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Lewis Blood Group Determination in Bloodstains by Planimetric Measurement of Eluted Monoclonal Antibodies

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ABSTRACT: Planimetric measurements were employed for reading the results of an elution test to determine Lewis blood groups in dry human bloodstains. In the absorption-elution test, two varieties of indicators were used to detect eluted Lewis antibodies. First, 64 bloodstains aged between 2 to 8 months were tested with glutaraldehyde (GLA)-treated erythrocytes (planimetric hemagglutination assay, PMHA). This method demonstrated that dry stains weighing approximately 0.4 mg (equivalent to 3 μL of whole blood) were sufficient for detection of Le^a or Le^b antigen. Results were obtained within 1 h. Then, 37 of these stains were tested with Lewis substance-coated latex particles (planimetric latex agglutination assay, PMLA). The presence of Le^a and Le^b antigen were detected from dry stains weighing 0.1 mg (equivalent to 1 μL of whole blood) within 3 h. Both these assays are faster and simpler with accuracy than the enzyme-linked immunosorbent assay (ELISA). Latex particles coated with Lewis substance are, in particular, strongly agglutinated and show agglutination patterns more clearly than erythrocytes. The blind tests using these two methods properly classified 7 Le(a+b−) and 23 Le(a−b+) bloodstains; whereas, 5 Le(a−b−) stains were undetermined by the criteria for these tests. These results indicate the usefulness of the PMHA and PMLA for typing Lewis blood groups from small bloodstains.

KEYWORDS: forensic science, genetic typing, planimetry, blood, bloodstains, Lewis blood group system, latex, enzyme-linked immunosorbent assay

It is a well-established fact that there is a correlation between secretor status and the Lewis antigens found on the red cell [*1,2*]. Lewis blood grouping of dry human bloodstains has consequently been used to ascertain the ABO secretor status in forensic science practice.

Recently, an enzyme-linked immunosorbent assay (ELISA) using monoclonal anti-Le^a and anti-Le^b antibodies, with an avidin-biotin complex [*3*], or a modification of this method using nitrocellulose paper [*4*], was reported. These methods, however, seem to be complicated, and the determination of the Lewis blood group of dry bloodstains through these methods is more difficult than identifying that of fresh red cells, saliva, or other nonblood body fluids.

We have already reported a simple and reproducible planimetric immunoassay method using latex particles [*5*]. That method was modified and applied to the blood grouping of dry bloodstains using the absorption-elution test with glutaraldehyde (GLA)-treated

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erythrocytes [6,7]. In this paper, we describe the application of this planimetric immunoassay method for the Lewis blood grouping of bloodstains and demonstrate its simplicity and sensitivity compared with conventional ELISA.

Materials and Methods

Bloodstains

Whole fresh venous blood samples were collected from medical students. Bloodstains were made on clean thin paper, dried, and stored for 2 to 8 months at room temperature. The ABO group and Lewis type of each donor's erythrocytes were determined by the standard technique using commercial antibodies.

Antibodies and Chemicals

Murine anti-Le^a and anti-Le^b were purchased from Biotest Diagnostics, Frankfurt. Biotinylated goat anti-mouse Ig (G + M + A) and avidin-peroxidase were purchased from Zymed Laboratories, San Francisco and Vector Laboratories, Burlingame, respectively. Lewis blood group substance and human anti-A and anti-B antisera were obtained from Ortho Diagnostic Systems, Raritan, New Jersey.

Absorption-Elution Method of Bloodstains

This procedure was carried out principally in accordance with the practice of a forensic science investigation [8,9]. A slip of blood stained paper was divided into two pieces, each stained with about 3 μL of blood, 0.5 by 0.5 cm in size, for planimetric hemagglutination assay. And for ELISA, another slip of the same dimension was used. For planimetric latex agglutination assay, one fourth of the bloodstain quantity was enough. Then each piece was sensitized with 25 μL of 1:200 diluted anti-Le^a and 1:500 diluted anti-Le^b monoclonal antibodies, respectively. Absorption was carried out at room temperature for 2 h and at 5°C overnight. Then the stains were washed 8 times by immersion in a large volume of ice cold $1/15M$ phosphate buffered saline (PBS), pH 7.3, and placed in wells of a microtiter-plate with 75 μL PBS containing 0.5% bovine serum albumin (BSA). Elution was carried out for 30 min at 55°C in an incubation chamber. Following elution, 25 μL of eluates were transferred into new wells. The elution assay was replicated at least three times for each sample, and control tests were carried out with all constituents and unstained portions of paper.

