Int J Leg Med (1991) 104:255–258

International Journal of
Legal Medicine


# ABO blood grouping of semen from mixed body fluids with monoclonal antibody to tissue-specific epitopes on seminal ABO blood group substance*

**Akihiko Kimura[1], Fuseo Matsumura[2], Kenichiro Sodesaki[2], Motoki Osawa[1], Haruhiko Ikeda[1], Seiji Yasuda[1], and Tsutomu Tsuji[1]**

[1]Department of Legal Medicine, Wakayama Medical College, 27 Kyuban-chou, 640 Wakayama, Japan
[2]Department of Forensic Science, Saga medical School, Saga, Japan

Received April 8, 1991 / Received in revised form July 15, 1991

**Summary.** Practical methods were developed for ABO blood grouping of semen from mixed body fluids. An monoclonal antibody (P6-5H) which recognizes a tissue-specific epitope on a seminal ABO blood group substance ($\alpha_2$-seminoglycoprotein) was used as a solid phase antibody for selective capture of the seminal ABO blood group substance. ABO blood group epitopes of secretor and non-secretor semen were detected in dilutions of $8 \times 10^3$–$3.2 \times 10^4$ and $8 \times 10^3$-fold, respectively, by sandwich ELISA. ABO blood group epitopes were also detected in dilutions up to $4 \times 10^3$-fold, irrespective of secretor status, by the sandwich absorption-elution test.

**Key words:** Monoclonal antibody – ABO blood grouping – Semen

**Zusammenfassung.** Eine praktikable Methode wurde entwickelt für die ABO-Bestimmung an Samen bei Vorliegen von gemischten Körperflüssigkeiten. Ein monoklonaler Antikörper (P6-5H), welcher ein gewebsspezifisches Epitop von Spermaflüssigkeits-ABO-Blutgruppensubstanz ($\alpha_2$-Seminoglycoprotein) erkennt, wurde als Fest-Phasen-Antikörper eingesetzt zur selektiven Bindung der ABO-Blutgruppensubstanz aus Samen. ABO-Blutgruppenepitope von Sekretoren und Nichtsekretoren wurde bis zu Verdünnungen $8 \times 10^3$ bis $3{,}2 \times 10^4$-fach bzw. $8 \times 10^3$-fach nachgewiesen mit Hilfe der Sandwich-ELISA-Technik. ABO-Blutgruppen-Epitope wurden ebenfalls nachgewiesen bis zu Verdünnungen von $4 \times 10^3$-fach, unabhängig vom Sekretorstatus mit Hilfe des Sandwich-Absorptions-Elutionstestes.

**Schlüsselwörter:** Monoklonale Antikörper – ABO-Blutgruppenbestimmung – Samenflüssigkeit

* This work was supported by Grant-Aid for Encouragement of Young Scientist 62770406, from the Ministry of Education, Science and Culture

*Offprint requests to:* A. Kimura

## Introduction

Although DNA fingerprinting has brought about innovations in the identification of individuals, conventional ABO blood grouping is still a very important procedure for individual identification in criminal investigations. ABO blood grouping is well established, however, procedures for the ABO blood grouping of each constituent body fluid in mixed body fluids remain to be developed. In particular, the ABO blood grouping of semen mixed with vaginal secretion is extremely important in the investigation of sexual assaults.

Recently, we developed methods for ABO blood grouping of saliva from mixed body fluids by sandwich methods using monoclonal antibodies (mAbs) to a tissue-specific epitope (TSE) on the core protein of an ABO blood group substance (BGS) in saliva [1, 2]. If seminal ABO-BGS also carries TSE on its core protein, ABO blood grouping of semen from mixed body fluids can be performed by sandwich methods using mAb to TSE of seminal ABO-BGS. In this report, we describe the production of mAb to TSE of seminal ABO-BGS and the application of this mAb to sandwich methods for ABO blood grouping of semen from mixed body fluids.

## Materials and methods

*Production of anti-seminal ABO-BGS mAb.* Seminal plasma (B Se) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [3] and the ABO blood group active fraction was eluted electrophoretically from the stacking gel. The ABO blood group epitope in the eluate was detected to a $10^4$-fold dilution by ELISA and this eluate was used as the immunogen. Immunization of mice with immunogen, fusion of mice lymph nodes cells with myeloma cells (P3U1) and establishment of antibody-producing hybridomas were performed essentially as described in the previous paper [1].

