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ABO blood grouping of bloodstains by sandwich ELISA using monoclonal antibody specific for human red cell band 3

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Summary. ABO blood grouping of human bloodstains was performed by a sandwich ELISA using a species-specific monoclonal antibody to the amino-terminal cytoplasmic domain of human red cell membrane band 3. In a blind trial, all A, B and O bloodstains (a 1 cm long thread) and AB bloodstains (a 1.5 cm long thread) were accurately typed by this method. Even when bloodstains were contaminated by other body fluids (e.g., semen and saliva), only the ABO blood group epitopes on band 3 of the red cell membrane were detected. Thus, identification of human blood and ABO blood grouping of bloodstains which were contaminated by other body fluids could be simultaneously performed by this method.

Key words: ABO blood grouping – Contaminated bloodstains – Sandwich ELISA – Monoclonal antibody – Red cell membrane band 3

Zusammenfassung. Die ABO-Blutgruppenbestimmung an menschlichen Blutspuren wurde mit Hilfe einer ELISA-Sandwichtechnik durchgeführt, indem ein Species-spezifischer monoklonaler Antikörper gegen die aminoterminale zytoplasmatische Domäne der menschlichen Erythrozytenmembran-Bande 3 benutzt wurde. In einem Blindversuch wurden alle A-, B- und O-Blutspuren (ein 1 cm langer Faden) und AB-Blutspuren (ein 1,5 cm langer Faden) mit dieser Methode richtig bestimmt. Auch wenn Blutspuren mit anderen Körperflüssigkeiten kontaminiert waren (z. B. Sperma, Speichel) wurden lediglich die ABO-Blutgruppen-Epitope auf der Bande 3 der Erythrozytenmembran nachgewiesen. Auf diese Weise konnte die Identifikation menschlichen Blutes und die Blutgruppenbestimmung von Blutspuren, welche mit anderen Körperflüssigkeiten kontaminiert waren, simultan mit dieser Methode durchgeführt werden.

Schlüsselwörter: ABO-Blutgruppenbestimmung – Kontaminierte Blutspuren – Sandwich ELISA – Monoklonale Antikörper – Erythrozytenmembran-Bande 3

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Introduction

Species identification and ABO blood grouping of bloodstains constitute the main subjects in medicolegal practices. The methods used for species identification and ABO blood grouping, for example, immunodiffusion with anti-human hemoglobin antibody and absorption-elution test using ABO blood group antisera, are well established and highly reliable. However, methods of distinguishing between species, especially between human and other primates, and of ABO blood grouping of bloodstains contaminated by other body fluids are not yet satisfactory. In our study of species-specific epitopes on human red cell membrane, we produced monoclonal antibodies (mAbs) to species-specific epitopes on human red cell membrane band 3. One (P3-9H) of these mAbs could even discriminate human red cells from those of chimpanzees [1]. Since red cell membrane band 3 carries ABO blood group active carbohydrate chains, P3-9H may be applicable not only to human blood identification but also to ABO blood grouping.

In this paper, we describe the applications of P3-9H for ABO blood grouping from bloodstains contaminated by other body fluids.

Materials and methods

1) Antibodies. Anti-human red cell membrane band 3 mAb (P3-9H) was produced using human red cell membranes as an immunogen as described previously [1]. The anti-saliva blood group substance mAb (P4-5C) and the anti-seminal blood group substance mAb (P6-5H) have been described previously [3, 4]. Anti-A and anti-B mAbs were obtained from Ortho Diagnostics (N.J, USA) and anti-H mAb from CHEMBIOMED (Ab, CANADA). P6-2B (anti-H mAb) was produced using α2-seminoglycoprotein as the immunogen (A. Kimura, unpublished results). 1E3 (anti-H mAb) produced using blood group O secretor saliva as immunogen [2], was a gift from Dr. K. Furukawa (Department of Legal Medicine, School of Medicine, Gunma University). Anti-H antibody obtained from chicken yolks was a gift from Dr. I. Ohya (De-

partment of Legal Medicine, Gifu University School of Medicine). Peroxidase-conjugated goat anti-mouse IgG and anti-mouse IgM antibodies were purchased from CALTAG Lab. (CA, USA). Peroxidase-conjugated rabbit anti-chicken IgG antibody was obtained from Zymed Lab. Inc (CA, USA).

2) Buffers. The constitutents of dilution, washing, blocking buffers and phosphate buffered saline (PBS) were as described previously [3]. Extraction buffer was PBS containing 1% Triton X-100 and 1 m*M* EDTA.