Planimetric Hemagglutination Assay (PMHA)

Papain-treated and GLA-treated Le(a + b −) and Le(a − b +) erythrocytes were prepared as in a previous report [7]. A 0.5% suspension of erythrocytes was added immediately to the eluates. After 30 min to 1 h, the area of precipitated patterns on the bottom of each well was measured by the planimetric system. The principle of planimetric measurement has previously been described in detail [5]. The planimetric system, in brief, consists of a microscope connected to a television unit and a computer with graphic capabilities. The agglutination pattern on the bottom of each well is magnified with a microscope under transmitted light, and this pattern is converted to a digitalized form to allow the area to be measured as numbers of dots on the screen. About 750 dots are equivalent to 1 mm^2. The titer of anti-Le^a and anti-Le^b was defined as significant at the highest dilution of antibodies, 10% [2 × maximum coefficient of variation (CV)] above the value without antibody addition. We compared the strengths of anti-Le^a and anti-

Le^b monoclonal antibodies eluted from stained paper, and the dot numbers of both anti-Le^a and anti-Le^b precipitated areas were added. Only in those cases where two anti-Le potencies were more than 10% (of the sum) apart from each other was the Lewis blood group determined.

Planimetric Latex Agglutination Assay (PMLA)

Latex particles (Type HD-08, 0.9 μm in diameter, specific gravity of 1.50) were obtained from Takeda Chemical Industries, Osaka. One volume of 1% latex particle suspension in 0.1*M* glycine-buffered saline (GBS), pH 8.2, was coated with an equal volume of 1:20 diluted Lewis substance in GBS as described previously [*10*]. A 0.1% suspension of Lewis substance-coated latex particles was added to the eluates, in place of indicator erythrocytes, and left for 3 h at room temperature to settle the latex particles. The settling patterns of latex particles were measured in the same way as those of erythrocytes.

ELISA Procedure

The procedure of ELISA we used is shown in Fig. 1. Assays were carried out in wells of flat-bottomed microtiter-plates (Greiner, West Germany). The wells of the plate were coated with 1:20 diluted Lewis substance in 0.01*M* carbonate-bicarbonate buffer (pH 9.6) and left overnight at 5°C. The plate was washed with 0.01*M* PBS and then filled with PBS containing 1% BSA for blocking and left 2 h at room temperature. After removal of the BSA solution, the plate was washed with PBS. Lewis antigen-coated plates were stored at −25°C until used. PBS containing 0.05% Tween 20 and 1% BSA (PBS-T-B) was used as the washing buffer. ABTS [2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid)diammonium salt (Sigma)] was used as a substrate [0.3 mg/mL in citrate buffer (pH 5.0) containing 0.1 μL of hydrogen peroxide], and the absorbancy at 405 nm was measured.

Blind Test of Bloodstains

"Blind" samples, in which the Lewis type was unknown to the two examiners, were experimentally prepared. One examiner tested these samples by PMHA and the other by PMLA. Thirty-five samples, with Lewis (a − b −) type included, were tested to verify this method. The samples were 2 to 4 months old. Here, again, the Lewis blood group was determined only in those cases where two anti-Le potencies were more than 10% (of the sum) apart from each other. Eluted anti-Le potencies with a more than 10% increase over controls were considered significant. Results were summarized by the person who did not take part in the test.

Results

Figure 1 presents a comparison of the PMHA, PMLA, and ELISA assay procedures. A planimetric immunoassay based on erythrocyte or latex particle agglutination does not require washing steps and does not use second antibodies. Results are simpler and easier to obtain with PMHA and PMLA than with ELISA.

Figure 2 compares titration curves with serially diluted murine anti-Le^a and anti-Le^b obtained by three different assay methods: (*a*) PMHA, (*b*) PMLA, and (*c*) ELISA. The sensitivities obtained by these methods are almost equivalent: anti-Le^a was 1:12 800 to 1:25 600 and anti-Le^b was 1:25 600 to 1:51 200. Of the three methods, planimetric latex agglutination showed the highest detection sensitivity and a clear difference between positive and negative reactions.

