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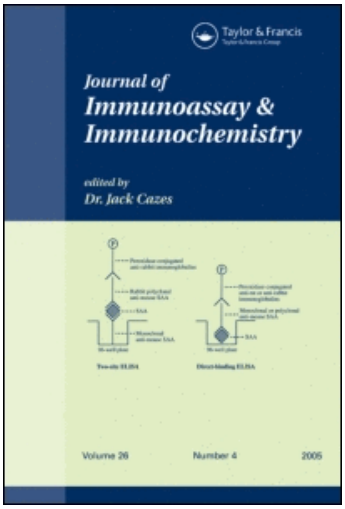

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JOURNAL OF IMMUNOASSAY, 18(3), 215-233 (1997)

BISPECIFIC RABBIT Fab'-BOVINE SERUM ALBUMIN CONJUGATE USED IN HEMAGGLUTINATION IMMUNOASSAY FOR β-MICROSEMINOPROTEIN

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ABSTRACT

A polyclonal bispecific (bifunctional) antibody was prepared to develop a hemagglutination immunoassay for β-microseminoprotein (β-MSP), a predominant seminal protein. Three types of $F(ab')_2$ fragments of rabbit IgG, affinity-purified anti-human red blood cell (RBC) $F(ab')_2$, nonaffinity-purified anti-β-MSP $F(ab')_2$ and nonspecific (nonimmunized) $F(ab')_2$, were mixed to obtain a $F(ab')_2$ mixture containing 10% anti-RBC molecules and 10% anti-β-MSP molecules. Fab' was obtained from the $F(ab')_2$ mixture, and then reacted with maleimide-activated bovine serum albumin (BSA) at a molar ratio of 10:1. As estimated by the decrease in the maleimide content, approximately 7 Fab' molecules were introduced per one BSA molecule. The bispecific (anti-β-MSP and anti-RBC) Fab'-BSA conjugate thus prepared was incubated successively with a human RBC suspension and with samples. In the presence of β-MSP, RBCs become agglutinated, providing a test simple for forensic semen identification.

(KEY WORDS: prostatic secretory protein of 94 amino acids (PSP94, PSP_{94} or PSP), prostatic inhibin peptide (PIP))

INTRODUCTION

Forensic semen identification (demonstration of the presence of semen) usually involves two steps. The first step (presumptive test) is screening for abundant seminal components, and the next step (confirmatory test) is confirmation

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of spermatozoa and/or an antigen highly specific to semen. β-microseminoprotein (β-MSP)[1] is a nonglycosylated protein of 94 amino acids (1,2,3). β-MSP constitutes three predominant proteins in the prostatic ejaculate (4). An earlier attempt to introduce β-MSP as a marker for confirmatory test (5) has been controversial due to its nonprostatic production (2,6). We believe that a seminal concentration of 0.1-3 mg/mL (6,7,8) is sufficiently high for β-MSP to be used as a marker for the presumptive test.

AGEN Biomedical Ltd. (Queensland, Australia) (9) has developed a homogeneous hemagglutination immunoassay system that is applicable to both antigens (10) and antibodies (11). The procedure is simple, the mixing of whole blood samples with a bispecific reagent. The reagent used for the detection of antigens is a bispecific monoclonal $F(ab')_2$ directed against human red blood cells (RBCs) and an antigen of interest (e.g., detection of cross-linked fibrin degradation products by SimpliRED™ D dimer (10)). When the antigen is present, hemagglutination occurs through bridges formed by RBC-bispecific $F(ab')_2$-antigen-bispecific $F(ab')_2$-RBC.