*Buffers.* Compositions of buffers used in this study were described in the previous paper [1].

*Enzyme-linked immunosorbent assay (ELISA).* ELISA was performed as described in the previous paper [1], except that biotin-conjugated F(ab')$_2$ fraction of sheep anti-mouse IgG(X2, 500 dil.; Cappel, Pa, USA) was used as secondary antibody.

*Immunoblotting.* Immunoblotting was carried out by separating the proteins on a SDS-polyacrylamide gel and electroblotting on to a PVDF membrane (Millipore, Ma, USA) as described by Towbin et al. [4]. The membrane was incubated with anti-seminal ABO-BGS mAb P6-5H (2 μg/ml) over night at room temperature and the antigen-antibody complexes were identified with biotin conjugated F(ab')$_2$ of sheep anti-mouse IgG (X2,500 dil.) and avidin-HRP (X5,000 dil.). 4-Chloro-1-naphthol was used as substrate for color development.

*Sandwich ELISA.* Preparation of P6-5H coated plate for sandwich ELISA was performed in the same way as described in the previous paper [2], except that purified P6-5H solution (2 μg/ml) was used for coating the plate. Specimens of mixed body fluids (50 μl) were added to the wells and incubated for 1 h at 37°C. The plate was then washed and to each well 50 μl of monoclonal anti-A, anti-B (X10 dil.; Ortho diagnostics, N.J., USA) or anti-H(X10 dil.; CHEMBIOMED, Alb, Cad) antibody was added. After 1 h incubation at room temperature, the plate was washed five times with washing buffer and incubated with 50 μl of HRP conjugate anti-mouse IgM (X1,000 dil.; CALTAG Laboratories, CA, USA) for 1 h at room temperature. Color development of the enzyme substrate reaction was measured as for the ELISA procedure.

*Sandwich absorption-elution test.* Preparation of P6-5H coated plate and incubation with specimens were performed as for sandwich ELISA. The absorption-elution test on the plate was carried out as described previously [2].

## Results

### *Production and properties of mAb to TSE on seminal BGS*

Hybridomas were obtained by fusion of myeloma P3U1 and lymph node cells from mice immunized with seminal BGS. Culture supernatants were screened by ELISA on the plate coated with seminal BGS(B Se). Culture supernatants of the positive wells were re-screened on the plate coated with saliva BGS(B Se) to discriminate the semen-specific clones from the cross-reactive and blood group specific clones. Of the many hybridomas producing antibodies, several produced antibodies which bound only to seminal BGS. One of these hybridomas (P6-5H) was highly specific for seminal BGS but not specific for ABO blood groups.

Although the amounts of seminal BGS varied between individual, they were detected to a dilution of at least 60,000 by ELISA with P6-5H, irrespective of ABO blood group and secretor status (Fig. 1).

Figure 2 shows that P6-5H bound not only to the immunogen but also to $\alpha_2$-SGP which carries the ABO blood group epitope [5]. Furthermore, high molecular weight glycoprotein which corresponded to $\alpha_2$-SGP, was stained with P6-5H on the blot of seminal plasma proteins (Fig. 3). These results suggest that the electrophoretically purified ABO-BGS for immunogen was $\alpha_2$-SGP.

The tissue specificity of P6-5H was investigated by immunoblotting (Fig. 4). Body fluids (saliva and vaginal

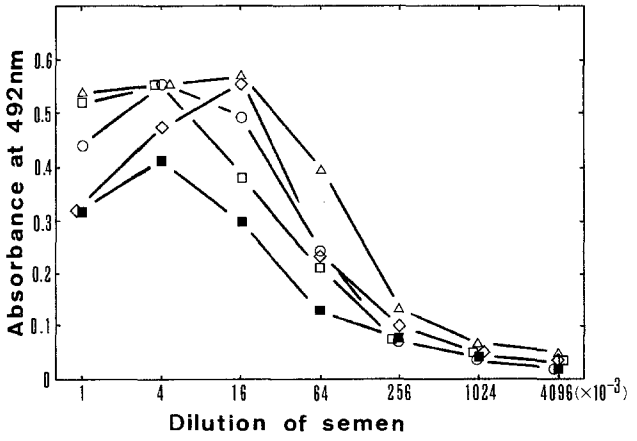


**Fig. 1.** Detection of semen by ELISA with P6-5H. Serial dilutions of semen on plate were used as the solid phase antigen. P6-5H (2 μg/ml) was used as primary antibody. ELISA was performed as described in Materials and Methods. ○: A Se, △: B Se, □: O Se, ■: O Se, ◇: AB Se

