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Production and characterization of a monoclonal antibody to ABH-carrying α_2-seminoglycoprotein for ABO grouping of semen by ELISA*

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Summary. BALB/c mice were immunized with α_2-seminoglycoprotein (A2SGP), the lymph node cells were fused with P3U1 myeloma cells and cultured by the conventional technique. Four antibody-producing hybridoma clones were established and antibody-containing ascitic fluid obtained. The antibody was directed to the protein backbone of A2SGP and not to ABH antigenic determinants and did not cross-react with saliva or vaginal secretions. When tested in an indirect ELISA the anti-A2SGP antibody had a titer of 512000. The anti-A2SGP was used in a capture ELISA (or sandwich ELISA) in which wells were coated with this antibody to capture A2SGP in semen, and the captured A2SGP was detected with anti-A, anti-B or anti-H-peroxidase conjugate and peroxidase-labeled second antibody. This ELISA allowed correct ABO grouping even of 1:12800 or higher dilutions of semen. When the ELISA was applied to ABO grouping of seminal fluids mixed with vaginal secretions only the seminal ABH antigens could be detected. The results strongly suggest the potential usefulness of monoclonal anti-A2SGP in the investigation of rape cases.

Key words: Personal identification – ABO – Semen – Monoclonal anti-α_2-seminoglycoprotein – ELISA

Zusammenfassung. BALB/c-Mäuse wurden mit α_2-Seminoglykoprotein (A2SGP) immunisiert; die Lymphknotenzellen wurden fusioniert mit P3U1 Myelomzellen und mit konventioneller Technik kultiviert. 4 Antikörper-produzierende Hybridoma-Klone wurden etabliert und Antikörper-enthaltende Ascitesflüssigkeit gewonnen. Der Antikörper war gegen das Protein-Rückgrat von A2SGP gerichtet und nicht gegen die ABO-Antigen-Determinanten und er reagierte nicht mit Speichel oder Scheidensekret. Im indirekten ELISA-Test hatte der Anti-A2SGP-Antikörper einen Titer von 512000. Der Anti-A2SGP-Antikörper wurde getestet in einem „Capture-ELISA" (oder „Sandwich-ELISA"), in welchem Vertiefungen mit diesem Antikörper beschichtet wurden, um A2SGP aus Spermaflüssigkeit zu binden und wurde dann mit einem zweiten Anti-A, Anti-B und Anti-H-Antikörper detektiert, welcher mit Peroxydase konjugiert oder markiert war. Dieser ELISA-Test erlaubte eine korrekte ABO-Bestimmung, selbst bei 1:12800 oder höheren Verdünnungen der Spermaflüssigkeit. Bei Gemischen von Spermaflüssigkeit mit Vaginalsekret war der ELISA-Test zum ABO-Nachweis imstande, nur die ABH-Antigene der Spermaflüssigkeit zu detektieren. Die Ergebnisse suggerieren eindrucksvoll die potentiellen Möglichkeiten des monoklonalen Anti-A2SGP-Antikörpers für die Untersuchung von Vergewaltigungsfällen.

Schlüsselwörter: Spermaflüssigkeit – Monoklonales Anti-α_2-Seminoglykoprotein – ABO

Introduction

Tsuda et al. [1] recently isolated a glycoprotein with blood group A specificity from the semen of group A individuals, which they designated α_2-seminoglycoprotein (A2SGP). This has been shown to: (1) have a molecular weight of about 472000, (2) contain 76% sugars and

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24% amino acids, (3) occur in seminal fluid in concentrations up to 1010 mg/100 ml (average 277), (4) neutralize anti-A antibody, and (5) induce the formation of anti-A antibodies in rabbits. In addition, tandem-crossed immunoelectrophoresis of A2SGP and seminal fluid gave a reaction of identity. These results indicate that A2SGP is also the ABH substance in semen.

Thus, it is possible for antibodies to the protein backbone of A2SGP to capture A2SGP in a mixture of semen and vaginal secretion for correct ABO grouping of the semen component. Using potato lectins or polyclonal anti-semen antibodies in a sandwich enzyme-linked immunosorbent assay (ELISA), Mukoyama et al. [2] were able to identify the ABO group in semen mixed with vaginal secretions. Kimura et al. [3] prepared a mouse monoclonal antibody to partially purified seminal proteins and successfully used it in a sandwich ELISA. Iki et al. [4] carried out ABO grouping on semen in mixed body fluids by an adsorption-elution technique after capturing A2SGP with rabbit anti-A2SGP serum immobilized on nitrocellulose filters.

Similarly four hybrid cell lines secreting a monoclonal anti-A2SGP antibody were established in this study. The production, serological characterization and ELISA application of the monoclonal antibody (MAb) are described.

Materials and methods

Isolation of A2SGP. As previously described [1], A2SGP was isolated from seminal fluid from volunteers of blood group A by a sequence of column chromatography procedures. Purity was tested by disc electrophoresis, crossed immunoelectrophoresis and ultracentrifugation.