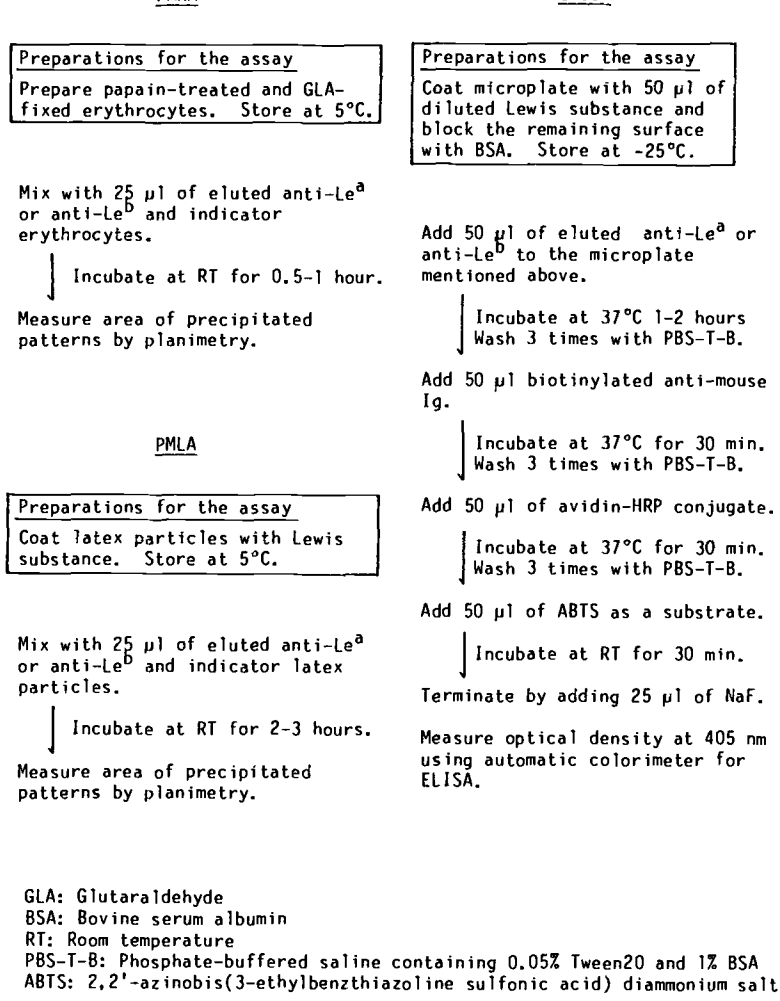

FIG. 1—*Procedures of planimetric hemagglutination (PMHA), planimetric latex agglutination (PMLA), and enzyme-linked immunosorbent assay (ELISA) methods for determining anti-Le^a or anti-Le^b agglutinin in eluates.*

Figures 3 and 4 show the corresponding results obtained for 12 bloodstains by the absorption-elution test using GLA-treated erythrocytes and Lewis substance-coated latex particles as indicators. The different combinations of ABO and Lewis groups revealed by the two indicator systems show a close similarity between the two. The use of latex particles as an indicator produces clear positive or negative patterns that can be read with the naked eye. Using GLA-treated erythrocytes produces the settling patterns more rapidly than latex particles. These two indicators could be preserved for about 2 years at 5°C.

Figure 5 shows the comparative strength of eluted anti-Le^a and anti-Le^b antibodies which were obtained by an absorption-elution test. A total of 64 bloodstain samples [34 Le(a+b−), 23 Le(a−b+), and 7 Le(a−b−)], ranging in age from 2 to 8 months and whose ABO and Lewis blood groups were already known, were determined by PMHA,