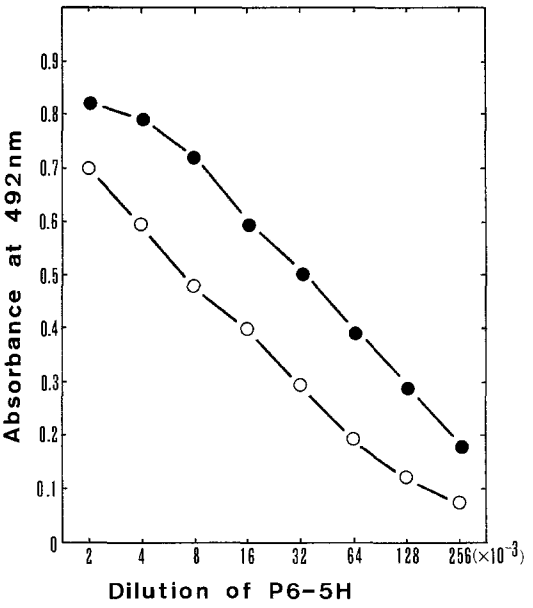


**Fig. 2.** Binding properties of P6-5H to immunogen and $\alpha_2$-SGP. Immunogen (ca. 10 μg/ml) and $\alpha_2$-SGP (10 μg/ml) were used as the solid phase antigen. Serial double dilutions of P6-5H (ascites) were used as primary antibody. ELISA was performed as described in Materials and Methods. ●: immunogen, ○: $\alpha_2$-SGP

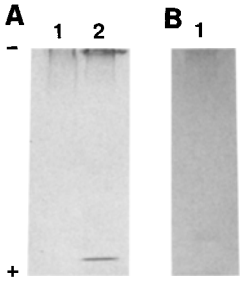


**Fig. 3A, B.** Identification of the seminal component defined by P6-5H. Semen and $\alpha_2$-SGP were separated by SDS-PAGE (5% acrylamide gel) and stained with periodic acid-schiff (PAS) **A**. Seminal components separated by SDS-PAGE were transfered to PVDF membrane and the blot was stained with P6-5H **B**. 1: $\alpha_2$-SGP, 2: semen

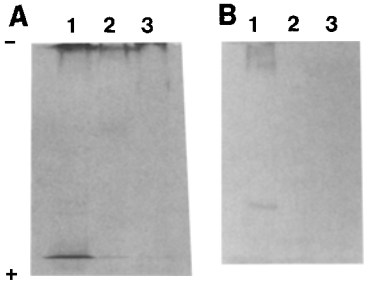


**Fig. 4A, B.** Analysis of tissue specificity of P6-5H. Semen, saliva and vaginal secretions were separated by SDS-PAGE (5% acrylamide gel) and transfered to PVDF membrane. The gel was stained with PAS **A** and the blot was stained immunologically with P6-5H **B**. 1: semen, 2: saliva, 3: vaginal secretion

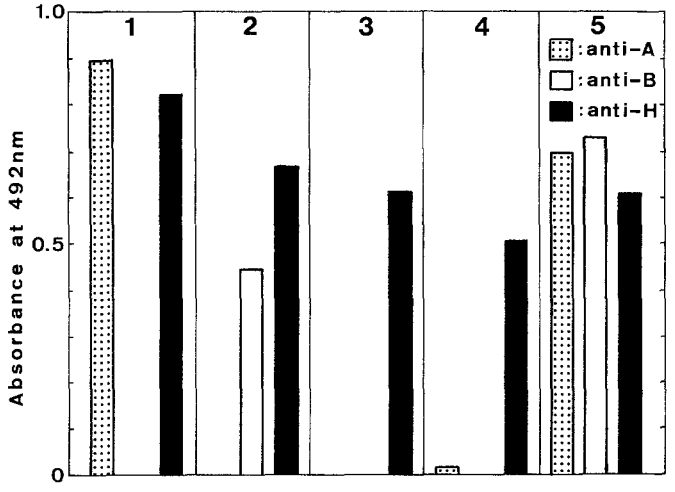


**Fig. 5.** ABO blood grouping of semen from mixed body fluids by sandwich ELISA. Specimens were prepared as follows: specimen 1, 25 µl of A Se semen (X250 dil) + 25 µl of B Se saliva (X250 dil); specimen 2, 25 µl of B Se semen (X250 dil) + 25 µl of extract from a A Se vaginal swab; specimen 3, 25 µl of O Se semen (X250 dil) + 25 µl of extract from a A Se vaginal swab; specimen 4, 25 µl of O se semen (X250 dil) + 25 µl of extract from a A Se vaginal swab; specimen 5, 50 µl of AB Se semen (X500 dil). Procedures are described under Materials and Methods. Absorbance values are obtained after subtraction of the blank values (ca. 0.05) from the measured ones

