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Blood Grouping of Mixed Bloodstains Using Immunocytochemical Methods

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ABSTRACT: Immunocytochemical methods to determine the ABO blood group of each blood of mixed bloodstains have been developed. Mixed bloodstains were made on surgical blades and a cedar board. The blades were dipped into blood and then dipped into blood of a different group at intervals of 30, 20, 15, 10 and 5 s. Two drops of blood were dropped on a cedar board and then two drops of blood of a different group were dropped there at the same intervals. The bloodstains were dried for a week. The blood samples were removed from the blades or the cedar board and processed according with a routine histological method. Three serial thin sections were obtained. After deparaffinization, the sections were treated in papain solution for 2 h at 36°C, to unmask antigenic sites on red cell membranes. The labeled streptavidin-biotin (LSAB) and peroxidase-anti-peroxidase (PAP) methods were used to detect A and B antigens, and an indirect immunocytochemical method for H antigen. These immunocytochemical methods showed specific immunologic reactions and allowed determination of the blood group of each blood of mixed bloodstains. Further, these methods indicated a possibility to determine who was stabbed first, in cases where two or more victims were stabbed with a single knife.

KEYWORDS: forensic science, genetic markers, ABO system, blood group, blood stain, immunocytochemistry

ABO blood grouping of human bloodstains is an indispensable part of routine medicolegal examinations. For blood grouping of stains, various assay systems such as precipitation in liquid media or gels, absorption-elusion grouping, direct or indirect hemagglutination, latex agglutination, isoelectric focusing and enzyme-linked immunosorbent assay are used. However, if the bloodstains in question are mixed ones, those conventional assay systems detect summed-up blood groups of concomitantly present contaminants. Thus, it is impossible, in the case where the blood group of a bloodstain is AB, to determine whether it is genuinely AB or a mixed blood group of A and B. Recently ABO genotyping has been introduced as a reliable and sensitive method of providing blood group determination (1,2). But the method could not resolve the present problem. Thus it is desired to develop a method which can determine the blood group of each blood of a mixed bloodstain.

Brinkmann et al. (3) and Scheithauer and Hofmann (4) employed immunocytochemical methods for detection of cellular ABH antigen in mixtures of body fluids and proved their usefulness in blood grouping of mixed stains. But immunocytochemical methods have not been used widely for blood grouping of blood (5). The present paper describes immunocytochemical methods, the peroxidase-anti-peroxidase (PAP) and labeled streptavidin-biotin (LSAB) methods, for the detection of ABH antigens on the red cell in mixed bloodstains and discusses their advantages and disadvantages.

Materials and Methods

Materials

Group A, B, O and AB bloods were obtained from healthy volunteers.

The antibodies and products used in this study were as follows: bovine serum albumin (BSA) and 3,3′-diaminobenzidine 4HCl (DAB)(Nacalai, Japan), papain (Merck, Germany), monoclonal mouse anti-A and anti-B (Seraclone, Biotest, Germany), Ulex europaeus anti-H (EY, US), anti-Ulex-europaeus and a peroxidase-anti-peroxidase kit (ZYMED, USA), and an Universal DAKO LSAB 2 kit (peroxidase, DAKO, USA).

The following buffer solutions were prepared: 0.01 M phosphate buffer saline (PBS) (pH 7.2) as washing solution, 1/150 M phosphate buffer saline (pH 7.3) for papain solution and 0.05 M Tris-HCl buffer for DAB solution.

Methods

Two kinds of bloodstains were formed: bloodstains on surgical blades and those on a cedar board. A surgical blade was dipped into blood. After 30, 20, 15, 10 or 5 s, the blade was dipped into blood of a different blood group; e.g., a blade was dipped into group A blood and then dipped into group B blood. Two drops of blood were dropped on a cedar board and the same amount of blood of a different group was dropped on the former blood at the same intervals. At each interval, three sets of specimens were made. Those specimens were dried for a week at room temperature. Bloodstains of group A, B, O or AB blood were also made as controls.