Production of monoclonal anti-A2SGP antibody. BALB/c mice were each immunized by s.c. injection of 20 μg A2SGP in Freund's complete adjuvant into each hind foot. After 10 days, popliteal lymph node cells were fused with P3U1 myeloma cells and cultured in autoclavable serum-free medium ASF-103 (Ajinomoto, Tokyo, Japan) in 96-well culture plates. The cultures were screened for antibody production by the indirect ELISA described below. After limiting-dilution cloning, four hybridoma clones were established and monoclonal anti-A2SGP antibodies were obtained as ascitic fluid. The antibodies were precipitated with ammonium sulfate at 50% saturation and stored at 5°C until use.

Semen and vaginal secretion. Semen and vaginal swabs were provided by the gynecological clinics of Nagoya University School of Medicine and Medical College of Oita. Vaginal secretions were extracted from the swabs with 0.01 *M* phosphate-buffered saline (pH 7.6) containing 0.15 *M* NaCl. ABO grouping and secretor status determination were carried out by routine procedures.

Indirect ELISA. Polystyrene ELISA plates were coated with 100 μl A2SGP (10 μg/ml) in 0.05 *M* carbonate buffer (pH 9.6) for antibody screening, or with serial doubling dilutions of seminal fluid, saliva, or vaginal secretion for the testing of anti-A2SGP. Distilled water was used for washing and the diluent was 0.5% bovine serum albumine (BSA) and 0.05% Tween 20 in 0.02 *M* Tris-buffered saline (TBS), pH 7.5. The protein coats were allowed to react (30 min) with 100 μl of culture supernatants or serial doubling dilutions of anti-A2SGP precipitates. After washing, the bound antibodies were allowed to react (30 min) with 100 μl of 1:1,000 dilution of anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Zymed Laboratories, San Francisco, Calif.). The plates were washed and 100 μl of a substrate mixture was added to each well. The substrate mixture contained 50 mg *o*-phenylenediamine dihydrochloride and 20 μl of 30% hydrogen peroxide in 0.05 *M* phosphate-0.024 *M* citric acid buffer 100 ml, pH 5.0. The enzyme reaction was stopped with 100 μl of 12.5% sulfuric acid and the plates were read at 492 nm on a Micro Plate Reader MPR-A4 (Toso, Tokyo, Japan).

In order to determine the isotype of the anti-A2SGP the bound antibody was detected with a set of rabbit anti-mouse Ig and HRP-labeled anti-rabbit IgG (Zymed Laboratories).

Capture ELISA. Wells were coated with 100 μl of serial doubling dilutions of anti-A2SGP in carbonate buffer. The antibody coats were allowed to react with 100 μl of serial doubling dilutions of semen. After washing, the captured A2SGP molecules were allowed to react (30 min) with 100 μl of 1:20 dilutions of monoclonal anti-A, anti-B, or diluent alone, followed by 30 min incubation with 1:1,000 dilution of HRP-labeled anti-mouse IgM (Zymed Laboratories) or HRP-anti-H lectin (E-Y Labs, San Mateo, Calif.). Substrate mixtures were added and the plates were read as described previously.

For ABO grouping of semen mixed with vaginal secretions, wells were coated with 150 μl of 1:500 dilution of anti-A2SGP, followed by incubation with 1% BSA in TBS (30 min). After washing, the antibody coats were allowed to react with 100 μl of 1:10 mixtures of semen and vaginal secretion (60 min). The captured A2SGP molecules were detected with monoclonal anti-A or anti-B and the enzyme-labeled second antibody.

Results and discussion

After recloning and testing of cultures for antibody production by indirect ELISA, four antibody-producing hybridoma clones were established. Intraperitoneal growth of these hybridomas resulted in the production of high-titer anti-A2SGP antibodies of IgG1 isotype. When ELISA plates were coated with semen at a 1:200 dilution, the semen coats could be detected with anti-A2SGP at dilutions of 1:128000 to 1:512000. When plates were coated with serial doubling dilutions of semen the lower limit of detection with 1:1000 dilution of anti-A2SGP was 1:10240. When coated with serial doubling dilutions of saliva or vaginal secretion wells gave no reactions with anti-A2SGP while anti-A, anti-B and anti-H reacted strongly with the corresponding antigen coats irrespec-