secretions) other than semen were not stained with P6-5H which shows that P6-5H recognizes TSE of seminal BGS.

*Blood grouping of semen by sandwich method with P6-5H*

Since P6-5H recognized TSE of seminal BGS, P6-5H seemed to be a suitable antibody to use for the capture of seminal ABO-BGS from mixed body fluids. Therefore, we examined the applicability of P6-5H as a capture antibody for ABO blood grouping of semen by sandwich methods.

Specimens of mixed body fluids were incubated with purified P6-5H. The ABO blood group epitopes of seminal BGS captured by P6-5H were detected with anti-

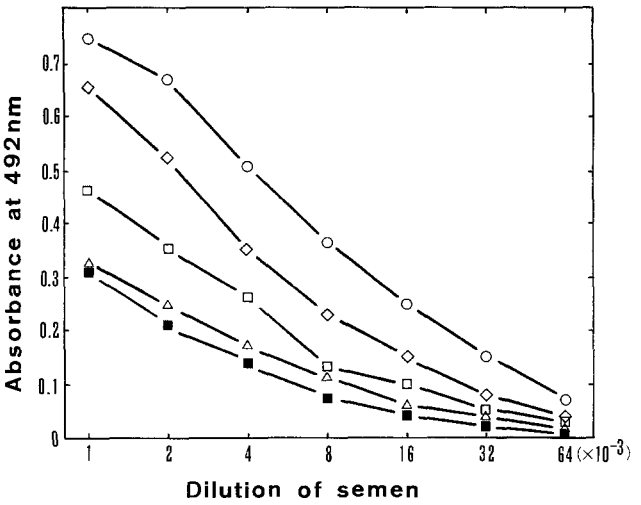


**Fig. 6.** The limits of detectable ABO blood group from semen by sandwich ELISA. Serial doubling diutions of semen were used as specimens. Procedures are described under Materials and Methods. ○: A epitope of A Se semen, △: B epitope of B Se semen, □: H epitope of O Se semen, ■: H epitope of O se semen, ◇: B epitope of AB Se semen. Absorbance values are obtained after subtraction of the blank values (ca. 0.05) from the measured ones

ABO blood group antibodies by ELISA and absorption-elution test, without interference by other body fluids.

ABO blood grouping of 500-fold dilutions of semen mixed with saliva or vaginal secretions was performed by sandwich ELISA (Fig. 5). Although the ABO blood groups of these specimens were detected as the sum of blood groups of each body fluid by the conventional absorption-elution test, only the ABO blood group of semen was detected by the present method. The same results were also obtained with the sandwich absorption-elution test (data not shown).

Figure 6 shows the limits of detection of ABO blood group epitopes from semen by sandwich ELISA. In this method, ABO blood group epitopes were detected in 8,000 to 32,000-fold and in 4,000-fold diluted semen from secretors and non secretors, respectively. These detection limits varied significantly between individuals.

In the sandwich absorption-elution method, detection limits were in the range 4,000 to 8,000-fold dilutions, irrespective of secretor status (data not shown).

## Discussion

In recent years several attempts to identify the ABO blood group of semen from mixed body fluids have been reported including an immunohistochemical procedure [6]. In other reports, polyclonal anti-seminal plasma antibodies were employed as capture antibodies in sandwich ELISA or as precipitating antibody in the immunodiffusion method [7, 8]. However, there were uncertainties in these methods, because the polyclonal antibodies employed were not specific for seminal BGS but for seminal plasma. Therefore, we attempted to achieve the same purpose by using mAb which was specific to semi-

