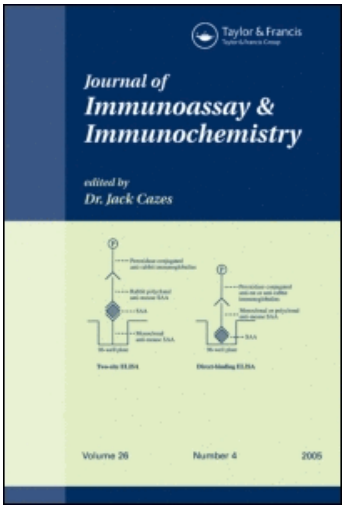

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SANDWICH CAPTURE ENZYME IMMUNOASSAY FOR WATER SOLUBLE BLOOD GROUP B SUBSTANCE IN SECRETOR SALIVA

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ABSTRACT

Sandwich capture enzyme immunoassays (EIA I and EIA II) are described. Water soluble B substance was reacted simultaneously with affinity-purified dinitrophenyl goat anti-B IgG and affinity-purified goat anti-B IgG-peroxidase conjugate. The complex formed of B substance, dinitrophenyl IgG and IgG-peroxidase conjugate was trapped onto a polystyrene ball coated with affinity-purified goat anti-dinitrophenyl bovine serum albumin IgG. After washing, peroxidase activity bound to the ball was assayed by fluorometry (the sandwich capture EIA I). In the sandwich capture EIA II, the complex was, after thorough washing, eluted from the ball with dinitrophenyl-L-lysine, and then peroxidase activity in the eluate was assayed. The thorough washing and elution processes improved the sensitivity 3.3-fold, and B substance in saliva samples from type B and AB secretors could be detected 200- to 500-fold more sensitively than hemagglutination inhibition, a method commonly used in forensic practices.

(KEY WORDS: Immunoassay, ABO blood-group system, Saliva)

INTRODUCTION

Blood group ABH(O) antigens are oligosaccharides, and biological molecules that carry the antigens are called ABH(O) substances. Predominant ABH(O) substances are membranous glycoproteins and glycolipids on red blood cells, and

are water soluble glycoproteins in body fluids (1,2,3). About 76% of the population are found to secrete abundant ABH(O) antigens into the body fluids (secretors), and the remainder secrete a little or almost no antigens (non-secretors) (3,4,5). According to the current genetic model (6), it seems more likely that the difference in the number of the ABH(O) antigens per one ABH(O) substance molecule rather than the number of the substance molecules themselves is responsible for the difference between secretors and non-secretors. On the basis of this assumption, Katsumata and coworkers (7) firstly developed a two-site (sandwich) EIA for the water soluble A and B substances from secretors, in which method A or B substance was trapped onto solid phase coated with anti-A lectin or goat anti-B antiserum and then detected by the combination of human (anti-A or anti-B) antiserum and alkaline phosphatase-labelled anti-human IgM antibody.

Besides complexity in performance, sandwich EIAs in general seem preferable for forensic practices because the forensic specimens are often contaminated by other body fluids that contain endogeneous enzyme activities (e.g., alkaline phosphatase in the intestinal contents and peroxidase like activity of hemoglobin in blood), which interfere with the sandwich EIAs less than one-site EIAs that immobilize any potentially adsorptive materials to solid phase (ELISAs in a narrow sense). However, the sensitivities by the earlier sandwich EIA (7), for B substance in particular, were not enough for routine practices, and this was probably due to low binding capacity of crude polyclonal antisera (8). Katsumata and coworkers (9) and we (10) therefore employed affinity-purified antibodies and achieved higher sensitivities (the detection limits were about 10^6-fold dilution for secretor saliva samples), which were comparable to those achieved by the ELISAs (8,11,12).

In this paper, we describe two types of EIA for water soluble B substance (named sandwich capture EIA I and EIA II), which offered more simplicity or a higher sensitivity.