The bloodstains were sampled according to the following procedure (Fig. 1) (see also Table 1 for the immunocytochemical procedure). First, the bloodstain on the surgical blade was covered with a paper tape. Then, the bloodstain was stripped from the blade with a new blade and transferred onto the paper tape. After that, the bloodstain's surface toward the original stained blade was covered with another paper tape of a different thickness. The samples

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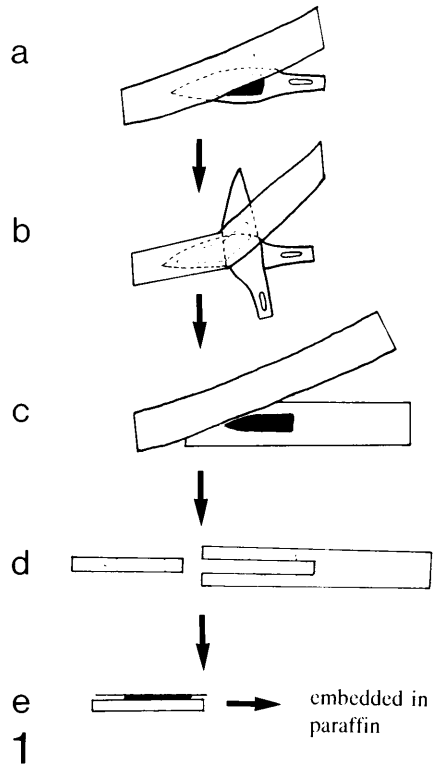

FIG. 1—*A schema of the procedure to obtain a bloodstain sample from a blade. (a) The bloodstain on a surgical blade was covered with a paper tape. (b) The bloodstain was stripped with a new blade and transferred onto the paper tape. (c) The bloodstain's surface toward the blade was covered with another paper tape of different thickness. (d) A bloodstain sample was obtained. (e) The sample was processed according with a routine procedure and embedded in paraffin.*

TABLE 1—*Procedure of immunocytochemical method for the detection of A and B antigens.*

1.	Deparaffinization
2.	Two washings in PBS
3.	Place in 1% papain solution for 2 h at 37°C
4.	Washing in distilled water
5.	Place in 10% H_2O_2/methanol for 30 min
6.	Washing in distilled water
7.	Three washings in PBS for 5 min each
8.	Apply 3% bovine serum albumin for 30 min
9.	Apply the primary antibody for 15 min Slide 1—anti-A antibody Slide 2—anti-B antibody
10.	Three washings in PBS for 5 min each
11.	Apply the linking agent for 15 min
12.	Three washings in PBS for 5 min each
13.	Apply peroxidase-anti-peroxidase for 15 min (Apply peroxidase conjugated streptavidin for 15 min)
14.	Three washings in PBS for 15 min each
15.	Place in DAB solution for 10 min

of the bloodstains on a cedar board were obtained in a similar manner. Then, bloodstain samples were processed according to a routine histological procedure and embedded in paraffin in order to obtain the whole layer of the samples (paper tape, bloodstain, paper tape). Three serial sections of 4 μm thickness were obtained and picked on silanized slides (DAKO, Japan).

Deparaffinization was performed by four changes of xylene and in the third change the slides were placed in fresh xylene for 1 h with gentle stirring. Then the slides were washed in the PBS and incubated in 1% papain (30 000 USP-U/mg) solution at 37°C for 2 h. After that, the slides were washed in distilled water and placed in 10% H_2O_2/methanol solution for 30 min to inhibit peroxidase-like activity of the hemoglobin. The slides were washed in distilled water and then in PBS, three times. A solution made in a 3% BSA-PBS mixture was dropped on the slides for 30 min to suppress nonspecific background staining. Without washing, the primary antibodies were applied for 15 min as follows: anti-A onto slide 1, anti-B onto slide 2 and Ulex europaeus anti-H onto slide 3. Anti-A, anti-B and anti-H were diluted 1:75, 1:40 and 1:100, respectively. After washing with ice-cold PBS three times for 5 min each, slides 1 and 2 were processed according to the technical manual of the PAP or LSAB kit and stained with DAB. Slide 3 was processed according to a usual indirect immunocytochemical method (6) and stained with DAB. Anti-Ulex europaeus was diluted 1:100.