We attempted to apply the system to detection of β-MSP. In this paper, we describe a method to prepare bispecific conjugate by using two polyclonal rabbit

[1] This protein has a few names. Tsuda et al. (5) purified this protein from seminal plasma and termed it "β-MSP" based on its immuoelectrophoretic property. Independently, Sheth et al. (32) isolated this protein and its amino acid sequence was elucidated by Seidah et al. (33) who termed it "β-inhibin", followed by confirmation by Johansson et al. (34). The sequence of β-MSP was then determined (1), and the identity of β-MSP and β-inhibin was established. Later, a highly purified protein was reported to lack inhibin-like activity (suppression of follicule-stimulating hormone) (35,36,37). Some investigators (2,3) and Japanese forensic investigators (including ourselves) have thus favored "β-MSP", whereas some have introduced "prostatic secretory protein of 94 amino acids (PSP94, PSP_{94})" (7,38) or "(PSP)" (37). Others however have been emphasizing the inhibin nature and use "prostatic inhibin peptide (PIP)" (39).

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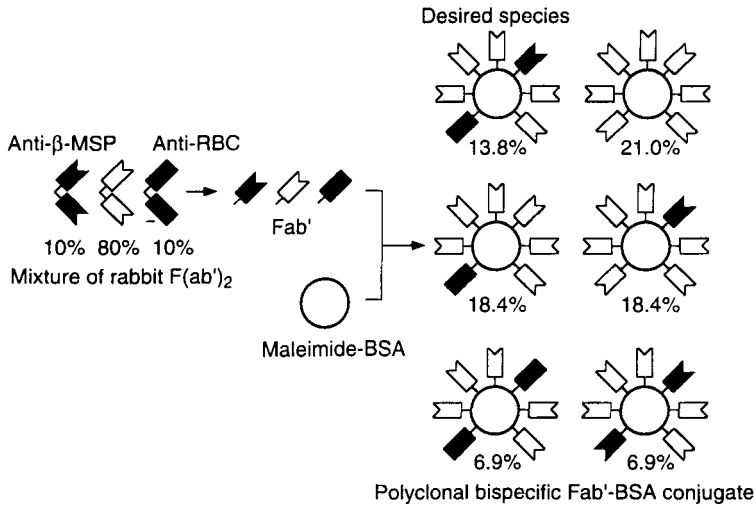

FIGURE 1 Preparation of a bispecific Fab'-BSA conjugate. The six most likely species are shown, provided that 7 Fab' fragments and 1 BSA molecule constituted the Fab'-BSA conjugate. Their percentages P(i,j) were expected to be $P(i,j) = ({}_{0.1n}C_i \times {}_{0.1n}C_j \times {}_{0.8n}C_{7-(i+j)}) \div ({}_nC_7)$, $0 \le i \le 7$, $0 \le j \le 7$, $0 \le i + j \le 7$, where i and j are the number of anti-β-MSP and anti-RBC Fab' fragments, respectively, in the conjugate, and 0.1n, 0.1n and 0.8n implied that the Fab' mixture subjected to the conjugation contained 10% of anti-β-MSP, 10% of anti-RBC and 80% of nonspecific molecules. The values in the figure were calculated by the approximation of $n \rightarrow \infty$.

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IgG antibodies, previously raised anti-β-MSP (8) and commercially available anti-human RBC.

MATERIALS AND METHODS

Figure 1 illustrates the principle of the method. It seems that a polyclonal antibody can be regarded as a mixture of specific antibody molecules that bind to the targeted antigen (closed symbols) and nonspecific antibody molecules that do not bind to the antigen (open symbols), though the nonspecific antibody may bind to other antigens of no interest. Here, the terms "specific" and "nonspecific" are used, respectively; the term "titer" is used as the percentage of specific antibody molecules in polyclonal antibodies.

β-MSP, Rabbit Antibodies and BSA

β-MSP (12) and rabbit anti-β-MSP IgG (13,14) were the same as prepared previously (8). Rabbit anti-human RBC IgG raised by immunizing a 10% (v/v) RBC (a mixture of ABO-blood group A and B types) suspension in saline was obtained from Intercell Technologies, Hopewell, NJ. Nonspecific IgG was prepared from a nonimmunized rabbit serum (14). $F(ab')_2$ fragments were prepared separately from the anti-β-MSP, anti-human RBC and nonspecific IgG by digestion with pepsin (14). Regarding the bovine serum albumin (BSA), PENTEX® (crystallized, Miles, Kankakee, IL) was used for the preparation of maleimide-BSA, and fraction V grade (Miles) for other purposes.