MATERIALS AND METHODS

Blood Group Substances and Samples

Artificially constructed blood group substances (Syntagen^R, Chembiomed, Edmonton, Canada) were produced by coupling chemically synthesized A, B, H(O) type 1 and H type 2 trisaccharides to bovine serum albumin (BSA) at a molar ratio (trisaccharides/BSA) of 14 to 16 (13). The amount of the substances was calculated as the amount of BSA molecule at an absorbance of 280 nm (10).

Saliva samples were gathered from secretor and non-secretor volunteers. After filtrating (0.45 μm), they were stored at –80°C until use as described previously (10).

Preparation of Affinity-Purified Dinitrophenyl Goat Anti-B IgG and Affinity-Purified Goat Anti-B IgG-Peroxidase Conjugate

A goat anti-B antiserum was a gift from Tokyo Standard Serum, Tokyo, Japan. According to the manufacture, a crude antiserum was raised by immunization with tortoise red cells that carried B antigen, and the antiserum was then incubated with human type A and O red blood cells to remove antibodies that elicit nonspecific hemagglutination. After removing the cell particles by centrifugation, the supernatant was diluted with saline to give a hemagglutination titer of 256, and shipped as a final product. Together with a rabbit anti-A antiserum, these antisera have been used in Japan instead of hazardous antisera of human origin. IgG was fractionated from the anti-B antiserum by salt precipitation followed by DEAE-cellulose column chromatography (14).

The anti-B IgG was mercaptosuccinylated using *S*-acetylmercaptosuccinic anhydride and was subsequently reacted with maleimide-dinitrophenyl-L-lysine, which had been prepared by reacting *N*-succinimidyl-6-maleimidehexanoate (Dojindo Laboratories, Kumamoto, Japan) on dinitrophenyl-L-lysine (Tokyo Kasei Kogyo Co., Tokyo, Japan) (15). On average, 15 dinitrophenyl groups were introduced into one IgG molecule. The amount of the dinitrophenyl IgG was calculated from absorbances of 280 and 360 nm (15).

The anti-B IgG was conjugated with horseradish peroxidase (grade I, Boehringer Mannheim GmbH, Mannheim, Germany) (10) through the reaction between SH groups introduced into the IgG (14) and maleimide groups introduced into the peroxidase (16). On average, 2.5 peroxidase molecules were introduced into one IgG molecule. The amount of the conjugate was calculated from its peroxidase activity (14).

The anti-B dinitrophenyl IgG (10.5 mg) was affinity-purified by elution at pH 2.5 from a column (3 × 7 mm) of B substance-Sepharose 4B, which had been prepared by coupling artificially constructed B substance (1 mg) to CNBr-activated Sepharose 4B (0.1 g, Pharmacia Biotech, Uppsala, Sweden) (10). The amount of affinity-purified dinitrophenyl IgG was 190 μg (1.8%), and, in order to eliminate trace amount of the B substance that were leaked from the affinity column, the dinitrophenyl IgG was subjected to gel filtration on a column (1.0 × 45 cm) of Ultrogel AcA 34 (IBF Biotechnics, Villeneuve-la-Garenne, France).

The anti-B IgG-peroxidase conjugate (23.2 mg) was also affinity-purified by elution at pH 2.5 from another B substance-Sepharose 4B column (3 × 7 mm). The amount of affinity-purified IgG-peroxidase conjugate was 220 μg (0.95%), and the conjugate was applied to an Ultrogel AcA 34 column (1.5 × 100 cm).

Buffer

A buffer consisting of 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl (NaPB-NaCl) was used regularly in the following procedures.

Preparation of Polystyrene Balls Coated with Affinity-Purified Anti-Dinitrophenyl BSA IgG

IgG was fractionated (14) from goat anti-dinitrophenyl BSA antiserum (Miles Laboratories, Elkhart, IN, USA). The anti-dinitrophenyl BSA IgG (26.2 mg) was affinity-purified by elution at pH 2.5 from a column (3 × 12 mm) of dinitrophenyl BSA-Sepharose 4B column, which had been prepared by coupling dinitrophenyl BSA (10 mg, the average number of dinitrophenyl groups per BSA molecule was 9.9) to CNBr-activated Sepharose 4B (1 g) (17). The amount of the affinity-purified IgG was 0.89 mg (3.4%).