Blood group was determined according to the staining reaction of the red cells to anti-A and anti-B antibodies. Group A blood showed positive reactions in slide 1 but not in slide 2. Group B blood showed positive reactions in slide 2 but not in slide 1. Group O blood showed a positive reaction in neither slide. Group AB blood showed positive reactions in both slides.

Results and Discussion

First observation using controls: Bloodstains of group A, B or O blood were tested to reveal whether immunoreactions were observed with the PAP and LSAB methods. Without papain treatment, the sections looked homogeneous without revealing clear cellular rims of the red cells and showed a positive reaction with neither method (Fig. 2). So, the proteolytic enzyme papain was used to unmask antigenic sites. After treatment of papain for 2 h at 36°C, cellular rims of the red cells became apparent and the PAP method showed positive reactions on the red cell membranes without apparent nonspecific background staining (Fig. 3). However, the sections treated with papain and stained with the LSAB method showed nonspecific background staining. If the duration of papain treatment was longer, the background staining became

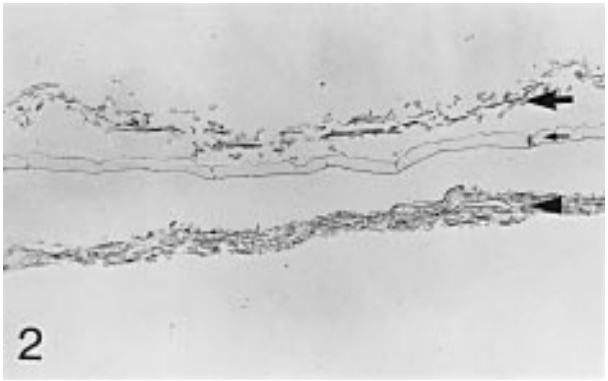

FIG. 2—*Result obtained from a control bloodstain sample made on a blade. The section was not treated with papain. No immunoreaction was observed. Arrow shows a paper tape covering the bloodstain first. Arrowhead shows another paper tape covering the bloodstain after stripping. Small arrow shows the bloodstain. Group A blood with anti-A. LSAB.* ×*35.*

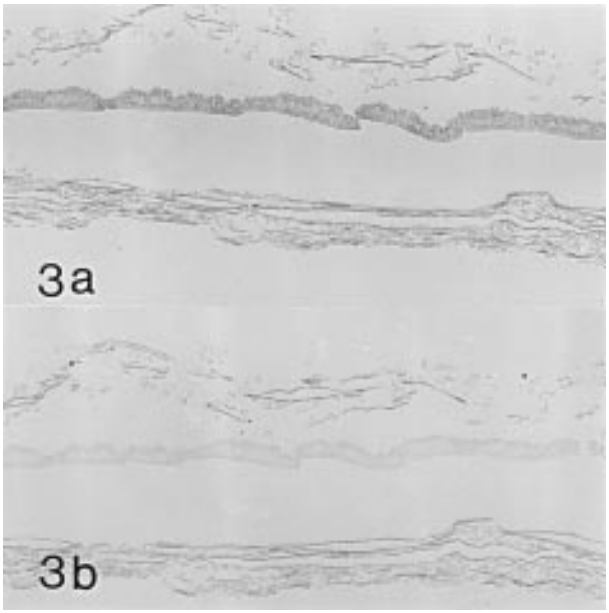

FIG. 3—*Results obtained from a control bloodstain sample made on a blade. The sections were treated with papain. Specific immunoreactions were observed. (*a*) Group A blood with anti-A. (*b*) Group A blood with anti-B. PAP. ×30.*

stronger and it was frequently difficult to determine exact blood group. Further observations revealed that the nonspecific background staining with the LSAB method was also observed in sections in which the primary antibody was avoided. But, if the linking agent was diluted 1:4–8, the papain-treated sections showed clear specific immunoreactions without any background staining (Fig. 4). These results suggest that long papain treatment allows the sections to obtain nonspecific background staining even in concentrations of antibodies in which the usual histological sections do not obtain background staining by the LSAB method. Thus, in the present procedure, concentrations of applied antibodies, especially that of the linking agent, should be checked carefully using control sections.