The extinction coefficients at 280 nm ($g^{-1} \cdot L \cdot cm^{-1}$) were 1.56 for β-MSP (7,8,15), 1.48 for $F(ab')_2$ and Fab'(14), and 0.667 for BSA (16). The molecular weight of Fab' was 46,000 (14) and that of BSA was 66,400 (17).

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Human RBC Membrane-Sepharose 4B

The RBC membranes (ghost) were prepared from blood (100 mL) of ABO-blood group O type (18). The membranes obtained (about 1.2 mL) were solubilized with 7.1 mL of 1 g/L sodium dodecyl sulfate (SDS) in 0.1 mol/L $NaHCO_3$ containing 0.1 mol/L NaCl to give a protein concentration of 1.5 mg/mL, which was determined by a modified Lowry's method (19) using BSA as the standard. The solubilized proteins were coupled to CNBr-activated Sepharose 4B (1.24 g, Pharmacia Biotech, Uppsala, Sweden) using the same SDS solution and 2 h incubation at room temperature. About 90% of the proteins were immobilized.

Affinity-purification of Anti-Human RBC $F(ab')_2$

Anti-human RBC $F(ab')_2$ (14.0 mg) was affinity-purified by elution at pH 2.5 (23) from a human RBC-Sepharose 4B column (1.0 mL, 1 × 1.3 cm). The amount of affinity-purified $F(ab')_2$ obtained was 0.342 mg (yield of 2.44%)[3].

Maleimide-BSA

BSA (8 mg) in 1.08 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0 was reacted with 0.12 mL of 70 mmol/L *N*-succinimidyl 6-maleimidohexanoate (Dojindo, Kumamoto, Japan) in *N*,*N*-dimethylformamide (14,21,22) at 30°C for 45 min. The excess reagent was removed by passage through a Sephadex G-25 medium column (1 × 30 cm, Pharmacia Biotech) using 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L ethylenediaminetetraacetate (NaPB-6-EDTA). The average number of maleimide groups introduced per one BSA molecule was 13.5-15.2, determined as described below. These values remained essentially unchanged in the same buffer at a BSA concentration of 15 μmol/L at 4°C for 3 days.

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Titer of anti-β-MSP

The percentage of specific molecules in the polyclonal anti-β-MSP molecules (titer of anti-β-MSP) was determined by applying anti-β-MSP Fab'-peroxidase conjugate (Fab'-POD) (8) to a β-MSP-Sepharose 4B column, followed by comparing peroxidase activity bound to the column with that in the effluent. This method, devised by Ishikawa and colleagues (23), relies on the understanding that Fab'-POD prepared by their "maleimide-hinge method" (14,21) is monomeric (reaction of one Fab' molecule with one POD molecule) and usually retains full antigen-binding activity of the Fab'.

β-MSP-Sepharose 4B column (β-MSP column) was prepared by coupling β-MSP (1 mg) to CNBr-activated Sepharose 4B (0.1 g). Anti-β-MSP Fab'-POD (1 ng) in 20 μL of 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl and 1 g/L BSA (POD buffer) was passed through a β-MSP column (50 μL, n=2) using 1 mL of the same buffer. 1) *POD activity applied and in the effluent.* Aliquots (0.1 mL) of the applied solution diluted 50-fold, the effluent (1.02 mL),

