Takehiko Takatori,[1] *M.D.; Yoshimitsu Tustsubuchi,*[1] *B.Sc.; and Koichi Terazawa,*[1] *M.D.*

Lewis Typing of Human Bloodstains by Enzyme-Linked Immunosorbent Assay (ELISA) Using Monoclonal Anti-Le^a and Anti-Le^b

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ABSTRACT: The Lewis blood grouping of human dried bloodstains could be determined by an enzyme-linked immunosorbent assay (ELISA) using monoclonal anti-Le^a and anti-Le^b antibodies with an avidin-biotin complex (ABC). The bloodstains aged 1 year were used as samples, and approximately 1 mg of the stains was enough to type each Lewis antigen reliably by this method. The Lewis substances of 106 individual stains were correctly typed regardless of their ABO blood group system.

KEYWORDS: pathology and biology, genetic typing, antigens, Lewis antigens, bloodstains, ELISA, monoclonal antibodies, avidin-biotin complex (ABC)

Lewis blood grouping has been used in forensic science practice to confirm the ABO secretor status of an individual. Fresh red blood cells are routinely typed for their Lewis phenotypes, but the literature contains conflicting studies on the presence of Lewis substances in secretions by use of traditional Lewis typing procedures, such as absorption-inhibition and absorption-elution tests [*1,2*]. Although the Lewis antigens in saliva are reported to be typed by radioimmunoassay or peroxidase-antiperoxidase immunoassay using polystyrene beads [*3*], the Lewis grouping of dried bloodstains is well-known to be most difficult.

Davie [*4*] reported that the Lewis system of bloodstains over two months old was untypable by absorption-inhibition on microtiter plates. Sallee et al. [*5*] also reported that neither the conventional absorption-inhibition test nor the absorption-elution method was suitable to type the Lewis system of dried bloodstains correctly. Takizawa et al. [*6*] prepared highly titrable anti-Le^a and anti-Le^b sera by immunization of rabbits with human salivas [*7*], and reported that even their antisera were not enough to type the Lewis system of considerably large amounts of bloodstains by the absorption-elution test.

The use of the avidin-biotin complex (ABC) method in an enzyme-linked immunosorbent assay (ELISA) was indicated to be more sensitive than conventional enzyme immunoassays using reagents directly labeled enzymes [*8-10*]. In this paper we successfully applied the ABC technique with monoclonal anti-Le^a and anti-Le^b antibodies in ELISA to Lewis typing of one-year-old bloodstains.

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[1]Professor, research student, and associate professor, respectively, Department of Legal Medicine, Hokkaido University School of Medicine, Sapporo, Japan.

Materials and Methods

Chemicals

Flat-bottomed polystyrene microtiter plates (Immuno Plate I) were obtained from Nunc, Denmark. Murine anti-Le^a and anti-Le^b monoclonal antibodies were supplied by Biotest-Serum-Institut GmbH, West Germany. Biotinylated horse anti-mouse IgG(H + L) and biotinylated rabbit anti-goat IgG(H + L) which were purified by affinity chromatography and a standard Vectastain ABC kit were purchased from Vector Laboratories, Burlingame, CA. Lewis substances from human saliva, goat anti-Le^a and anti-Le^b polyclonal antisera against human saliva, and human anti-A and anti-B antisera were obtained from Ortho Diagnostic Systems, Raritan, NJ. All other reagents were obtained from Nakarai Chemical, Ltd., Japan.

Samples

One hundred and six whole fresh blood samples were collected venously from healthy individuals. Bloodstains were prepared by drying the blood samples in crucibles at room temperature, and stocked for a year at room temperature.

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

ELISA procedure

The principle of the reaction system of the ELISA using the ABC technique was described in detail previously [9]. The standard ELISA procedure was carried out as follows.

Step 1—Wells of a flat-bottomed microtiter plate were first coated with the Lewis substance as antigen, 100 μL/well of a twofold solution in 0.01*M* carbonate buffer (pH 9.6), and left for 2 h at room temperature. The plate was washed once with 0.01*M* phosphate-buffered saline (PBS) containing 0.02% sodium azide (pH 7.4), and then filled with PBS containing 1% gelatin for blocking and left for 10 min at room temperature.