In the present study, Ulex europaeus was used for the detection of H antigen. The purpose to detect H antigen is to confirm whether papain treatment is enough. If the treatment was not enough, foci without positive reactions were observed. One may consider that the strengths of positive reactions of group O red cells, group A or B red cells, and group AB red cells to anti-H decrease in this order and the strength of a reaction to anti-H would help to determine blood group, especially group O. In the present study, however, blood grouping could not be determined based on the strength of reaction to anti-H. The strength of group O red cells to anti-H was similar to that of group A and B red cells and the strengths of immunocytochemical reactions were not constant. These results indicate that it is unreliable to determine blood group based on strengths of reactions to anti-H. Exact blood grouping should be made based on reactions to anti-A and B antibodies.

Immunocytochemical observations of mixed bloodstains on surgical blades and a cedar board were carried out using three serial sections. The immunocytochemical methods revealed specific positive reactions on the red cell membranes and could determine the blood group of each blood of mixed bloodstains in all samples (Figs. 5 and 6). Blood grouping of mixed stains is an important forensic subject. Some investigators have developed methods of blood grouping of mixed body fluids or contaminating bloodstains (7,8). However, no reliable method has been developed to determine the blood group of each blood of mixed bloodstains. The present method provides a practical procedure for ABO blood grouping of each blood of mixed bloodstains.

In the present study, mixed bloodstains made experimentally on the blades at intervals of 30 and 20 s showed apparent layered structures (Figs. 5 and 6) and it was possible to determine which group of blood adhered first. In those made at an interval of 15 s, layered structures were also observed, but some small groups of red cells adherent first on blades were seen in the opposite layer. In those made at intervals of 10 and 5 s, layered structures became obscure and in some specimens it was impossible to determine which group of blood adhered first. Mixed bloodstains made on the cedar board at each interval showed layered structures and it was possible to determine which group of blood adhered first, although in specimens made at intervals of 15, 10 and 5 s, some small groups of red cells adherent first on the board were seen in the opposite layer (Fig. 7). This result suggests another advantage of this method. In some instances, two or more victims would be stabbed by a single knife and it would become necessary to define who was stabbed first. In other instances, two or more individuals

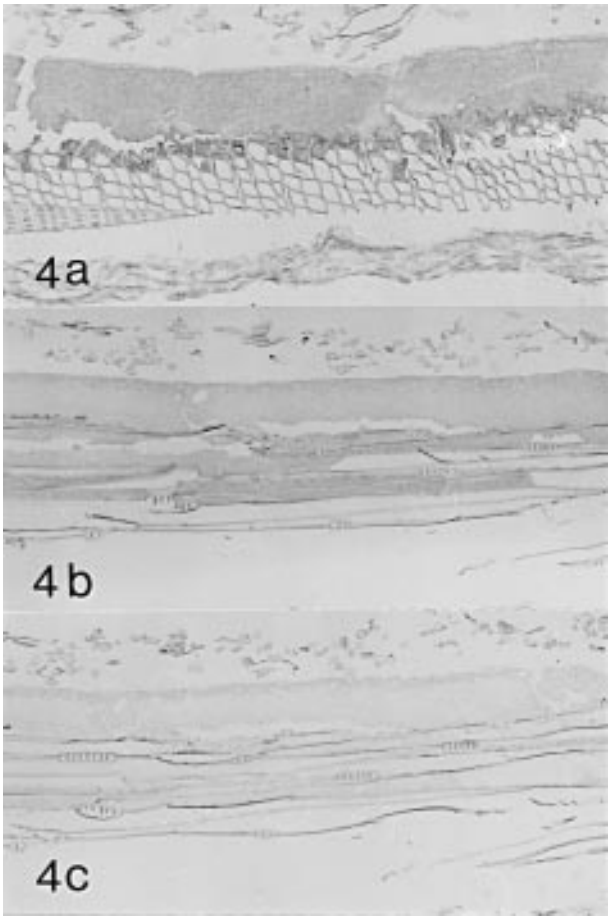

FIG. 4—*Results obtained from control bloodstain samples made on a cedar board. The sections were treated with papain. (*a*) The linking agent was not diluted. Strong background staining was observed. Group O blood with anti-A. (*b*) The linking agent was diluted 1:8. Specific immunoreactions were observed. Group A blood with anti-A. (*c*) The linking agent was diluted 1:8. No background staining was observed. Group A blood with anti-B. LSAB. ×45.*