and the POD buffer as the reagent blank were separately mixed with a mixture of 15 g/L of 3-(4-hydroxyphenyl) propionic acid in 0.1 mol/L sodium phosphate buffer, pH 7.0 (40 μL) and 0.075% (v/v) H_2O_2 in the same buffer (10 μL) (hydrogen donor and peroxide solution). After incubation at room temperature for 30 min, the enzyme reaction was terminated with a mixture of 0.1 mol/L glycine-NaOH buffer, pH 10.3 (2.0 mL) and distilled water (0.5 mL). 2) *POD activity bound to the column.* The β-MSP column was filled with a mixture of the hydrogen donor and peroxide solution (50 μL) and the POD buffer (100 μL). After incubation at room temperature for 10 min, the column was quickly washed out with 0.5 mL of distilled water, and the passed solution (0.65 mL) was immediately mixed with the glycine-NaOH buffer (2.0 mL). 3) The fluorescence intensity (FI) was measured using 320 nm for excitation and 405 nm for emission by taking FI of the glycine-NaOH buffer as 0 and that of 1 mg/L quinine in 50 mmol/L H_2SO_4 as 100. 4) The physical adherence of anti-β-MSP Fab'-POD to columns was estimated by applying anti-β-MSP Fab'-POD to a BSA-Sepharose 4B column (BSA column, n=1) and also by applying a nonspecific (nonimmunized) Fab'-POD to β-MSP column (n=1).

The measured FI values were as follows. Anti-β-MSP Fab'-POD: applied = 145, effluent from and bound in one β-MSP column = 89.0 and 212. The reagent blank = 13.2. These FIs were adjusted by variables (e.g., dilution factors and incubation times): (145 - 13.2) × 50-fold × (20 μL ÷ 100 μ L) = 1318 for applied, (89 - 13.2) × (1.02 mL ÷ 100 μ L) = 773 for effluent, and, (212 - 13.2) × (30 min ÷ 10 min) = 596 for bound. Thus, the percentage of anti-β-MSP Fab'-POD bound to the β-MSP column was 596 ÷ (596 + 773) = 43.5%, with a recovery of (596 + 773) ÷ 1318 = 104%. The portion of anti-β-MSP Fab'-POD bound to another β-MSP column and that bound to the BSA column were 41.8%

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and 1.1%, respectively. The portion of nonspecific Fab'-POD bound to the other β-MSP column was 0.97%. The titer was determined to be 42%.

Fab'

Anti-β-MSP $F(ab')_2$ (0.708 mg), which was estimated to contain 0.297 mg (42%) of the specific $F(ab')_2$, affinity-purified anti-human RBC $F(ab')_2$ (0.297 mg) and nonspecific $F(ab')_2$ (1.97 mg) were mixed (total 2.97 mg). The $F(ab')_2$ mixture was then reduced with 2-mercaptoethylamine (14) to yield 2.6 mg of Fab'. The number of SH groups per one Fab' molecule was 1.20, determined with 4,4'-dithiodipyridine (14,24).

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Conjugation of Fab' to Maleimide-BSA

Fab' (2.60 mg) was mixed with maleimide-BSA (0.374 mg) in NaPB-6-EDTA and then concentrated with Centricon 30 (Amicon, Danvers, MA) to a final volume of 0.38 mL. The concentrations of Fab' and maleimide-BSA were 150 μmol/L and 15 μmol/L, respectively. After incubation at 4°C for 16 h, the remaining maleimide and SH groups were blocked with 2-mercaptoethylamine and *N*-ethylmaleimide (14). The reaction mixture was then applied to an Ultrogel AcA 22 column (1.5 × 45 cm, IBF Biotechnics, Villeneuve-la-Garenne, France) using 10 mmol/L sodium phosphate buffer, pH 6.5, containing 0.145 mol/L NaCl (Figure 2). The amount of the conjugate obtained was 1.34 mg, which was calculated by assuming the extinction coefficient at 280 nm to be 1.38 $g^{-1} \cdot L \cdot cm^{-1}$ (the derivation of this value is described in "RESULTS AND DISCUSSION").

