Z Rechtsmed (1989) 103:21–25

Zeitschrift für
Rechtsmedizin
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Determination of MN blood group from blood stains by electrophoresis and immunoblotting

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Summary. The determination of blood groups from blood stains is extremely important in medicolegal practice, but there is the possibility of an error in the determination of MN phenotypes by the absorption-elution test. We investigated a new method applying electrophoresis and immunoblotting. As a consequence of various experiments, the most appropriate pretreatment of blood stains was as follows. Blood stains were immersed in physiological saline for 0.5 to 1 h and centrifuged. The supernatant was discarded. The sediment was dissolved in sample buffer (TRIS-buffered physiological saline containing 2% sodium dodecyl sulfate) and followed by thermodegradation. It was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a nitrocellulose membrane by Western blotting, MN phenotypes could be determined accurately from blood stains by an enzyme immunoassay (EIA) using commercially available polyclonal anti-M and anti-N sera. For blood stains more than 1 month old it was not easy to determine the MN phenotypes.

Key words: MN blood group in blood stains – Bloodstains – MN identification

Zusammenfassung. Es ist selbstverständlich, daß in der Gerichtsmedizin die Blutgruppenuntersuchung aus dem Blutfleck außerordentlich wichtig ist. In Bezug auf MN-Gruppen gibt es jedoch die Gefahr der falschen Beurteilung bei dem Absorptionstest und Elutionstest. Wir haben eine neue Methode mit Hilfe von Elektrophorese und Immunoblotting untersucht. Zuerst haben wir verschiedene Methoden der Vorbehandlung von Blutflecken überprüft. Daraus ergab sich, daß jene Methode am besten ist, bei der der Blutfleck in physiologischer Kochsalzlösung 0.5 bis 1 Stunde eingetaucht und dann zentrifugiert wird. Nach der Beseitigung der überstehenden Flüssigkeit wird er in dem Probenpuffer (2% Natriumlaurylsulfat in TRIS-gepufferter physiolo-

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gischer Kochsalzlösung) aufgelöst und mit Hitze zersetzt. Dann haben wir mit den vorbehandelten Proben die Natriumlaurylsulfat-Polyacrylamidgel-Elektrophorese (NLS-PAGE) durchgeführt, durch Westernblotting auf Nitrozellulosemembran abgedruckt und mit dem handelsüblichen polyclonal Anti-M-Serum und Anti-N-Serum Enzymimmunoassay (EIA) vorgenommen. Damit konnte man sicher im Blutfleck MN-Gruppen beurteilen. Die MN-Antigene, die bei Raumtemperatur aufbewahrt werden, schienen aber nicht stabil zu sein. Nach ca. einem Monat waren die Gruppen schwer zu beurteilen.

Schlüsselwörter: MN-Blutgruppen – Untersuchung im Blutfleck – Blutspuren, MN-Nachweis

Introduction

The MN blood group discovered by Landsteiner and Levine [4] is very important for medicolegal identification and paternity tests, etc. However, there is the possibility of an error being made in the determination of MN phenotypes from blood stains when using the conventional absorption-elution test. Such an error can come about as a result of the presence of a small amount of δ-glycophorin that shows N-antigenic activity on MM red blood cells [1] and the nonspecific binding of commercially available polyclonal anti-M and anti-N.

Lekov [5] has recently attempted to determine MN phenotypes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting and enzyme immunoassay (EIA). However, in this method large amounts of self-made monoclonal anti-M and anti-N sera are used. After various experiments, we have developed a reliable and practical method using commercially available polyclonal anti-M and anti-N sera.

Materials and methods

Seraclone anti-M and anti-N (Biotest Diagnosis, Frankfurt, FRG) were used as monoclonal antisera and Ortho anti-M and anti-N (Ortho Diagnostic System, New Jersey, USA), as polyclonal antisera. Fifteen different blood samples were used that were known MN types. Of each blood sample 1 to 10 µl was adhered to bleached cotton cloth and stored at room temperature (20°–30°C) for 1 day to 3 months.