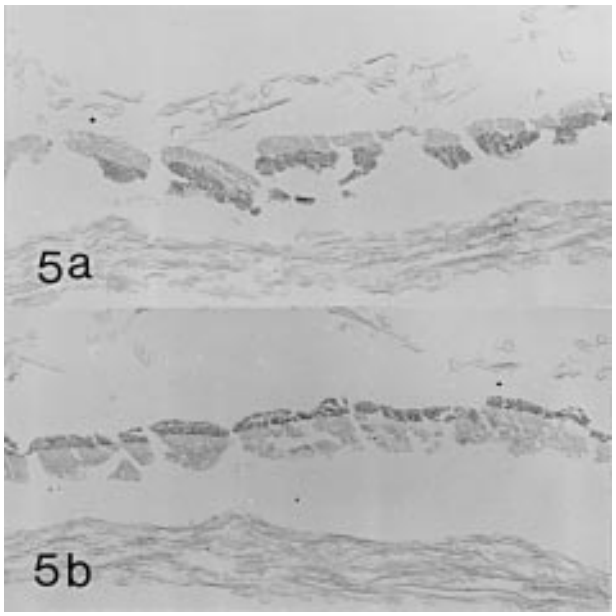

FIG. 5—*Results obtained from a mixed bloodstain made on a blade which was first dipped into group A blood and then into group B blood 20 s later. (*a*) Result of anti-A. A clear layered structure was observed and the layer toward the blade showed a positive reaction. (*b*) Result of anti-B. The superficial layer of the stain showed a positive reaction. PAP. ×85.*

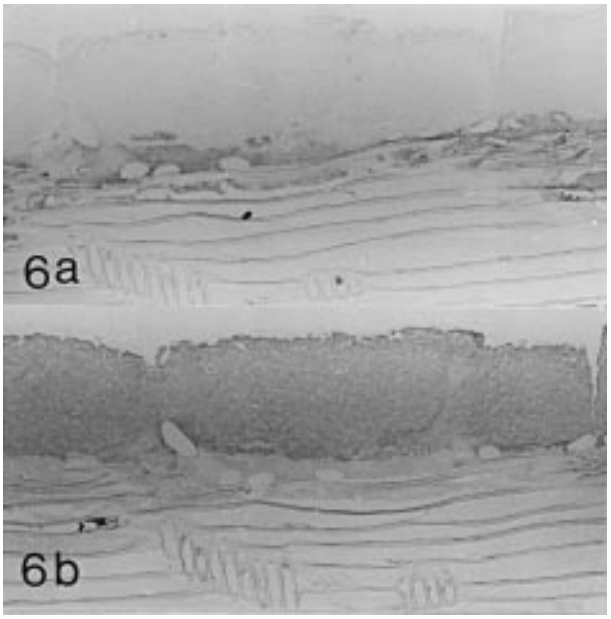

FIG. 6—*Results obtained from a mixed bloodstain made on a cedar board on which group A blood was first dropped and then group B blood was dropped 20 s later. (*a*) Result of anti-A. A clear layered structure was observed and the layer toward the board showed a positive reaction. (*b*) Result of anti-B. The superficial layer of the stain showed a positive reaction. PAP. ×85.*

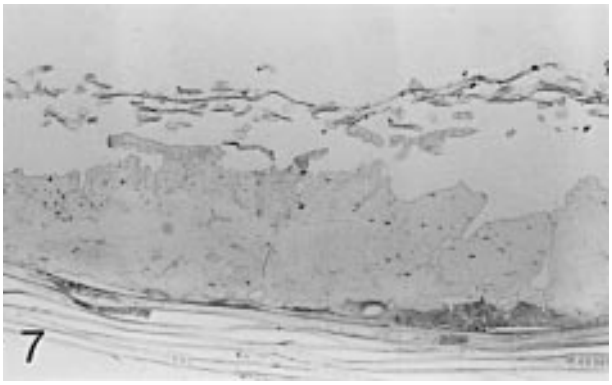

FIG. 7—*Results obtained from a mixed bloodstain made on a cedar board on which group A blood was first dropped and then group O blood was dropped 10 s later. A clear layered structure was observed and the layer toward the board showed a positive reaction, but small groups of group A red cells were also observed in the opposite layer. PAP. ×85.*