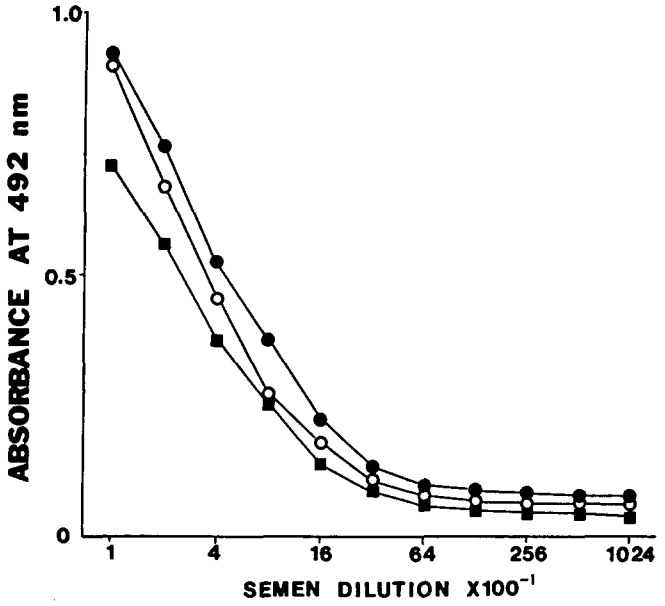

Fig. 1. Results of capture ELISA for ABO grouping of semen. Wells were coated with 1:200 (●), 1:1600 (○), or 1:12800 (■) dilution of anti-A2SGP, and incubated with serial doubling dilutions of group B secretor semen. Captured A2SGP was detected with anti-B and HRP-anti-mouse IgM

Table 1. ABO grouping of seminal fluids mixed with vaginal secretions using the capture ELISA technique

Seminal fluid	Vaginal secretion	Number	Reaction with anti-A[a]	Reaction with anti-B[a]
A	B	12	0.303	0.009
B	A	9	0.016	0.226
O	AB	5	0.038	0.019
AB	A	5	0.300	0.243
AB	B	5	0.230	0.212

[a] Values indicate average absorbances at 492 nm

tive of secretor status. The results indicate that the ascitic antibody preparations had no anti-ABH activity or cross-reactivity with saliva or vaginal secretions.

The anti-A2SGP was used in a capture ELISA (sandwich ELISA) to capture exclusively the ABH epitope-carrying protein in semen. Figure 1 shows the results of an experiment in which a semen sample from a group B secretor was captured by anti-A2SGP antibody on the solid phase and subsequently detected with anti-B and HRP-anti-mouse IgM. The anti-A2SGP could be diluted 1:12800 for use in this ELISA. Even a very low concentration ($\geq$ 1:12800) of semen could be detected and correctly grouped.

This capture ELISA was then applied to experimental mixtures of seminal fluids and vaginal secretions. Table 1 lists the ABO combinations tested and typical results: in each combination only the ABH antigens from semen could be detected. In exceptional cases, however, conflicting results were obtained. For example, an experimental mixture of a group B secretor semen and group A secretor vaginal secretion was grouped as AB. Since it was quite possible that the vaginal secretion was contaminated with semen of group A the anti-A2SGP coat was allowed to react with the vaginal secretion alone. As a result the sample was grouped as A. Thus, it is reasonable to conclude that the discrepancy was due to contamination with semen. Cases where a semen sample cannot be correctly grouped should be interpreted in a similar manner.

One of the major seminal proteins of forensic interest is γ-Sm [5] or p30 [6] and has long been used as a marker for semen identification. The present study demonstrated that A2SGP is another sensitive marker when detected by an indirect ELISA method.

Since the individualization of seminal fluids or stains is the final goal, especially in connection with rape cases, a number of forensic scientists have attempted to determine the ABO groups of semen mixed with vaginal secretions. Success has been claimed after separation of seminal and vaginal components, but none of the claims were convincing: no conclusive evidence of complete separation was presented in the absence of information on the carrier of ABH epitopes in semen. Although an increasing number of genetic markers are being used in semen analysis, the ABO system remains most important because of the high concentration and stability of the ABH antigens. Increasingly wider use of ELISA methods in recent years has stimulated a new approach to the ABO grouping of semen. Mukoyama et al. [2] devised a sandwich ELISA using potato lectins or anti-semen serum for capture of seminal ABH antigens. Seminal ABH antigens of secretors and non-secretors could be detected at dilutions of 1:12800 to 1:51200 and 1:400 to 1:800, respectively. We obtained comparable results. They also stated that the ELISA method was successful for grouping seminal fluids mixed with vaginal secretions for all ABO combinations. Using the same principle, Sagisaka et al. [7] reported a similar "double-sandwich ELISA" with absorbed rabbit antisera against human semen. To our knowledge, Kimura et al. [3] were the first to use an MAb prepared after immunization of mice with partially purified proteins of semen in a sandwich ELISA. In isolating A2SGP by a combination of column chromatography methods, Tsuda et al. [1] discovered by biochemical and serological analysis that it carries ABH epitopes. Immunizing rabbits with A2SGP, Iki et al. [4] raised anti-A2SGP serum, but it was necessary to absorb with human red blood cells and serum before use. Although monospecific, the absorbed polyclonal anti-A2SGP serum may pose problems in the study of casework material. To avoid the potential hazard of cross-reactions we produced anti-A2SGP MAbs of well-defined specificity which appeared to work well in the capture ELISA described here.

In the last few years, DNA analysis has been increasingly applied to the individualization of seminal fluids and stains and has given reliable results. However, not all forensic scientists can afford to use the technique at the present stage. Thus, the capture ELISA with the monoclonal anti-A2SGP merits use in forensic science practice.

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