Blood stains were immersed in 5 ml physiological saline alone or in 5 ml saline containing protease inhibitors such as soybean trypsin inhibitor, *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride, iodoacetamide, diazoacetyl-DL-norleucine methyl ester, and disodium ethylenediaminetetraacetate, respectively. After centrifugation at 3,500 rpm for 5 min, the supernatant was discarded. Twenty to 50 µl of sample buffer (0.0625 *M* TRIS-HCl physiological saline, pH 6.8, containing 2% sodium dodecyl sulfate) was added to the sediment. After sufficient stirring and leaching, thermodegradation was carried out at 100°C for 1.5 min and 5 µl of glycerin was added. Ten to 20 µl of this mixture was loaded on the gel. SDS-PAGE was performed using a Marysol Microslab electrophresis device (Marysol, Tokyo, Japan) and a gel containing 10% acrylamide (1-mm thick) was prepared as described by Laemmli [3]. Electrophoresis was completed after about 2 h at a constant voltage of 125 V.

Electrophoretic blotting was performed using a Marysol Gel-Membrane blotting device (distance between electrodes, 5 cm). After the completion of electrophoresis, the gel was

immersed in the buffer for blotting which was prepared as described by Towbin et al. [7] for 5 to 10 min and placed on filter paper moistened with the same buffer. Nitrocellulose membranes that had been immersed in the same buffer were placed on the gel and further filter paper moistened with the same buffer was placed over the nitrocellulose membranes. Blotting for 0.5 h was most appropriate at a constant current of 200 mA.

EIA was carried out by the application of a Bio-Rad Immunoblot assay (Bio-Rad Laboratories, California, USA). After electrophoretic blotting, the nitrocellulose membranes were incubated with Seraclone anti-M and anti-N or Ortho anti-M and anti-N diluted 1:10 to 1:1,000 with sterile saline at room temperature (20°–30°C) for 2 h and then in a refrigerator (about 5°C) for about 12 h. Further incubation with Bio-Rad horseradish peroxidase-conjugated goat antimouse immunoglobulin G or Bio-Rad horseradish peroxidase-conjugated goat antirabbit immunoglobulin G diluted 1:1,000 with sterile saline was carried out at room temperature (20°–30°C) for 2 h. Finally, M antigen and N antigen were visualized by immersing the membranes in 0.05% 4-chloro-1-naphthol solution containing 0.015% H_2O_2.

Results and discussion

When polyclonal anti-M and anti-N were used in EIA, M antigen and N antigen bands appeared with relative clearness, and it was possible to determine MN phenotypes, although they showed many nonspecific binding reactions and their specificities were weak (Fig. 1). They showed bindings to M and N antigens on a nitrocellulose membrane even in 1:100 to 1:200 dilutions of the sera. In addition, it was quite possible to determine MN phenotypes from a small amount of blood stain equivalent to 3 µl of fluid blood (Fig. 2). M antigen and N antigen bands became weaker after 1 month and it became impossible to determine MN phenotypes after 2 months. It was considered that MN activities decrease relatively quickly at room temperature (20°–30°C). When blood stains were directly pretreated by sample buffer, nonspecific bands around M antigen and N antigen bands and tailing became more marked after a few days, and it became impossible to determine MN phenotypes. This was improved considerably by immersing blood stains in physiological saline in order to remove serum proteins etc. as much as possible.

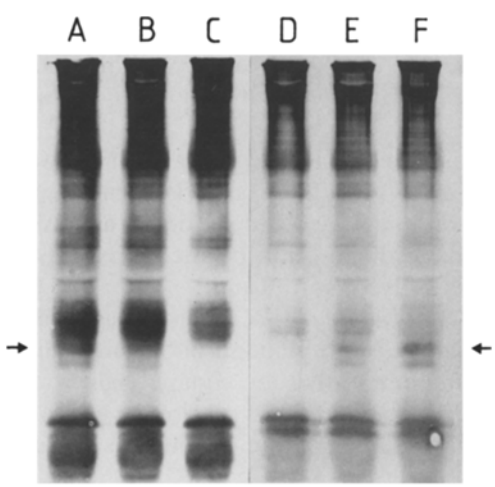

Fig. 1. EIA of MM, MN, and NN blood stains using polyclonal anti-M *(A, B, C)* and anti-N *(D, E, F)*. Samples: (A, D) MM blood stain, 7 days old; *(B, E)* MN blood stain, 7 days old; *(C, F)* NN blood stain, 7 days old. *Arrows* indicate M or N antigen