Polystyrene balls (3.2 mm in diameter, Immuno Chemical, Okayama, Japan) were coated with the affinity-purified IgG by physical adsorption (18). Balls were immersed in 0.1 mg/mL of the IgG in 0.1 mol/L sodium phosphate buffer, pH 7.5 at 4°C for 16 h. After removing the IgG solution (this solution was able to be used for further twice coating), the balls were stored in NaPB-NaCl containing 1 g/L BSA and 1 g/L NaN_3 at 4°C. Just prior to use, the balls were washed several times with NaPB-NaCl containing 1 g/L BSA to remove NaN_3.

Sandwich Capture EIA I and EIA II

The common process to the two EIAs: B substance in NaPB-NaCl containing 1 g/L BSA (150 μL) was incubated with affinity-purified dinitrophenyl anti-B IgG (100 fmol, 15 ng) and affinity-purified anti-B IgG-peroxidase conjugate (100 fmol, 25 ng) in the same buffer (10 μL) at 20°C for 3 h and at 4°C for 16 h.

Subsequently, the reaction mixture was incubated with one affinity-purified anti-dinitrophenyl BSA IgG-coated polystyrene ball at 20°C for 3 h with continuous gentle shaking.

Sandwich capture EIA I: Following the common process, the ball was washed twice by addition and aspiration of 2 mL of NaPB-NaCl, and then peroxidase activity bound to the ball was assayed in another test tube at 30°C for 60 min by fluorometry using 3-(4-hydroxyphenyl) propionic acid (Nakalai Tesque, Kyoto, Japan) as a hydrogen donor (18). The enzyme reaction was initiated by the addition of 0.015% (V/V) H_20_2 (50 μL) to the ball in 100 μL of 6 g/L 3-(4-hydroxyphenyl) propionic acid in 0.1 mol/L sodium phosphate buffer, pH 7.0, and was terminated with 2.5 mL of 0.1 mol/L glycine-NaOH buffer, pH 10.3. The fluorescence intensity was measured relative to 0.2 mg/L quinine in 50 mmol/L H_2S0_4 using 320 nm for excitation and 405 nm for emission in a fluorospectrophotometer (650-60, Hitachi, Tokyo, Japan). In this assay condition, 1 fmol of peroxidase generated a fluorescence intensity of 1,800.

Sandwich capture EIA II: Following the common process, the ball was washed once by addition and aspiration of 2 mL of NaPB-NaCl, and then incubated in 2 mL of NaPB-NaCl containing 1 g/L BSA at 30°C for 10 min with continuous gentle shaking. After washing once again with 2 mL of NaPB-NaCl, the ball was transferred to another test tube and incubated with 1 mmol/L dinitrophenyl-L-lysine in NaPB-NaCl containing 0.1 g/L BSA (100 μL) at 20°C for 30 min with continuous gentle shaking. Finally, the ball was removed, and peroxidase activity in the eluate (100 μL) was assayed at 30°C for 60 min. The enzyme reaction was initiated by the addition of a mixture of 30 g/L 3-(4-hydroxyphenyl) propionic acid in 0.1 mol/L sodium phosphate buffer, pH 7.0, (20 μL) and 0.025% (V/V) H_20_2 (30 μL), and was terminated with 2.5 mL of 0.1

mol/L glycine-NaOH buffer, pH 10.3. The fluorescence intensity was measured relative to a mixture of 1 mmol/L dinitrophenyl-L-lysine in NaPB-NaCl containing 0.1 g/L BSA (100 μL) and 0.2 mg/L quinine in 50 mmol/L H_2SO_4 (2.55 mL) as described above.

Hemagglutination Inhibition

Hemagglutination inhibition on a "U" bottom micro titer plate (96 wells) using mouse monoclonal anti-B IgM (Bioclone^R, Ortho Diagnostic System, Raritan, NJ, USA) was performed according to Fiori (19) as described previously (10). The method is based on the phenomenon that B substance was capable of neutralizing the hemagglutination activity of the anti-B IgM (3).

