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Detection of Le^a Substance in Saliva Stains by Enzyme-Linked Immunosorbent Assay (ELISA) Using Anti-Gum Arabic Serum

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ABSTRACT: It is known that rabbit anti-gum arabic (GA) serum has cross-reactivity with Le^a antigen, and that, by using this cross-reactive anti-Le^a antibody, the presence of Le^a antigen in red blood cells and saliva can be demonstrated with accuracy. We have devised a rapid and highly sensitive method for detecting Le^a substance in human saliva by the enzyme-linked immunosorbent assay (ELISA) method using an anti-Le^a antibody isolated from anti-GA serum by affinity chromatography on Synsorb Le^a. The ELISA plate, coated with the specific anti-Le^a antibody, adsorbed the Le^a substance in saliva which was subsequently identified by adding enzyme labeled anti-Le^a IgG in that order. The method could detect the Le^a substance in Le(a+) saliva stains as small as 0.1 by 0.1 cm in size that had been stored at room temperature for three weeks and in Le(a+) saliva stains 0.7 by 0.7 cm in size that had been stored for ten years. This method seems to be useful for quantitative analyses of the Le^a substance in various body fluids.

KEYWORDS: pathology and biology, immunology, enzyme-linked immunosorbent assay, saliva, Lewis blood group system, gum arabic

It is known that a Le^a-like substance is present in gum arabic (GA) and that anti-GA serum has cross-reactivity with the Le^a antigen [*1,2*]. Narita [*3*] has shown that a specific anti-Le^a antibody can be isolated from rabbit anti-Le^a serum by affinity chromatography on Synsorb Le^a. Using this cross-reactive anti-GA serum, the presence of Le^a antigen in red blood cells and saliva can be demonstrated with 100% accuracy.

On the other hand, because of its simplicity and high sensitivity, the enzyme-linked immunosorbent assay (ELISA) has increasingly been used in the field of legal medicine [*4-7*].

In this paper, we have attempted to devise a new and rapid ELISA method for the detec-

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tion of Le^a substance in a trace amount of human saliva using a specific anti-Le^a antibody isolated from anti-GA serum.

Materials and Methods

Saliva Samples

Fifty-six saliva samples were collected from healthy individuals. The saliva was placed in a boiling water bath for 30 min immediately after collection and then centrifuged. The clear supernatant was removed and stored at 4°C. Saliva stains were made on pieces of filter paper (Whatman, No. 2), 0.1 by 0.1, 0.3 by 0.3, 0.5 by 0.5, 0.7 by 0.7, and 1.0 by 1.0 cm in size. Three-week-old saliva stains were extracted with 150 μL of 0.01*M* phosphate buffered saline (pH 7.2) containing 0.05% Tween 20 (PBS Tween) for 18 h. Some saliva stains 0.7 by 0.7 cm in size that had been stored at room temperature for two, four, and ten years were also extracted with 150 μL of PBS Tween for 18 h.

Blood Grouping

The ABO group and Lewis type of each donor's red blood cells were determined by the use of standard serological techniques with commercial antisera (Ortho Diagnostic Systems, Raritan, New Jersey).

Anti-GA Sera

Gum arabic (GA) was purchased from Sigma Chemical Co., St. Louis, MO. Anti-GA sera were raised in rabbits by intramuscular injection of 1 mL of 30% GA solution emulsified in Freund's complete adjuvant. Sera were collected after five injections had been given at one-week intervals.

Isolation of the Specific Anti-Le^a Antibody from Rabbit Anti-GA Sera

Bio-synsorb Le^a was obtained from Chembiomed LTD., Edmonton, Alberta, Canada. Anti-GA serum (3 mL) was applied to a Bio-synsorb Le^a column (size: 1.4 by 5.8 cm) equilibrated with PBS. Unbound proteins were washed with PBS and specifically bound antibodies were eluted with 2% ammonium hydroxide from a Bio-synsorb Le^a column. The eluted fractions were dialyzed against PBS for 18 h. The final protein concentration in the eluted fractions was 0.70 mg/mL [*3,8*].

Coupling of Alkaline Phosphatase to Immunoglobulin G (IgG) in the Anti-Le^a Antibody

Immunoglobulin G (IgG) in the anti-Le^a antibody isolated from anti-GA serum was purified by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). The eluted fraction from the Synsorb Le^a column was applied to a protein A column. IgG purified as described previously [*9*] was dialyzed and made into crystals by freeze-drying.