Number of Fab' Fragments in Fab'-BSA conjugate

The average number of Fab' molecules conjugated to one BSA molecule was estimated by comparing the average number of maleimide groups per one BSA molecule (maleimide content) before and after conjugation. To save specific

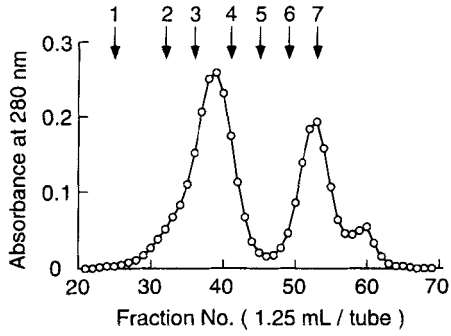

FIGURE 2 Elution profile of the reaction mixture of rabbit Fab' and maleimide-BSA on an Ultrogel AcA 22 column. Fractions containing Fab'-BSA conjugate (fractions 32-43) were pooled and concentrated with Centricon 30 of to a concentration 0.42 mg/mL for increasing stability (40). The conjugate was preserved at 4°C after addition of NaN_3 and BSA, each at a final concentration of 1 g/L. Arrow 1 indicates the void volume of the column, and arrows 2-7 indicate the elution volumes of IgM (molecular eight of 900,000; #2), thyroglobulin (669,000; #3), ferritin (440,000; #4), catalase (232,000; #5), $F(ab')_2$ (92,000; #6) and Fab' (46,000, #7), respectively.

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antibodies, this experiment was conducted by only using nonspecific (nonimmunized) Fab'. 1) *After conjugation.* Nonspecific Fab' was reacted with maleimide-BSA in NaPB-6-EDTA at a concentration of 150 µmol/L and 15 µmol/L, respectively. The reaction mixture was incubated at 4°C for 16 h, and then the maleimide content was determined. 2) *Before conjugation.* Maleimide-BSA was allowed to stand in NaPB-6-EDTA at a concentration of 15 µmol/L at 4°C for 16 h, and then the maleimide content was determined.

The maleimide content was determind by reacting samples with a known amount of 2-mercaptoethylamine followed by measuring the remaining SH groups (14) with 4,4'-dithiodipyridine (24). The regularly used buffer was NaPB-6-EDTA. After appropriate dilution of samples, the following mixtures were prepared: 1) buffer (0.55 mL) and 0.5 mmol/L 2-mercaptoethylamine (2-ME, 0.05 mL), n=4. 2) sample (0.55 mL) and 2-ME (0.05 mL), n=3-4. 3) sample

(0.55 mL) and buffer (0.05 mL), n=2. 4) buffer (0.6 mL), n=2. After incubation at 30°C for 20 min, 20 μL of 5 mmol/L 4,4'-dithiodipyridine was added. After further incubation at 30°C for 10 min, the absorbance at 324 nm was measured for each mixture (A1 to A4), and the maleimide content was calculated by ((A1 - A2) + (A3 - A4)) ÷ (19,800 $mol^{-1} \cdot L \cdot cm^{-1} \times 1$ cm) × (0.62 mL ÷ 0.55 mL) ÷ BSA concentration (mol/L) in diluted samples where 19,800 was the molar extinction coefficient of 4-mercaptopyridine.

Result for one run was as follows. BSA concentration of samples = 15 μmol/L. Dilution factors = 12-fold (before conjugation, maleimide-BSA) and 6-fold (after conjugation, a mixture of maleimide-BSA and Fab'). Absorbance: A1 = 0.796, A2 = 0.500 (before) and 0.763 (after), A3 = 0.082 (before) and 0.307 (after), A4 = 0.072. The maleimide content before and after conjugation were calculated to be 13.9 and 6.1, respectively. In another trial, the maleimide content before and after conjugation were 13.3 and 6.0, respectively. The number of maleimide groups decreased throught the conjugation was determined to be 7.55