RESULTS

Detection Limit of Artificially Constructed B Substance

The sensitivities of the sandwich capture EIAs were assessed by artificial antigen techniques introduced by Le Pendu et al. (4,5). Artificially constructed B substance (chemically synthesized B trissacharides-BSA complex) was serially diluted to a final volume of 150 μL and measured by the sandwich capture EIA I. In this assay, the complex formed of B substance, dinitrophenyl anti-B IgG and anti-B IgG-peroxidase conjugate was trapped onto a polystyrene ball coated with anti-dinitrophenyl BSA IgG, and peroxidase activity bound to the ball was assayed by fluorometry. The detection limit[1] of artificially constructed B substance was determined as 0.67 pmol/L (0.1 fmol per 150 μL) (Figure 1). This was the same as that by the previous sandwich EIA (10) and 300-fold lower than that by the hemagglutination inhibition (600 pmol/L, 30 fmol per 50 μL).

In the sandwich capture EIA II, the complex trapped on the ball was, after thorough washing, further eluted with dinitrophenyl-L-lysine, and the peroxidase

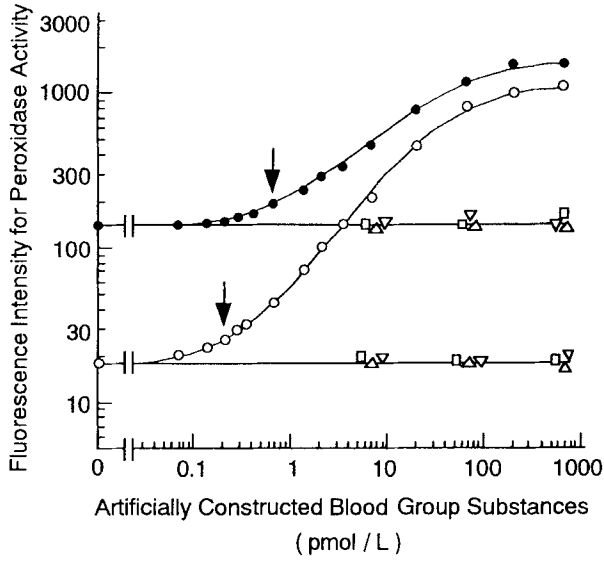

FIGURE 1 Dose-response curves of artificially constructed substances by the sandwich capture EIA I (closed symbols) and EIA II (open symbols). Squares, circles, triangles and inverted triangles indicate the curves for A, B, H(O) type 1 and H type 2 substances, respectively. The detection limits are indicated by arrows.

activity in the eluate was assayed. The detection limit (0.2 pmol/L, 0.03 fmol per 150 μL)[2] was improved 3.3-fold as compared with the EIA I (Figure 1). As the result, the detection limit of the EIA II was 1,000-fold lower than the hemagglutination inhibition.

Specificity of the Sandwich Capture EIAs

Artificially constructed A, H(O) type 1 and H type 2 substances were measured by the sandwich capture EIA I and EIA II. There was no increase in peroxidase activity with up to 670 pmol/L (100 fmol per 150 μL) of the substances in both EIAs (Figure 1).

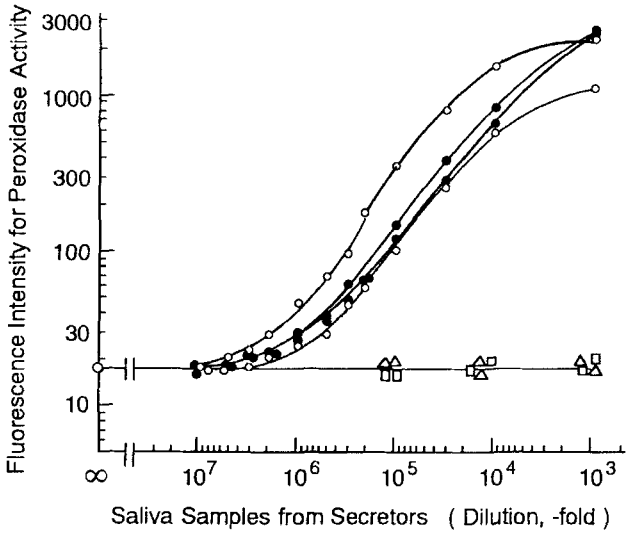

FIGURE 2 Dilution curves of saliva samples from secretors by the sandwich capture EIA II. Squares, open circles, closed circles and triangles indicated the curves for type A, B, AB and O individuals, respectively.