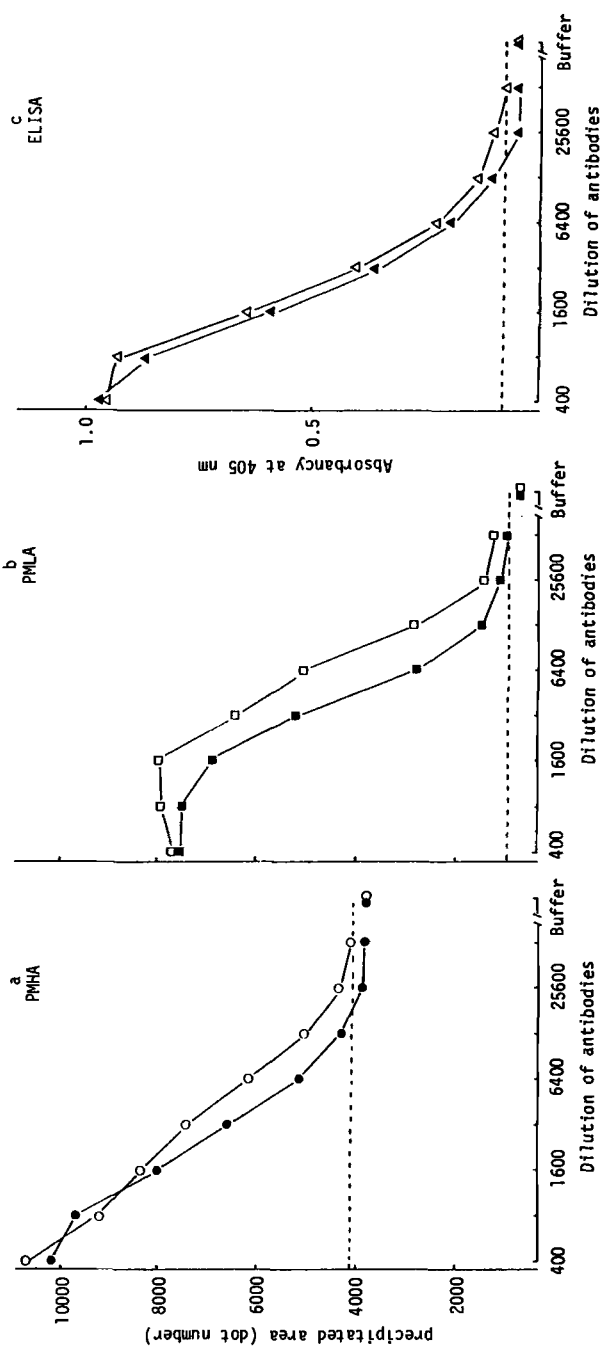

FIG. 2—*Quantitation curves of anti-Le^a and anti-Le^b monoclonal antibodies obtained by three different methods:* (a) *PMHA reacted with glutaraldehyde (GLA)-treated erythrocytes,* (b) *PMLA reacted with Lewis substance-coated latex particles, and* (c) *ELISA with avidin-biotin complex. A mean of two measurements is plotted for each point. The symbols used are* ○, △, □ *for anti-Le^a and* ●, ▲, ■ *for anti-Le^b monoclonal antibodies. The broken line marks the cutoff values. Points above the line are considered significant increases.*

Sample No.	ABO blood group	Anti-Le^a Dilution of eluate 1:1	2	C	Anti-Le^b Dilution of eluate 1:1	2	C	Determined Lewis blood group
21	O							Le(a-b+)
27	O							Le(a+b-)
28	O							Le(a-b+)
29	B							Le(a-b+)
31	A							Le(a-b+)
32	A							Le(a-b-)
35	O							Le(a-b+)
36	B							Le(a+b-)
37	O							Le(a-b+)
40	B							Le(a-b+)
42	O							Le(a+b-)
41	A							Le(a-b+)

FIG. 3—*Precipitated patterns of GLA-treated erythrocytes reacted with eluates obtained by an absorption-elution test from 12 bloodstains 0.5 by 1.0 cm in size (0.5 by 0.5 cm for either anti-Le^a or anti-Le^b). Sample Nos. 21, 27, 28, 29, 31, 32 are 2 months old; Nos. 35, 36, 37, 40 are 4 months 17 days old; Nos. 42 and 41 are 8 months old. Agglutination can be determined by careful naked-eye examination in some cases (Nos. 32 and 41). C = control tests.*

Sample No.	ABO blood group	Anti -Le^a	-Le^b	C	Determined Lewis blood group
21	O				Le(a-b+)
27	O				Le(a+b-)
28	O				Le(a-b+)
29	B				Le(a-b+)
31	A				Le(a-b+)
32	A				Le(a-b-)
35	O				Le(a-b+)
36	B				Le(a+b-)
37	O				Le(a-b+)
40	B				Le(a-b+)
42	O				Le(a+b-)
41	A				Le(a-b+)

FIG. 4—*Precipitated patterns of Lewis substance-coated latex particles reacted with eluates obtained by an absorption-elution test from 12 bloodstains 0.25 by 0.5 cm in size (0.25 by 0.25 cm for either anti-Le^a or Le^b). Note the clear discrimination between Le positive and Le negative patterns. C = controls.*