Coupling of alkaline phosphatase (ALP) (Type VII-S, Sigma) to the anti-Le^a IgG was done by the use of glutaraldehyde according to Engvall and Perlman [*10*]. In brief, ALP (5.3 mg/mL) was first dialyzed against PBS for 18 h. Glutaraldehyde in PBS was added to the ALP solution. The final concentration of glutaraldehyde was 0.2%. The incubation was done at 25°C for 1 h. The coupling was then performed by incubating 2 mg of anti-Le^a IgG with the glutaraldehyde treated enzyme at 25°C for 1 h. The reaction mixture was added to an amount of PBS four times greater in volume and was finally fractionated on a 1.5- by

90-cm column of Sepharose CL-6B (Pharmacia) in 0.05*M* Tris-hydrochloric acid (HCl) (pH 8.0) buffer.

Figure 1 shows the fractionation of a preparation of enzyme labeled anti-Le^a IgG. The chromatogram revealed three protein peaks (P_1, P_2, and P_3). ALP recorded by its enzyme activity (absorbance at 405 nm) was fractionated from P_1 to P_2. Preliminary experiments revealed that fractions between P_1 and P_2 (fraction number 30 to 49) used in the ELISA system showed the greatest reaction against Le(a+b−) saliva. So these fractions (protein concentration: 0.073 mg/mL) were used in the ELISA reaction system.

ELISA Procedure

Figure 2 illustrates the principle of the present ELISA method. The wells of a flat bottomed polystyrene microtiter plate (129B, Dynatech Ltd., Sussex, England) were coated with 100 μL of a 40-fold diluted solution of the specific anti-Le^a antibody isolated by Synsorb

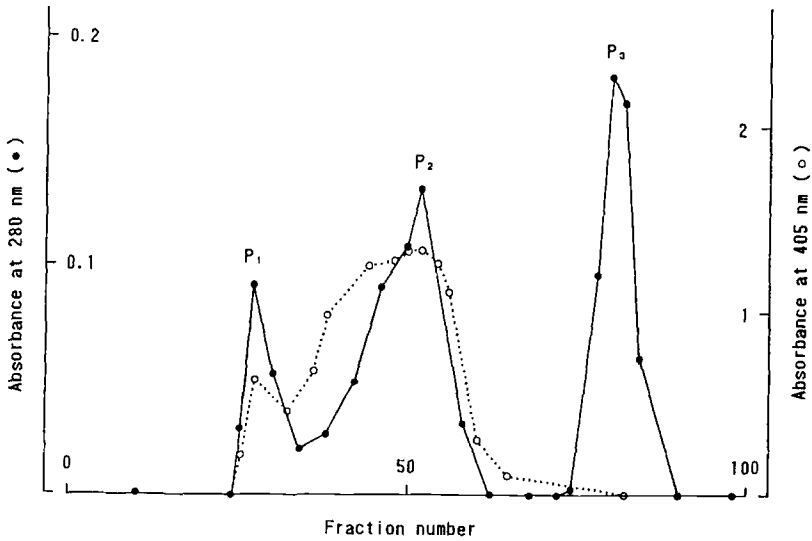

FIG. 1—*Fractionation of a preparation of enzyme labeled anti-Le^a IgG on a 1.5 by 90-cm column of Sepharose CL-6B. Fraction volume: 4 mL, flow rate: 15 mL/h.*

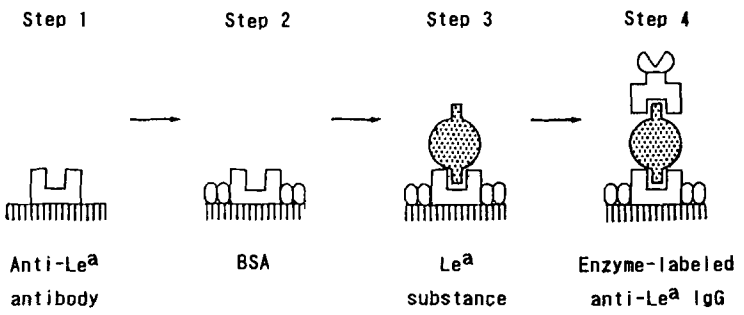

FIG. 2—*Principle of the present ELISA method.*

Le^a column chromatography in 0.05*M* sodium carbonate (pH 9.6) buffer and left at 4°C for 18 h (Step 1). The plate was washed 3 times with PBS Tween, then filled with 150 μL of blocking buffer (PBS Tween containing 1% bovine serum albumin), and left at 25°C for 1 h (Step 2). Following removal of the blocking buffer, 100 μL of PBS Tween containing extracts of either saliva stains or of a 10-fold diluted solution of Ortho Lewis Blood Group Substance (Lot LS114) as standard was added to each well and incubated at 25°C for 2 h (Step 3). After washing 3 times with PBS Tween, 100 μL of a 40-fold diluted solution of IgG coupled with ALP in PBS Tween was added to each well and incubated at 25°C for 1.5 h (Step 4). The plate was washed 5 times with PBS Tween. Finally, a reaction medium (100 μL; 1*M* diethanolamine containing 1m*M* magnesium chloride, pH 9.3) and a substrate solution (10 μL; 0.1*M* *p*-nitrophenyl phosphate in 0.001*N*√HCl) were added to the wells and incubated at 37°C for 2 h. The enzyme reaction was stopped by adding 100 μL of 0.5*N* sodium hydroxide (NaOH). Absorbances were measured at 405 nm with a Dynatech MICROELISA autoreader. The amount of Le^a substance in the extracts of the saliva stains was calculated as: (absorbance at 405 nm of the extracts/that of standard) × 100 (%).