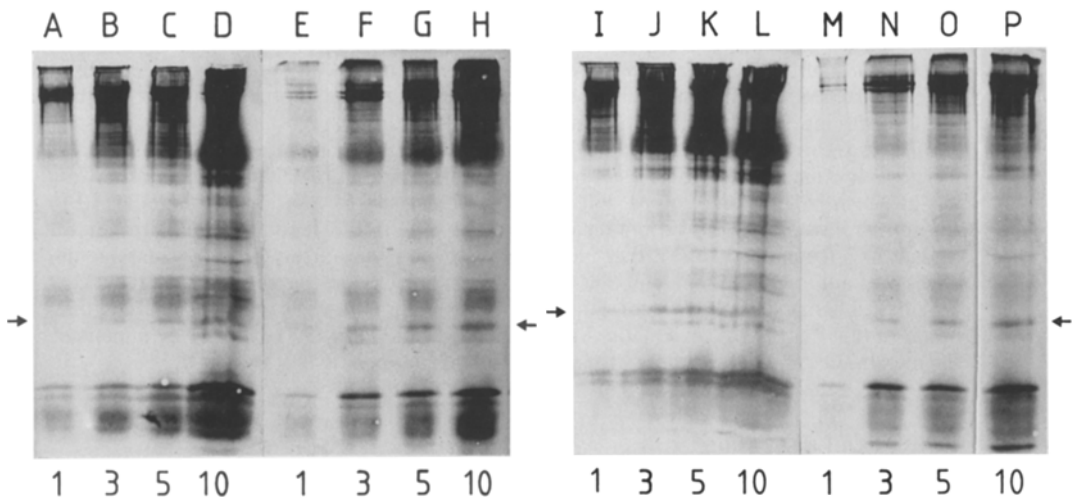

Fig. 2. EIA of MM, MN, and NN bloodstains using polyclonal anti-M *(A, B, C, D, E, F, G, H)* and anti-N *(I, J, K, L, M, N, O, P)*. Samples: *(A, B, C, D)* MM blood stain, 4 days old; *(E, F, G, H, M, N, O, P)* MN blood stain, 2 days old; *(I, J, K, L)* NN blood stain, 4 days old. *Arrows* indicate M or N antigen and each figure represents the amount of blood (unit, μl)

In our experiment, monoclonal anti-M and anti-N were highly specific to M antigen and N antigen on a nitrocellulose membrane, but showed the binding only in dilutions of 1:10 or less. This may be unsuitable as a routine method since large amounts of antisera are required.

The MN activities are known to be diminished by proteases [8]. The effects of soybean trypsin inhibitor and *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride as serine protease inhibitors, iodoacetamide as cysteine protease inhibitor, diazoacetyl-DL-norleucine methyl ester as aspartic protease inhibitor, and disodium ethylenediaminetetraacetate as iron protease inhibitor were investigated. The use of soybean trypsin inhibitor and a contrary effect on the MN activities and M antigen and N antigen bands disappeared. In the case of other protease inhibitors, however, no changes were observed in the appearance of M antigen and N antigen bands when they were used singly or in combination. It seems that the MN activities do not decrease after pretreatment of blood stains without protease inhibitors.

Incidentally, δ-glycophorin, which has N antigenic activity, can be separated from M and N antigens by SDS-PAGE [2, 6]. Thus, there is no risk of mistyping MM blood stains as MN in our method, although we could not detect a δ-glycophorin band because polyclonal anti-N showed many nonspecific bands and because monoclonal anti-N did not detect a δ-glycophorin band.

In conclusion, the use of monoclonal anti-M and anti-N seems to be unsuitable as a practical method because EIA requires large amounts of the antibodies. Our method, using polyclonal anti-M and anti-N that are commercially available, is considered useful in medicolegal practice since it is possible to use small amounts of antisera and blood stains. MN grouping of blood stains was possible for stains up to 1 month old.

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Received November 30, 1988