Step 2—The dilution of anti-Le^a (1:500) and anti-Le^b (1:5000) monoclonal antibodies was first made with PBS containing 0.1% gelatin (PBSG). Some amounts of the bloodstains were added to each tube containing either the diluted anti-Le^a or anti-Le^b antibody (250 μL), and no bloodstain was added to a control tube. The tubes were covered with a plastic film and absorption was done at 37°C for 2 h and then at 4°C overnight; this procedure had been started the day before the ELISA reaction.

After removal of the blocking solution from the wells, 100-μL aliquots of the incubation mixture (250 μL) containing the unabsorbed anti-Le^a or anti-Le^b antibody were transferred in duplicate into individual wells and incubated at 37°C for 1 h; the plates that the anti-Le^a and anti-Le^b antibodies were used in the reaction system were designated as the A and B plates, respectively.

Step 3—After washing three times with PBSG, 100 μL of biotinylated horse anti-mouse IgG(H + L) (10-μg protein/mL in PBSG) were added to the wells.

Step 4—Following incubation for 30 min at 37°C, the plate was washed three times with 0.01*M* Tris-buffered saline (pH 7.4, TBS), and 100 μL of the ABC solution, which was prepared by the addition of 5 μL each of A and B reagents of a Vectastain ABC kit to 1 mL of TBS, were added to the wells.

Step 5—Following incubation for 15 min, the plate was again washed with TBS and 150 μL of a chromogenic solution were added to the well as described elsewhere [9].

Step 6—After incubation for 30 min under a dark place, the enzymatic reaction of peroxidase was stopped by the addition of 50 μL of 2.5*N* sulfuric acid. The color developed in each

well was then read at 492 nm on a two-wavelength microplate photometer (Corona MTP-22, Japan).

The change in the absorbance of the sample well was represented as percentage with the following equation.

$$\% \text{ of absorbance} = I/I_0 \times 100$$

where I_0 = absorbance of the control well under the standard ELISA procedure, and I = absorbance of the sample well.

At the fourth and fifth stages of the reaction, TBS was used instead of PBS containing sodium azide because this preservative is known to decrease the activity of peroxidase.

When goat anti-Le^a and anti-Le^b polyclonal antisera were used as the primary antibody in the aforementioned ELISA system, biotinylated anti-goat IgG(H + L) was of course used as the secondary antibody.

Results and Discussion

In the present ELISA system, the addition of the Lewis substance from bloodstain to a constant amount of the corresponding antibody results in the competitive inhibition of the Lewis antigen (adsorbed to the surface of the well) -antibody complex. Therefore, if there is the Lewis blood group substance corresponding to the antibody, the final color development will be decreased, making it feasible to determine the Lewis group; the change in absorbance of less than 50% is judged to be a positive reaction.

The standard ELISA system used diluted anti-Le^a (1:500) and anti-Le^b (1:5000) without the stains as a control and gave approximately 1.0 of absorbance at 492 nm. The blank wells were incubated in the absence of either the primary antibodies or the Lewis antigen coated on the surface of the well; both of the absorbances were only 0.05 to 0.08. The 10-min blocking was therefore sufficient in the ELISA system, indicating that there was little nonspecific binding of not only the highly diluted antibodies but the ABC reagent.

Figure 1 shows relationships between amounts of dried bloodstains and changes in absorbance. Individual Le(a+b−) and Le(a−b+) bloodstains (0.75, 1.5, 3.0, and 6.5 mg each) were added to 250 μL of the diluted anti-Le^a or anti-Le^b antibody. The following ELISA procedure was done as described in the method.