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Hemagglutination Immunoassay

The working buffer was 10 mmol/L sodium phosphate buffer, pH 7.3, containing 0.145 mol/L NaCl and 2.2 g/L BSA. A suspension (1.2%, v/v) of washed RBCs of ABO-blood group O type was mixed with an equal volume of 4 μg/mL of bispecific Fab'-BSA conjugate, and incubated at room temperature for 20 min with intermittent gentle shaking. An aliquot (50 μL) of the RBC suspension thus sensitized was mixed with the same volume (50 μL) of a sample in a "U" bottom micro titer plate (96 wells, Fujirebio, Tokyo, Japan). After gentle shaking for 2 min, the test was allowed to stand at room temperature for 2 h for the sedimentation to occur, and the settling pattern was then determined macroscopically (25).

Sample preparation. 1) A semen sample was serially diluted with the working buffer and then assayed. The β-MSP concentration of the semen was 0.26 mg/mL as measured by a sandwich enzyme immunoassay (8). 2) The same semen was serially diluted with distilled water (2.7 mL), and then placed on cotton gauze (8 × 10 cm). After air-drying and storing at room temperature for a few weeks, a portion (about 5 mm × 5 mm) of the gauze was immersed in 300 μL of the working buffer at room temperature for 20 min under sonication. The extract was centrifuged at 7,000 rpm for 5 min, and the supernatant was assayed. 3) Stains were made from plasma of heparinized male blood (n=8) and male saliva (n=8). Nonpostcoital vaginal swabs (about 2 × 2 cm, n=12, taken from medicolegal autopsy cases and stored at 4°C for 2 months to 2 years) were also included. After extraction and centrifugation, the supernatant was serially diluted and assayed.

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RESULTS AND DISCUSSION

Development of the Method

Although most methods for preparation of bispecific antibodies, especially biotechnological methods, primarily rely upon monoclonal antibodies, the chemical method is theoretically applicable to both monoclonal and polyclonal antibodies (26). In the method by Glennie et al (27) and its modification (28), bispecific $F(ab')_2$ was constructed from two types of Fab' fragments using a homobifunctional maleimide reagent as a linker. When two types of polyclonal antibodies are subjected to their methods, the yield of functionally bispecific, "desired" $F(ab')_2$ depends on the titers of the polyclonal antibodies (when two polyclonal antibodies having titers of m% and n% are used, the yield of the desired $F(ab')_2$ is m% × n%). Thus, their methods are very effective if at least one of the two polyclonal antibodies is available in an affinity-purified form, which is regarded as 100% specific. However, it seems often difficult to obtain sufficient amount of affinity-purified

Fab' especially when commercial antibodies are used as the starting material. The present method was developed to alleviate this difficulty by incorporating nonspecific Fab' as a weight extender of affinity-purified Fab'.

Bispecific (anti-β-MSP and anti-RBC) Fab'-BSA Conjugate

By the conjugation with rabbit Fab' fragemt, the maleimide content of BSA (the average number of maleimide groups per one BSA molecule) was decreased by 7.55. Maleimide groups introduced into the BSA showed no disernable spontaneous decay in a condition adopted for the conjugation. The decrease in maleimide content was therefore, attributed to the thioether formation through the selective reaction with SH of the Fab'. The average number of the SH groups was 1.18 for the Fab'. Beacuse rabbit Fab' has one SH in the hinge, the redundant SH (0.18) of would be derived from intra- or inter-disulfide bonds (14, 23). If SH in the hinge participated only in the thioether formation, the average number of Fab' molecules conjugated to one BSA molecule would be 7.55. On the other hand, if all SH groups of Fab' underwent thioether formation, the average number would be 6.40, as given by (7.55/1.18) ÷ 2. We therefore, arbitrarily assume that the average number of Fab' molecules conjugated to one BSA molecule to be 7. The following comments are based on this supposition.