Results

Table 1 and Fig. 3 show the area (amount) effect of ELISA reactions of Le(a+b−), Le(a−b+), and Le(a−b−) saliva stains. The Le^a substances calculated were significantly higher in the Le(a+b−) ($n = 4$) than in the Le(a−b+) ($n = 3$) and Le(a−b−) ($n = 2$) saliva stains of every area prepared. The amount of Le^a substances in the Le(a−b+) stains were somewhat higher than in the Le(a−b−) stains, but not significantly. Therefore, the anti-Le^a antibody used in the ELISA system did not have cross-reactivity with the Le^b antigen. The present ELISA method could detect Le^a substance in extracts of Le(a+) saliva stains as small as 1 mm^2.

As shown in Table 2 and Fig. 4, the amount of Le^a substance in each Le(a+b−) saliva stain of 1 by 1 mm ($n = 17$) was higher than 20%, and that of each Le(a−b+) ($n = 28$) and Le(a−b−) ($n = 11$) stain was lower than 15%. Therefore, Le(a+b−) saliva stains could be differentiated from Le(a−b+) and Le(a−b−) by the present ELISA method. Le^a substances were not significantly different among the ABO blood groups. So, the anti-Le^a antibody isolated from anti-GA antibody did not have cross-reactivity with ABH antigens.

Table 3 shows the percentage of Le^a substances in saliva stains stored for a long period. When 7 by 7 mm of saliva stains were analyzed by the present ELISA method, Le^a substance was found in Le(a+b−) saliva stains stored for as long as ten years.

Discussion

Narita [3], one of the authors of this study, has shown that the anti-Le^a antibody isolated from anti-GA serum by affinity chromatography on Synsorb Le^a agglutinates all papain

TABLE 1—*Area (amount) effect of ELISA reactions of Le(a+) and Le(a−) saliva stains.*

Size of Stain, mm^2	Le(a+b−)		Le(a−b+)		Le(a−b−)	
	n	Le^a, %	*n*	Le^a, %	*n*	Le^a, %
100	4	77.0 ± 3.4	3	12.8 ± 6.3	2	7.0 ± 0.7
49	4	70.6 ± 1.2	3	12.1 ± 6.3	2	6.8 ± 0.4
25	4	67.0 ± 2.8	3	12.0 ± 3.2	2	6.8 ± 0.5
9	4	56.8 ± 2.9	3	9.7 ± 2.6	2	7.0 ± 0.4
1	4	37.6 ± 4.0	3	8.8 ± 1.1	2	6.7 ± 0.5

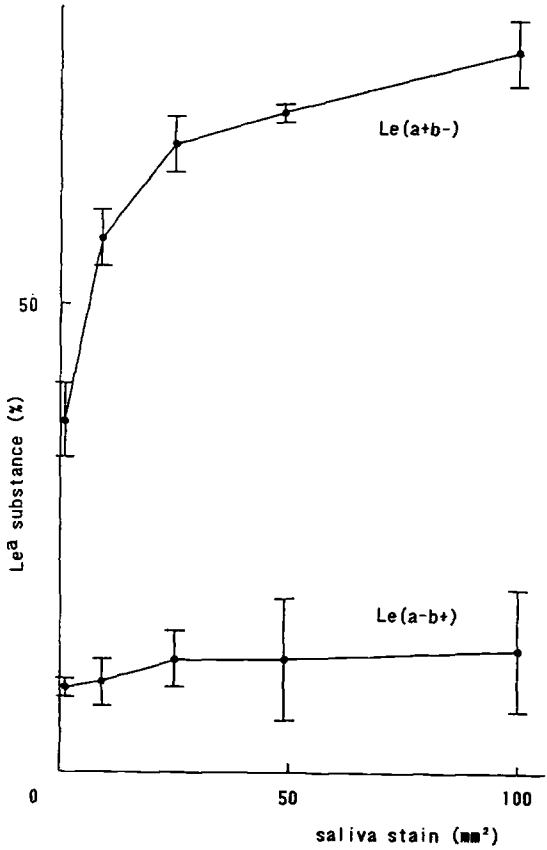

FIG. 3—*Area (amount) effect of ELISA reactions of Le(a+b−) and Le(a−b+) saliva stains.*