3) Specimens. Bloodstains were obtained by dropping whole blood onto cotton gauze, drying and storing in air. Contaminated bloodstains were made by overlaying body fluids onto the bloodstains.

4) Extraction of band 3 from specimens. Band 3 was extracted by immersing threads of specimens in extraction buffer for 30 min. In some experiments extraction was carried out by sonication with BRANSONIC 220 (Branson, Conn, USA) for 1 min in extraction buffer and standing at room temperature for 30 min.

5) Sandwich ELISA. Wells of flat bottom plastic plates (Nunc, IL, USA) were filled with 50 µl of P3-9H (10 µg/ml in PBS) and incubated overnight at 4°C. The mAb coated plate was washed three times with PBS and blocked with blocking buffer. The plate was stored at 4°C and washed twice with washing buffer before use. The extracts from specimens (50 µl) were added to wells and incubated for 1 hour at room temperature. The plate was washed five times with washing buffer and 50 µl of anti-A, anti-B mAbs (× 10 dil) or anti-H antibody (chicken, × 3,000 dil) was added. After 1 hour incubation at room temperature, the plate was washed five times with washing buffer and incubated with 50 µl of peroxidase-conjugated anti-mouse IgM (× 1,000 dil.) or peroxidase-conjugated anti-chicken IgG (× 2,000 dil.) for 1 hour at room temperature. Color was developed with o-phenylenediamine. Absorbance at 492 nm was measured with the EIA reader after adding 2 *N* H_2SO_4 (50 µl) to each well.

The Sandwich ELISA for ABO blood grouping of saliva and semen has been described previously [3, 4].

6) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-PAGE was performed by the method of Laemmli [5] and immunoblotting was performed as described by Towbin et al [6]. Colloidal gold staining was performed with Auro dye forte (Amersham International plc, Bucks, UK) according to the supplier's instructions.

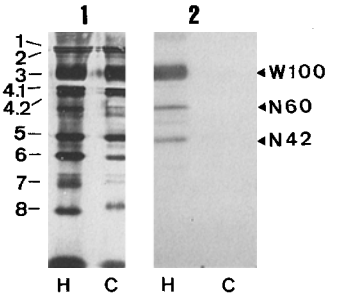

Fig. 1. Immunoblotting of the antigen defined by P3-9H. Proteins in erythrocyte red cell membranes from humans and chimpanzees were separated by SDS-PAGE (10% acrylamide gel) and transferred onto polyvinylidene difluoride membranes. The blots were stained with colloidal gold (panel 1) or P3-9H (panel 2). H = human red cells; C = chimpanzee red cell membranes; W100 = whole band 3; N60 and N42 = 60 and 42 kDa amino-terminal fragments of band 3, respectively. Nomenclature for human red cell membrane proteins is according to Steck [13]

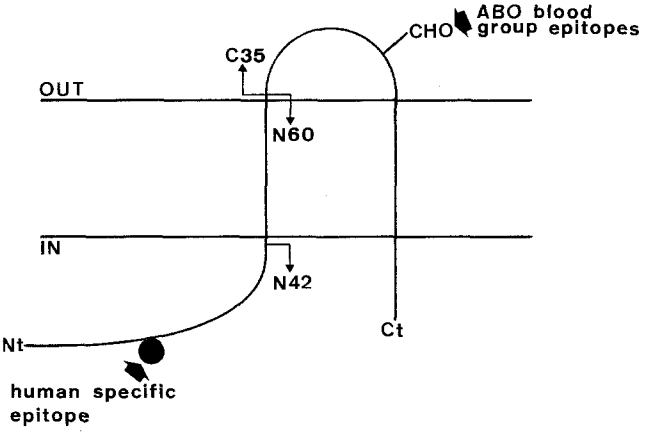

Fig. 2. Topological model of human specific epitope and ABO blood group epitopes on band 3. CHO = carbohydrate chain; Nt = amino-terminal; Ct = carboxyl-terminal; OUT = outside the membrane; IN = inside the membrane; C35 = 35 kDa carboxyl-terminal fragment; N42 and N60 = 42 and 60 kDa amino-terminal fragments

Results and discussion

Anti-human band 3 mAb P3-9H (IgG1) bound to whole band 3 and to its 60 and 42 kDa amino-terminal (N-60 and N-42) fragments in the human red cell membrane but not to those of chimpanzee (Fig. 1), indicating that the epitope defined by P3-9H was located on the N-42 fragment and that P3-9H could discriminate human blood from that of chimpanzee [1]. P3-9H did not crossreact with band 3 of other primates (spider monkey, capuchin monkey, Rhesus monkey or Japanese monkey) or other mammals (dog, cat, cow, pig and rabbit).