The extinction coefficient of the Fab'-BSA conjugate was assumed to be 1.38, as given by $((1 \times 0.667) + (7 \times 1.48)) \div (1 + 7) = 1.38$. The average molecular weight of the conjugate was expected to be 388,400, as given by $(1 \times 66{,}400) + (7 \times 46{,}000)$. In gel filtration using an Ultrogel AcA 22 column, the conjugate emerged in a region between molecular weight markers, 440,000 and 669,000 (Figure 2).

Since three kinds of Fab' fragments reacted with maleimide-BSA at random, many different Fab'-BSA conjugate species were likely produced. Figure 1

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shows the six most likely species, among which the "desired" species consisted of 1 anti-β-MSP Fab' fragment, 1 anti-RBC Fab' fragment and 5 nonspecific Fab' fragments. It should be noted that not necessarily all the molecules of the desired conjugate would be functionally bispecific for our purpose. When anti-β-MSP and anti-RBC are in close proximity to each other within desired conjugates, such conjugates could not become involved in bridging formation with RBC and the targeting antigen. On the other hand, other likely minority species (not shown in the Figure) containing 2 or more anti-β-MSP Fab' fragment and 1 anti-RBC Fab' fragment might be functional.

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Hemagglutination Immunoassay for β-MSP

The AGEN's original immunoassay system offers two advantages, rapidity and simplicity (29). Although the rapidity was sacrificed for the sake of achieving a higher sensitivity by a micro titer plate technique (25), our assay still seems simple enough for forensic application.

The sensitivity tended to improve as the concentration of the bispecific conjugate increased, but spontaneous agglutination in the absence of β-MSP occurred when 8 μg/mL or more of the conjugate was used (Table 1). The spontaneous agglutination might be caused by some Fab'-BSA conjugate species that carry two or more anti-RBC Fab' fragments. A concentration of 4 μg/mL was hence chosen, and, in this assay condition, complete agglutination was observed up to 1 ng/mL of β-MSP, which is a sensitivity comparable to that obtained by the widely-used hemagglutination immunoassays using antibody-labelled RBCs (reverse passive hemagglutination test) originally devised by Coombs (30).

No agglutination was observed in the lower dilutions of semen samples (Table 2). This was probably due to saturation of the anti-β-MSP antibodies (a prozone effect), indicating the need to dilute samples to exclude false negative

TABLE 1

Hemagglutination for β-MSP at Various Concentrations of the Bispecific Fab'-BSA Conjugate.

Conjugate prepared from a $F(ab')_2$ mixture containing 10% of anti-β-MSP.

	β-MSP (ng/mL)											
	50	20	10	5	2	1	0.5	0.2	0.1	0.05	0.02	0
0	–	–	–	–	–	–	–	–	–	–	–	–
0.25	–	–	–	–	–	–	–	–	–	–	–	–
0.5	±	±	±	±	±	–	–	–	–	–	–	–
1	+	+	+	+	±	±	±	–	–	–	–	–
2	+	+	+	+	+	±	±	–	–	–	–	–
4	+	+	+	+	+	+	±	±	–	–	–	–
8	+	+	+	+	+	+	+	±	±	±	±	±
16	+	+	+	+	+	+	+	+	±	±	±	±

Fab'-BSA Conjugate (μg/mL)

Conjugate prepared from a $F(ab')_2$ mixture containing 30% of anti-β-MSP.

	50	20	10	5	2	1	0.5	0.2	0.1	0.05	0.02	0
0	–	–	–	–	–	–	–	–	–	–	–	–
0.25	±	±	±	±	±	–	–	–	–	–	–	–
0.5	+	+	+	+	±	±	–	–	–	–	–	–
1	+	+	+	+	+	±	±	–	–	–	–	–
2	+	+	+	+	+	±	±	–	–	–	–	–
4	+	+	+	+	+	+	±	±	–	–	–	–
8	+	+	+	+	+	+	±	±	±	±	±	±
16	+	+	+	+	+	+	±	±	±	±	±	±

The interpretation of the settling pattern follows: A uniform salmon-pink disc covering the entire lower part of the well (complete agglutination +); Rings with increasing thickness and decreasing diameter (partial agglutination ±); A smooth round central button (no agglutination –).