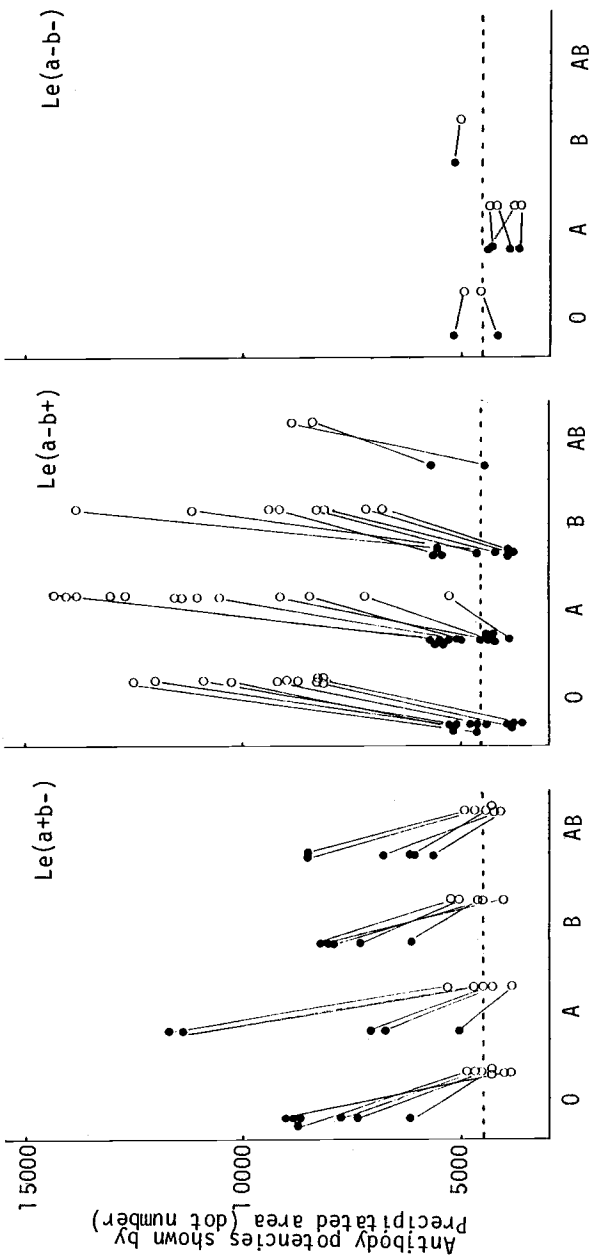

FIG. 5—*Comparative strength of eluted anti-Le^a (●) and anti-Le^b (○) monoclonal antibodies as determined by an absorption-elution test from 64 bloodstains 1.0 by 0.5 cm in size (0.5 by 0.5 cm for either anti-Le^a or anti-Le^b). The broken line marks the cutoff values. Dots above the line are considered significant increases. On the figure, some paired open and closed circles are connected with a line for clarity.*