Band 3 is a major component of the red cell membrane and is a transmembrane glycoprotein which acts as an anion exchanger [7] and plays a key role in the stability of the membrane [8]. Approximately 25% of the ABO blood group determinants in red cell membranes are distributed on the sugar chain which links to an asparagine residue on an exoplasmic domain of band 3 [9, 10]. Since the human-specific epitope and ABO blood group epitopes are located apart from each other on the band 3 molecule, the binding of the antibody to the appropriate epitope is not disturbed by the binding of the other antibody (Fig. 2). Therefore, it appeared appropriate to use P3-9H as a capture antibody in a sandwich ELISA for ABO blood grouping of bloodstains. Sakata at al. [11] reported a similar sandwich method for ABO blood grouping using a mAb which recognized the exoplasmic domain of human red cell band 3 and crossreacted with only some primates but not to other mammals. When P3-9H was used as a capture antibody in a sandwich ELISA for ABO blood grouping, A and B blood group epitopes were detected in extracts from minute bloodstains (at least 1 cm long) by anti-A and B mAbs (Fig. 3a). On the other hand, H-epitopes were undetectable by anti-H mAbs (Chembiomed, P6-2B, and 1E3) which reacted to the type II chain despite the fact that the structure of

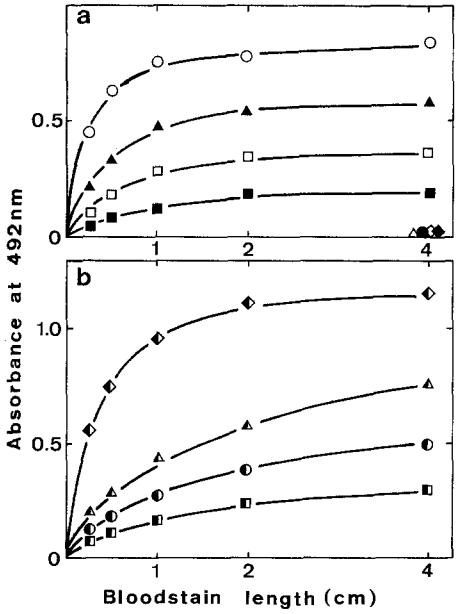

Fig. 3a, b. The limits of detectable ABO blood group from bloodstains by sandwich ELISA using P3-9H. ABO blood group antigens in extracts from bloodstained threads (1~4 cm long) were detected by sandwich ELISA on P3-9H coated plates. Procedures are described under Materials and methods. ○, A epitope of A bloodstain; ●, B epitope of A bloodstain; △, A epitope of B bloodstain; ▲, B epitope of B bloodstain; □, A epitope of AB bloodstain; ■, B epitope of AB bloodstain; ◇, A epitope of 0 bloodstain; ◆, B epitope of 0 bloodstain; ◐, H epitope of A bloodstain; ◭, H epitope of B bloodstain; ◧, H epitope of AB bloodstain; ◆, H epitope of 0 bloodstain. Data represent the mean values of duplicate experiments

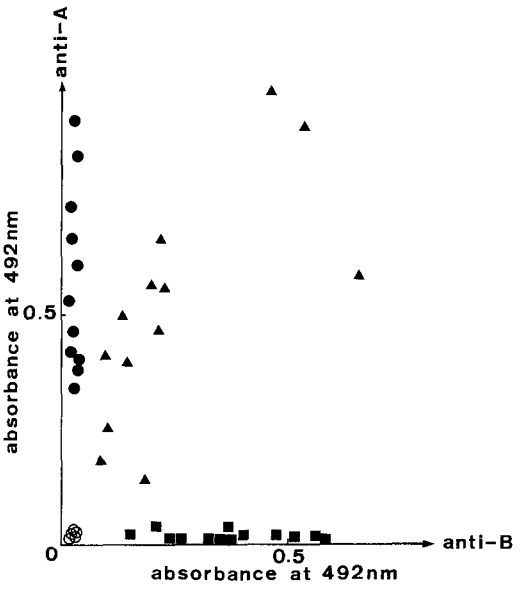

Fig. 4. Detection of ABO blood group antigens from bloodstains by sandwich ELISA using P3-9H. Extracts from bloodstained threads (1 cm long) were assayed. Procedures are described under Materials and methods. ●, A bloodstains; ■, B bloodstains; ▲, AB bloodstains; ○, 0 bloodstains. Data represent the mean value of duplicate experiments