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TABLE 2

Hemagglutination for Semen and Extracts of Some Biological Stains by Using the Conjugate Prepared from a $F(ab')_2$ Mixture Containing 10% of Anti-β-MSP.

A semen sample containing 0.26 mg/mL of β-MSP.

Dilution ($\times 10^3$ fold)												
1	2	5	10	20	50	100	200	500	1000	2000	5000	10000
–	±	±	+	+	+	+	+	±	±	–	–	–

Neat extracts of diluted seminal stains prepared from the same semen.

Dilution (fold)												
1	2	5	10	20	50	100	200	500	1000	2000	5000	10000
–	–	–	±	±	+	+	+	+	+	±	±	–

Diluted extracts of some neat biological stains.

	Dilution (fold)						
	1	2	5	10	20	50	100
one nonpostcoital vaginal swab	+	±	±	–	–	–	–
others	–	–	–	–	–	–	–

The concentration of the conjugate was 4 μg/mL.

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results. The body fluid specificity was therefore examined by using both neat and diluted extracts from eight plasma stains, eight saliva stains and twelve nonpostcoital vaginal swabs. The results were acceptable where no agglutination were observed at all dilutions except for one sample (Table 2).

The exceptional one was a vaginal swab which gave partial agglutination up to 5-fold dilution. When the Fab'-BSA conjugate prepared from $F(ab')_2$ mixture containing only 10% of anti-RBC (90% of nonspecific molecules) was used instead, it was not agglutinated, indicating the absence of both isoagglutinins and antibodies to the conjugate molecules. By a sandwich enzyme immunoassay for β-MSP (8),

an immunoreactive substance(s) corresponding to 420 pg of β-MSP was extracted from about 5 × 5 mm portion of the swab. In addition, nonpostcoital nature was comfirmed by the negative result for a sandwich enzyme immunoassay for γ-seminoprotein (also known as p30 or prostate specific antigen), a marker for the comfirmatory test (8). Weiber et al. (6) reported that cervical secretions occasionally contain high levels of β-MSP [median, 74 ng/mL; range, below the detection limit (1 ng/mL) to 5.7 μg/mL, n=7]. The agglutination observed in this particular swab was therefore thought to reflect the intrinsic β-MSP, by which we can not adop the detection of β-MSP as a comfirmatory test but as a presumtive test for forensic semen identification.

It was apparently better to use Rh minus blood (no D antigen on RBCs) as indicator cells because body fluids of Rh minus type occasionally contain antibodies against D antigen (29). However, we used Rh plus blood because of the infrequency (0.567%) of the Rh minus type in the Japanese population (31).

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Applicability of Other Polyclonal Antibodies against Antigens to be Detected

We also prepared another conjugate by increasing the percentage of specific anti-β-MSP in the $F(ab')_2$ mixture to 30% (10% of anti-RBC and 60% of nonspecific molecules). There was no significant difference in the sensitivity between 10% and 30% (Table 1), suggesting that $F(ab')_2$ mixture containing 10% of the specific antibodies may be sufficient. Therefore, our method may be applicable to other polyclonal antibodies containing at least 11.1% specific molecules [e.g., for a $F(ab')_2$ antibody with 11.1% specific molecules, mixing this with affinity-purified anti-RBC $F(ab')_2$ at a molar ratio of 9:1 without nonspecific $F(ab')_2$ gives a $F(ab')_2$ mixture containing 10% specific molecules]. In this regard, Ishikawa and colleagues (23) have found that the titer of typical polyclonal antibodies ranges from about 2 to 35%.

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