Sandwich Capture EIA II for Secretor Saliva

Saliva samples from type B and AB secretors were serially diluted to a final volume of 150 μL and subjected to the sandwich capture EIA II. The detection limit of B substance was determined as 5×10^5- to 2×10^6-fold dilution (Figure 2). This was 200- to 500-fold more sensitive than that by the hemagglutination inhibition (1×10^3- to 5×10^3-fold). No obvious increase in fluorescence intensity for peroxidase activity was observed in type A and O secretor saliva, indicating no cross-reaction with A and H(O) substances in secretor saliva (Figure 2).

Assay Variation of the Sandwich Capture EIA II

In the sandwich capture EIA II, the variation coefficient of intra-day assay and inter-days assay at three different dilutions (1×10^4- to 1×10^6-fold) of one saliva

sample from a type B secretor, for which sample the detection limit was determined as 2×10^6-fold dilution, were 7.8-11.7% (n=15) and 11.4-14.3% (n=12), respectively.

DISCUSSION

In a previous sandwich EIA for water soluble B substance (10), we had prepared goat anti-B Fab'-peroxidase conjugate and then affinity-purified it using a B substance-Sepharose 4B column. However, a yield of the affinity-purified conjugate was considerably lower (0.05%, unpublished data) than a value (2.2%) that obtained from the unmodified parent IgG (10). In contrast, the yields of affinity-purified IgG-peroxidase conjugate were 0.55% (10) and 0.95% in this experiment, which were acceptable by considering spatial interferences due to the introduced peroxidase molecules. Introduction of smaller molecules interfered less with the antigen-binding activity, resulting in a better yield (1.8%) for the affinity-purified dinitrophenyl IgG. Thus, we gave up the goat anti-B Fab'-peroxidase conjugate and used the IgG equivalent. Employment of Fab'-peroxidase conjugate was also not justified for rabbit anti-A antibody because a yield in affinity-purification (0.16%, unpublished data) was rather lower than that for the unmodified parent IgG (4.9%) and the IgG-peroxidase conjugate (1.1%) (10). The disappointing results for the Fab'-peroxidase conjugates may be due to lower functional affinity of monovalent Fab', but this seems questioned by Romano's findings that both bivalent anti-A IgG and its Fab fragment (not Fab') had similar functional affinity for type A red blood cells (20).

The background of the previous sandwich EIA (10), in which a polystyrene ball coated with affinity-purified anti-B IgG was successively incubated with B substance and with affinity-purified anti-B IgG-peroxidase conjugate, was about 10-fold higher than that usually observed for Fab'-peroxidase conjugates. The

detection limit of artificially constructed B substances (chemically synthesized B trisaccharides-BSA complex) was hence about 10-fold worse (0.1 fmol as BSA molecule per assay) than that attainable (0.005-0.01 fmol per assay) by employing affinity-purified Fab'-peroxidase conjugate (21, 22). The goal of this current assay format was to alleviate the drawback pertaining to the IgG-peroxidase conjugate, which we did with the sandwich capture EIA II format.

It had been hoped that a reaction of antibody to corresponding antigen would occur more efficiently when they were both within a solution than when either was immobilized to solid phase. This was a conception of developing the sandwich capture EIA I. However, the sensitivity was not improved (0.67 pmol/L, 0.1 fmol per assay) because an increase in the specific binding[3] of the IgG-peroxidase conjugate was cancelled out by a high background due to an unexpected increase in the nonspecific binding. Simplicity in performance (one washing step) was an advance of the EIA I over the previous sandwich EIA (two washing steps) (10).