would be injured and it would become necessary to determine whether bloodstains left at the scene were a mixed bloodstain or not, or whose blood adhered first to the walls or furniture. The previous methods have failed to solve this question. In the present study, except for the mixed bloodstains made on the blades at an interval of 10 or 5 s, most specimens showed clear layered structures and it was possible to determine which blood adhered first. This indicates a possibility that the present method provides a practical procedure to answer these questions in an examination of a mixed bloodstain.

Case Presentation

A male schizophrenic killed his father and mother by stabbing with a pair of scissors and a cooking knife. His father was stabbed seven times by the scissors and four times by the knife. His mother was stabbed five times by the scissors and more than three times by the knife. His father's and mother's blood groups were groups O and B, respectively, and the results of absorption-elution tests of the bloodstains on these materials were group B. A public procurator attempted to interview him. His illness, however, was so severe that the procurator failed to obtain any understandable account of the event. So, the scissors and the knife were sent to us to determine whether the bloodstains on these materials were mixed ones or not and which group of blood adhered first on those. On the scissors and the knife, only small and thin bloodstains scattered. Three bloodstain samples, two from the knife and one from the scissors, were obtained and processed according to the above-mentioned method, and papain-treated sections were stained with the PAP method. A microscopic observation revealed that all bloodstain samples obtained were composed of group B red cells and no group O red cells was observed (Fig. 8). From these results, we considered that first the schizophrenic stabbed his father and then stabbed his mother. The father's group O red cells which adhered first on the scissors and the knife were probably wiped off when he stabbed his mother.

In the present method, two kinds of paper tapes of different thickness were used to cover the bloodstains. In microscopic observation, the difference of the thickness of the paper tapes becomes important in determining which surface of the bloodstain is toward the blade (Fig. 1). A cellophane tape could not be used in place of a paper tape because thin sections usually tore between the

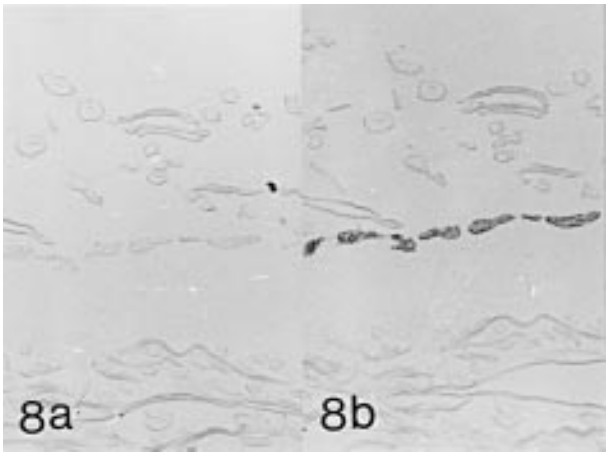

FIG. 8—*Results obtained from an evidence bloodstain sample on the knife. All red cells showed positive reactions with anti-B but not with anti-A. (*a*) Result of anti-A. (*b*) Result of anti-B. PAP. ×140.*

cellophane tape and the bloodstain. When a section with a tear was floated on water, small pieces of bloodstains frequently turned around and it became difficult to determine which surface of the bloodstain was toward the blade.

In the present study, inhibition of papain was observed in the bloodstain's surface layers toward the paper tapes. The surface layers looked homogeneous and no immunoreaction was obtained. The inhibition disappeared when the slides were placed in xylene for 1 h with gently stirring and the glue was dissolved into xylene. The inhibition of papain with glue should be checked carefully using control sections.

It should be stressed that this method has some disadvantages. Compared with previous methods, this method needs more time and is more expensive. The concentrations of applied antibodies should be checked carefully using control sections. Moreover, much skill is needed to obtain good serial thin sections. These disadvantages may suggest that the present method cannot be used as a routine procedure in forensic practice. The method, however, has excellent advantages and can be considered to be useful for forensic examinations of mixed bloodstains.

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