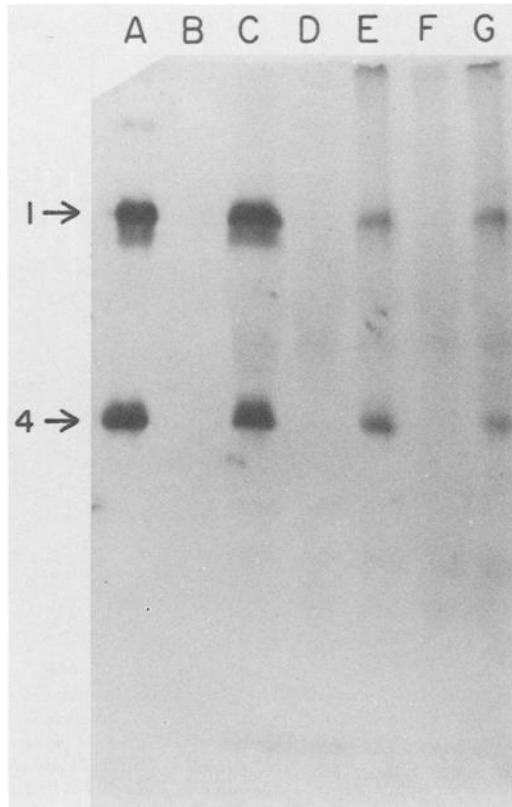
FIG. 2—*SDS/PAGE electrophoretic immunoblots of dried blood reacted with M specific monoclonal antibody. Lanes: (A) 1.3 µg of M α glycophorin standard; (B) 0.5 µg of N α glycophorin standard; (C) 2-µL MM blood, 10 days old; (D) 2-µL NN blood, 10 days old; (E) 2-µL MM blood, 5 months 17 days old; (F) 2-µL NN blood 5 months, 9 days old; (G) 2-µL MN blood, 5 months, 8 days old. Arrows point to the dimer (1) and monomer (4) of α glycophorin.*

Conclusion

For forensic science applications, identification of α glycophorin has the added significance that it offers additional evidence that the blood is of human origin. Among animal species, only the higher primates have been shown to exhibit M,N blood group activity.[4]

In summary, the technique described above has been used successfully to identify the MN blood group phenotype in samples of dried bloodstains, representing less than 1 µL of blood, aged to six months under laboratory conditions. Should it prove as reliable with known and casework bloodstains, it would provide a potentially routine procedure for the unequivocal determination of MN phenotypes in bloodstains.

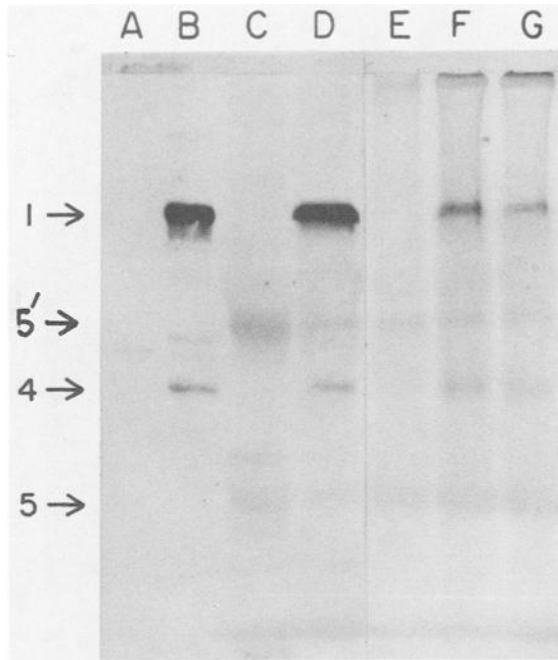[4]O. O. Blumenfeld, personal communication, Department of Biochemistry, Albert Einstein College of Medicine.

FIG. 3—*SDS/PAGE electrophoretic immunoblots of dried blood reacted with N specific monoclonal antibody. Lanes: (A) 1.3 μg of M α glycophorin standard; (B) 0.5 μg of N α glycophorin standard; (C) 2-μL MM blood, 10 days old; (D) 2-μL NN blood, 10 days old; (E) 2-μL MM blood, 5 months 17 days old; (F) 2-μL NN blood, 5 months, 9 days old; (G) 2-μL MN blood, 5 months, 8 days old. Arrows point to the dimer* (1) *and monomer* (4) *of α glycophorin and monomer* (5) *and dimer* (5') *of δ glycophorin.*

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Address requests for reprints or additional information to
Dr. Jerome S. Levkov
Iona College
715 North Ave.
New Rochelle, NY 10801