We then incorporated a short time immersion of a ball in a buffer within the washing step of the EIA I. This decreased the background more (1.9-fold) than the specific binding (1.3-fold, an average value calculated from 0.67 - 20 pmol/L of artificially constructed B substance). Further, the complex of B substance and anti-B antibodies (dinitrophenyl IgG and IgG-peroxidase conjugate), which had been captured onto the ball with anti-dinitrophenyl BSA IgG, was eluted with free dinitrophenyl groups. The peroxidase activity for the specific binding was well recovered with a decrease of 1.7-fold, whereas the background decreased further 4.1-fold. In all, the background in the EIA II was decreased 7.8-fold, but a decrease in the specific binding was 2.2-fold, resulting in 3.3-fold improvement of the final detection limit (0.2 pmol/L, 0.03 fmol per assay) (Figure 1).

The principles of the sandwich capture EIA I and EIA II for water soluble B

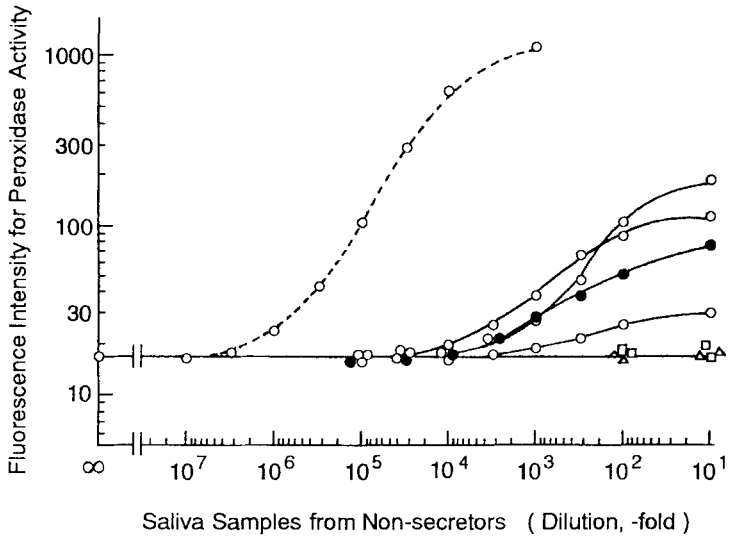

FIGURE 3 Dilution curves (solid lines) of saliva samples from non-secretors by the sandwich capture EIA II. Squares, open circles, closed circles and triangles indicated the curves for type A, B, AB and O individuals, respectively. For comparison, a curve (broken line) for a B secretor sample is also depicted.

substance appear to be applicable to the measurement of water soluble A substance in secretor saliva. However, in common with other sandwich EIAs (7,9,10), non-secretor saliva seems unsuitable for the sandwich capture EIAs because dilution curves depicted for type B and AB non-secretor saliva samples, though the number of samples examined was limited, were not sufficiently steep and did not parallel each other (Figure 3).

To conclude, we recommend the EIA I for actual forensic specimens (stain extracts) because it is more simple than both the previous sandwich EIA and the EIA II. When conclusive results cannot be obtained by the EIA I, the 3-fold enhanced sensitivity of the EIA II may be useful for further checking.

FOOTNOTES

1 The detection limit of B substance was expressed as the minimal amount of artificially constructed B substance or the maximal dilution of saliva that gave a peroxidase activity significantly in excess of that in the absence of B substance (background due to the nonspecific binding of the IgG-peroxidase conjugate). The existence of a significant difference from the background was confirmed by the Student's t-test ($n=5$, $p<0.01$).

2 When a more strict criterion ($p<0.001$) was applied, the detection limit was 0.27 pmol/L (0.04 fmol per assay) and the improvement was hence 2.5-fold.

3 The specific binding of anti-B IgG-peroxidase conjugate was calculated by subtracting fluorescence intensity for peroxidase activity nonspecifically bound in the absence of B substance (background due to the nonspecific binding of the conjugate) from that bound in the presence of the substance.

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