nal BGS. We prepared mAb(P6-5H) against TSE on seminal BGS which was specific for semen and could be used to distinguish between semen and other body fluids (saliva and vaginal secretions) as shown in Fig. 4. By using P6-5H as a capture antibody, ABO blood group epitopes on seminal BGS were selectively detected by the sandwich method with anti-ABO blood group antibodies (Fig. 5). These results supported our hypothesis on the structure of seminal ABO-BGS. Figures 3 and 4 show that P6-5H recognizes $\alpha_2$-SGP. Iki et al. (1988) reported that anti-$\alpha_2$-SGP antibody, as well as anti-ABO blood group antibody, was produced by injection of purified $\alpha_2$-SGP into rabbits, and that this anti-$\alpha_2$-SGP antibody could be used in the sandwich method for ABO blood grouping of semen. However, this polyclonal anti-$\alpha_2$-SGP antibody cross-reacted with saliva BGS [9]. In contrast to the polyclonal anti-$\alpha_2$-SGP, P6-5H did not cross-react with saliva BGS, demonstrating that both common and specific epitopes exist on seminal BGS and P6-5H recognized a specific epitope.

ABO blood grouping of semen by the sandwich method with P6-5H is highly sensitive. The sensitivity of the sandwich absorption-elution test is lower than that of sandwich ELISA, but is sufficient for practical use. The sandwich absorption-elution test can be performed without expensive reagents and equipment such as labeled second antibodies and EIA reader.

Since rape cases involving more than one assailant are not rare, it is important to identify the ABO blood groups of all assailants relevant to the sexual assault. When the eluate in our sandwich absorption-elution test was applied to sandwich ELISA using ABO blood group antibodies as capture antibodies [10], it was possible to discriminate whether the specimen is blood group AB semen or a mixture of blood group A and B semen. However, if the specimen is a mixture of blood group AB semen and other blood groups, all specimens will be identified as blood group AB semen. Therefore, blood grouping of individual semen in a specimen which is mixture of semen from multiple assailants is very restricted.

Based on our hypothesis on the structure of ABO-BGS in body fluids, we have also produced anti-human salival BGS mAbs. These mAbs recognized the core protein of salival BGS and were tissue and species specific [1], and could be employed for blood grouping of saliva from mixed body fluids [2]. We think that the sandwich method with mAb against TSE of BGS may be the universal method for ABO blood grouping of individual body fluids from a mixture of body fluids.

*Acknowledgement.* We wish to thank Dr. R. Tsuda, Department of Legal Medicine, Kurume University School of Medicine, for providing $\alpha_2$-SGP.

## References

1. Kimura A, Matsumura F, Sodesaki K, Tsuji T (1991) Production and characterization of monoclonal antibodies against tissue specific epitope on ABO blood group substance in saliva. Int J Leg Med 104:193–196
2. Kimura A, Matsumura F, Sodesaki K, Tsuji T (1991) ABO blood grouping of saliva mixed body fluids by sandwich methods using monoclonal antibodies to tissue specific epitopes on blood group substance in saliva. Int J Leg Med 104:189–192
3. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
4. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets – Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
5. Tsuda R, Ito Y, Hara M (1988) Immunochemical studies of a glycoprotein ($\alpha_2$-Seminoglycoprotein; $\alpha_2$-SGP) in seminal plasma – Forensic immunological studies of body fluids and secretions, report 33. Jpn J Leg Med 42:28–37 (in Japanese)
6. Brinkmann B, Kernbach G, Rand S (1986) Cytochemical detection of ABH antigens in human body fluids. Z Rechtsmed 96:111–117
7. Mukoyama H, Sakai K, Kasai K (1987) A sandwich-ELISA method for ABO typing in mixtures of semen and vaginal secretion using a solid-phase immunoabsorbent microplate. Jpn J Leg Med (Suppl) 739 (in Japanese)
8. Yoshioka N, Takahashi K, Shohji T (1986) ABO blood grouping on seminal fluids contaminated with vaginal secretions. Act Crim Jpn 52:236–242 (in Japanese)
9. Iki H, Minamitake H, Tsuda R, Hara M (1988) Detection of ABO blood group of seminal stains mixed with vaginal secretion or blood by a method using NCF coated with anti $\alpha_2$-SGP serum-Forensic immunological studies of body fluids and secretions, report 36. Jpn J Leg Med 42:504–508 (in Japanese)
10. Katsumata Y, Sato M, Sato K, Tsutsumi H, Yada S (1986) A novel method for ABO grouping of mixed stains of saliva using enzyme-linked immunosorbent assay (ELISA). Act Crim Jpn 50:167–172