In the A plate, the percent of inhibition of absorbance by the addition of 2.6 mg of the Le(a−b+) bloodstain per well was observed to be only 24, whereas the addition of 0.3, 0.6, 1.2, and 2.6 mg of the Le(a+b−) bloodstains per well produced, respectively, 54, 42, 31, and 13% as the change in absorbance (Fig. 1*a*). Likewise, the changes in absorbance in the B plate were shown in Fig. 1*b*; the percent of inhibition of absorbance by the same addition of 2.6 mg of the Le(a+b−) bloodstain was 47, whereas the addition of 0.3, 0.6, 1.2, and 2.6 mg of the Le(a−b+) bloodstains per well produced, respectively, 47, 36, 24, and 17% as the change in absorbance. From these results, when approximately 1 mg of dried bloodstains corresponding to the primary antibodies was used in the ELISA system, the change in absorbance in both the A and B plates was shown to be less than 50%. Therefore, in the present ELISA system, 1 mg of individual dried bloodstain samples per well was used.

Figures 2 and 3 show the percent of changes in absorbance in the A and B plates among 106 bloodstain samples. In the A plate the changes in absorbance for Le(a−) bloodstains were observed to be in the range of 61 to 99%, while those for Le(a+) substances were in the range of 19 to 45% (Fig. 2). Likewise, in the B plate, the changes in absorbance for Le(b−) bloodstains were maintained to be in the range of 63 to 92%, while those for Le(b+) samples were shown to be in the range of 11 to 47% (Fig. 3). These results indicate that the changes in absorbance against the Lewis antigens corresponding to both anti-Le^a and anti-Le^b antibodies were invariably less than 50%. For those bloodstains lacking Lewis antigens, corre-

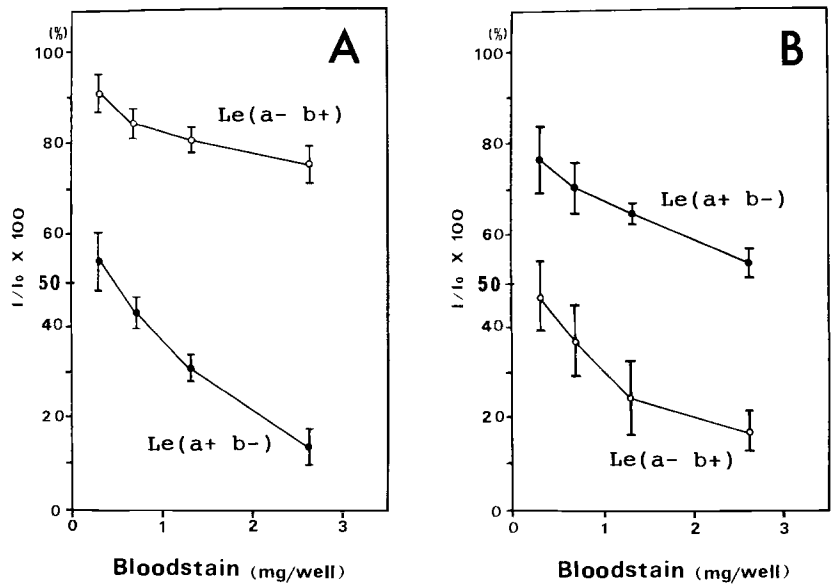

FIG. 1—*Percentage absorbance on the ELISA inhibition assay by the addition of increasing amounts of bloodstains to the A plate* (a) *and the B plate* (b). *Values reported are the mean ± standard deviations of five independent determinations.*

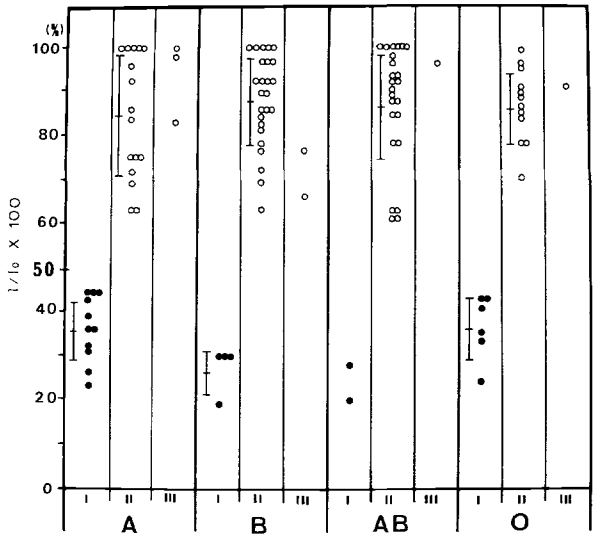

FIG. 2—*Percentage absorbance on the ELISA inhibition assay by the addition of 106 bloodstain samples among ABO blood groups to the A plate. Values reported are the mean ± standard deviations of 4 to 25 independent determinations.* ●: *Le(a+)*, ○: *Le(a−)*, I: *Le(a+b−)*, II: *Le(a−b+), and* III: *Le(a−b−)*.