TABLE 2—*Comparison of Le(a+) with Le(a−) saliva stains (0.1 by 0.1 cm in size) on the ELISA activity among ABO blood groups.*

	Le(a+b−)		Le(a−b+)		Le(a−b−)	
Blood Group	*n*	Le^a, %	*n*	Le^a, %	*n*	Le^a, %
A	6	33.5 ± 7.6	7	7.9 ± 0.7	5	7.1 ± 0.6
B	3	43.0 ± 6.5	8	8.1 ± 2.0	2	6.7 ± 0.3
O	5	37.0 ± 8.5	8	9.1 ± 2.8	2	6.3 ± 0.2
AB	3	35.7 ± 9.3	5	8.2 ± 1.2	2	7.0 ± 1.9
Total	17	36.6 ± 8.7	28	8.4 ± 2.0	11	6.8 ± 1.0

treated Le(a+b−) erythrocytes, but does not agglutinate papain treated Le(a−b+) or Le(a−b−) erythrocytes without exception. He has also shown that it only precipitates saliva samples in which the Le^a antigen is present. Our results indicate that the anti-Le^a antibody isolated from anti-GA serum does not have cross-reactivity with the ABH and Le^b antigens in samples of saliva stain (Tables 1 and 2 and Figs. 3 and 4). Therefore, it is evident that the antibody used in this study reacts specifically with the Le^a antigen in erythrocytes and saliva.

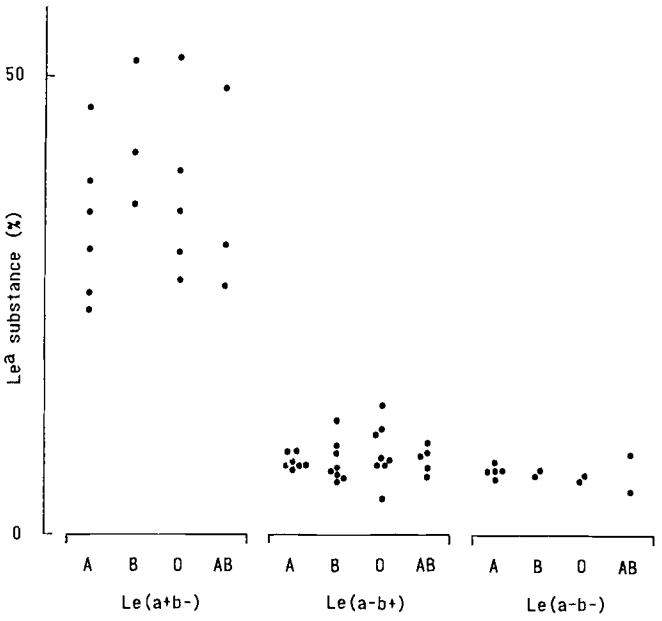

FIG. 4—*Comparison of Le(a+) with Le(a−) saliva stains (0.1 by 0.1 cm in size) on the ELISA activity among ABO groups.*

TABLE 3—*Comparison of Le(a+b−) with Le(a−b+) saliva stains (0.7 by 0.7 cm in size) stored at room temperature for several years.*

Saliva Stain (49 mm^2)	Le(a+b−)		Le(a−b+)	
	n	Le^a, %	*n*	Le^a, %
2-year-old	1	42.8	2	10.5 ± 0.6
4-year-old	3	32.3 ± 4.1	3	11.8 ± 3.1
10-year-old	4	31.9 ± 9.7	2	7.7 ± 0.4

On the other hand, ELISA methods for detecting ABH blood group substances in human saliva have been devised [5–7]. The results presented above indicate that the rapid and sensitive ELISA technique can be also useful for the detection of Le^a substance in a trace amount of saliva. The present ELISA method enables the detection of Le^a substance in samples of Le(a+) saliva stain as small as 1 mm^2 (Tables 1 and 2 and Figs. 3 and 4) or those stored for ten years (Table 3).

This method seems to have the following advantages. First, quantitative analysis of the Le^a substance can readily be made by using appropriate standards. Second, the method does not require any expensive reagents or antibodies. Third, this modified sandwich ELISA method can be performed even if only one antibody is prepared.

When saliva is used for the identification of Lewis blood groups, it should be taken into account that Le^a substance is also present in the saliva of the majority of Le(a−) persons [1,2], and some Le(a−) salivas seem to show relatively high amounts of Le^a substance [3,11–13]. However, usually Le(a+) saliva stains can be easily differentiated from Le(a−) stains using the present ELISA method.

All these advantages should encourage the wide use of the ELISA method for the detection of Le^a substance in various body fluids.

Furthermore, it is well known that Le^a-like substances are present in the saliva of nonhuman primates [*14*], cattle [*15*], and rabbits [*16*]. This ELISA method appears to be also useful for characterization of these Le^a-like antigens in body fluids of different species.

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