Table 1. Blind trials of ABO blood grouping of bloodstains by a sandwich ELISA using P3-P9

Bloodstains	Number	Correct
A	16	16
B	21	21
O	19	19
AB	13	11[a]

[a] Two specimens were typed as blood group A

ABO blood group active sugar chains on band 3 are type II, as described by Tsuji et al [10]. The reason why the anti-H mAbs did not react with H-epitopes on band 3 is unknown. However H-epitopes on band 3 could be detected with chicken anti-H antibody obtained from yolks of chickens immunized with blood group 0 human red cells (I. Ohya, unpublished results) (Fig. 3b). This antibody was suitable for detecting H-epitopes by ELISA, because it was highly sensitive but the background was low. The plateau levels of each blood group in Fig. 3 indicated that more H-epitopes on band 3 were converted to A-epitopes in A and AB blood groups than to B-epitopes in B and AB blood groups. This fact and the results of examining a number of bloodstains ($n = 54$) by this method showed that B-epitopes tend to be detected at a lower rate than A-epitopes (Fig. 4), suggesting the possibility that AB bloodstains could be typed as A in minute bloodstains. All bloodstains of A, B and 0 were correctly typed, however, 2 out of 13 AB bloodstains (1 cm long) were typed as group A in the blind trial (Table 1). These two incorrectly typed specimens could be accurately typed by increasing the amount of bloodstains (1.5 cm long). The sensitivity of this method seemed to be not so high, since only 25% of ABO blood group determinants on red cell membranes were detected by this method, however, it seems to be sufficient for practical use. Even when bloodstains were contaminated by other body fluids (e.g., saliva and semen), only the ABO blood group epitopes of red cells were detected by this method without interference from other body fluids. As shown in Table 2, the ABO blood group of red cells, saliva and semen in contaminated bloodstains was detected selectively from the same extract by sandwich ELISA using P3-9H, P4-5C (mAb to saliva ABO blood group substance) [3] and P6-5H (mAb to seminal ABO blood group substance) [4] as capture antibodies, respectively.

Since the extraction of band 3 from bloodstains is indispensable for this method, the age of the bloodstains seriously affects the sensitivity of this method. The limits of detection of ABO blood groups from aged bloodstains depended on the storage conditions of the stains. However, ABO blood grouping could be performed after several months. Although, ABO blood group epitopes were not detected by this method from aged bloodstains from which the hemoglobin was not extracted, when band 3 was extracted by sonication, ABO blood grouping could be performed (Table 3). This effect was also found in extraction of band 3 from freshly prepared bloodstains.

Table 2. Differential ABO blood grouping of bloodstains contaminated by other body fluids

Specimens	P3-9H		P4-5C		P6-5H	
	A	B	A	B	A	B
A Se semen + B blood	0.055	0.611	0.016	0.000	0.544	0.000
B Se semen + A blood	0.908	0.075	0.000	0.001	0.000	0.361
B Se saliva + A blood	0.525	0.042	0.024	0.939	0.007	0.005

Table 3. Effects of sonication on ABO blood grouping of aged bloodstains

Specimens	Without sonication		With sonication	
	A	B	A	B
3 months A blood	0.395	0.052	0.649	0.045
B blood	0.023	0.130	0.036	0.337
6 months A blood	0.190	0.054	1.108	0.070
B blood	0.092	0.083	0.042	0.285
10 months B blood	0.138	0.149	0.067	0.277

Yoshiuchi et al. [12] reported a method for ABO blood grouping of blood from contaminated bloodstains. They differentially extracted ABO blood group active glycolipids from red cell membranes with chloroform-methanol. This method was based upon differences in the chemical nature of ABO blood group substances between red cell membranes and body fluids. On the other hand, our method is based on immunological differences although we used the same principle as described previously for ABO blood group grouping of a specific body fluid from mixture of body fluids [3, 4].

Since the peptide backbone of band 3, on which the species-specific epitope defined by P3-9H exists, is more unstable than that of ABO blood group determinants, our method may not be applicable to bloodstains that have been stored for long periods (over several years), or are in bad condition, therefore, our method should be applied carefully.

This method provides a practical procedure for ABO blood grouping of blood from bloodstains contaminated by other body fluids. Furthermore, since P3-9H is specific for human red cell membrane band 3, it is evident that any ABO blood group epitopes detected using the present method will be of human blood. Therefore, human blood identification and ABO blood grouping of bloodstains could be performed simultaneously using the present method.

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