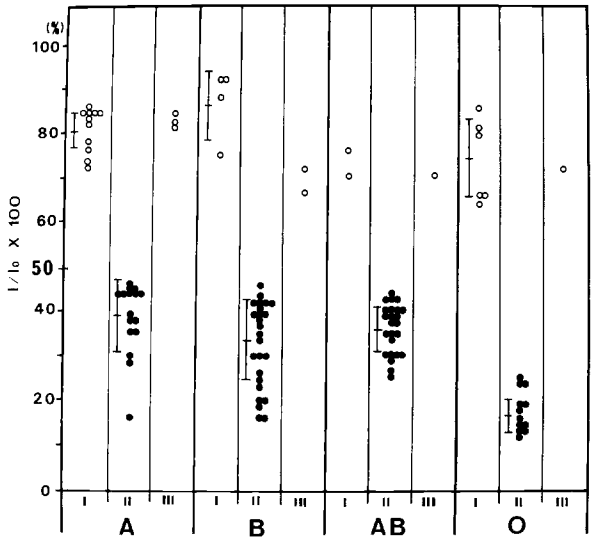

FIG. 3—*Percentage absorbance on the ELISA inhibition assay by the addition of 106 bloodstain samples among ABO blood groups to the B plate. Values reported are the mean ± standard deviations of 4 to 25 independent determinations.* ●: *Le(b+),* ○: *Le(b−),* I: *Le(a+b−),* II: *Le(a−b+), and* III: *Le(a−b−).*

spondence to the antibodies absorbance was always greater than 50%. This means that the Lewis substances of 106 individual bloodstains were typable regardless of their ABO blood group by the present ELISA.

In this experiment we also attempted to use goat anti-Le^a and anti-Le^b polyclonal antisera from Ortho Diagnostic System which were produced by immunization with human saliva. This ELISA system used diluted antisera (1:320) and gave about 1.0 of absorbance. Although some amounts of bloodstains were added to this ELISA reaction system, an effective decrease in absorbance was not observed. This may be due to the following reason. The antisera, which were absorbed with erythrocytes, are prepared to be usable for the hemagglutination test, but the various antibodies against substances such as normal saliva proteins still remain unabsorbed. The unabsorbed antibodies bind to the corresponding saliva antigens which are coated as the ELISA antigen on the surface of the well. It is therefore compatible that even though some amounts of bloodstains were added to the ELISA system, the percent of inhibition of absorbance was hardly observed. Hence, the polyclonal anti-Le^a and anti-Le^b antibodies against whole human saliva are inadequate in the present ELISA system.

Conclusion

Detectability of Lewis substance in aged bloodstains is of particular importance in forensic science casework. The bloodstains used in the ELISA system were stored at room temperature for one year. Typing of their Lewis substances was easily performed. Lewis substances were detected in fresh bloodstains more easily than aged ones, suggesting that the solubility of stains in the reaction mixture may be attributed to the reactivity of the ELISA.

Bässler [*11*] recently investigated secretor status of various stains of body fluids by using a hemagglutination inhibition test and reported that there are a fair number of stains judged to be not able to be evaluated or incorrect, and furthermore, there have been no reports that Lewis typing of bloodstains was successful by use of the conventional absorption-inhibition

or absorption-elution test [5]. These problems are based on the low antigen concentration in comparison with the ABO antigens [*12,13*].

In this paper, we demonstrated that the ELISA using the ABC technique can be used for Lewis typing of small amounts of aged bloodstains. As a result of this investigation it has been shown that Lewis phenotypes of dried bloodstains can be determined easily and correctly.

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Address requests for reprints or additional information to
Dr. Takehiko Takatori
Department of Legal Medicine
Hokkaido University School of Medicine
Sapporo 060, Japan