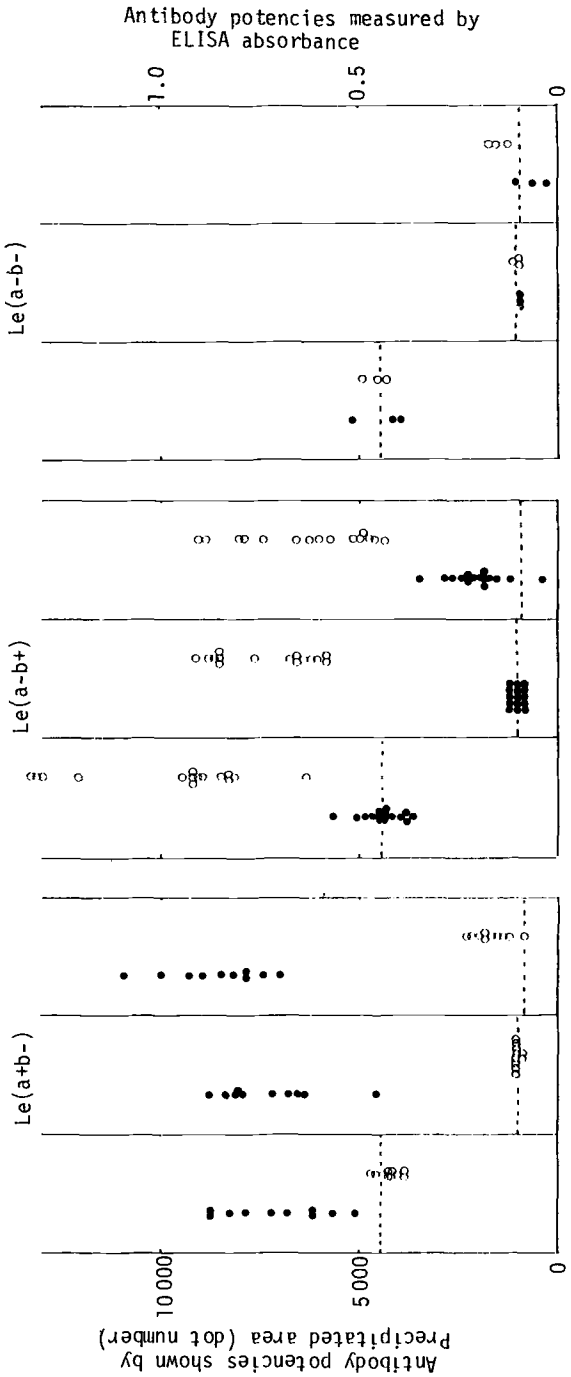

FIG. 6—*Comparison of three different detection methods for eluted anti-Le^a and anti-Le^b antibodies that were obtained by an absorption-elution test from 28 bloodstains. The same diluted antibodies (1:200 anti-Le^a, 1:500 anti-Le^b) are used in the same way. Symbols used are* ● *for anti-Le^a and* ○ *for anti-Le^b.*

and on 37 of these, PMLA was also carried out (part of these results are shown in Fig. 6).

Figure 6 compares the results of Lewis blood typing by the absorption-elution test. Twenty-eight bloodstains [10 Le(a+b−), 15 Le(a−b+), and 3 Le(a−b−)] were tested, and the eluted anti-Le^a and anti-Le^b were determined by PMHA, PMLA, and ELISA. The results obtained by the three methods agreed with each other; however, in the results obtained for PMHA and PMLA, there was a wide difference between positive and negative values (65 to 80%) when latex particles coated with Lewis substance were used as the indicator. A small difference (15 to 40%) was noted when GLA-treated erythrocytes were used.

Table 1 shows the results of a blind test of bloodstains in which Lewis types obtained by PMHA and PMLA are compared with those of fresh blood samples by standard hemagglutination. Thirty samples belonging to Le(a+b−) and Le(a−b+) were correctly typed. The remaining five Le(a−b−) samples were typed as "undetermined" because the difference of the eluted antibodies was below 10%, or a significant increase of eluted antibodies was not detected. All PMHA and PMLA results were in agreement.

Discussion

Various ELISA techniques for ABO and Lewis typing of secretions have replaced the traditional hemagglutination methods [*11–16*] because of the availability of highly specific monoclonal antibodies and automatic instruments. These methods currently use the absorption-inhibition technique, whose sensitivity is sufficient for detecting a large quantity of antigen, such as ABH or water-soluble Lewis antigen, in secretions. The technique, however, requires an extraction step and a relatively large stain size in order to obtain a large volume of stain extract. Moreover, in the case of old stains, effective extraction would be difficult. The absorption-elution technique, however, has superior sensitivity, and requires no extraction procedure. The absorption-elution technique obviously cannot be used with ELISA because of the lengthy procedure that would be involved.

Our method, previously reported under the title "Quantitation of Hemagglutinins by the Planimetry of Precipitated Erythrocyte Patterns" [*6,7*], is a rapid, simple, and sensitive method and shows the best results in ABO, MN, and Rh typing of bloodstains. The use of GLA-treated erythrocytes as indicator cells with this method offers good reproducibility and constant agglutinability, and it does not require preparation of indicator cells for each individual experiment. In addition, it is possible to use Lewis substance-coated latex particles as the indicator for successful Lewis blood group typing from small bloodstains since the latex particles used in the present method can adsorb the Lewis antigens firmly and densely. The Lewis substance-coated latex particle reagent we prepared was agglutinated even by small quantities of antibodies or antibodies with weak agglutinating abilities, which makes it easy to discriminate between Le-positive and Le-negative patterns. On the other hand, the GLA-treated indicator cells can be used with conventional

TABLE 1—*Results of blind study for Lewis typing.*

Lewis Type	Typing by Hemagglutination of Fresh Blood	Number of Samples by	
		PMHA Typing	PMLA Typing
Le(a+b−)	7	7	7
Le(a−b+)	23	23	23
Le(a−b−)	5	. . .	. . .
Undetermined	. . .	5	5
Total	35	35	35

serological reagents because of their specific agglutination reactions, and they have the advantage of saving reaction time.

The methods described here mostly produce clear positive or negative reactions that can be read macroscopically, but better sensitive and quantitative results were obtained by planimetric measurement. The results can be expressed numerically, and an objective and precise determination of Lewis blood groups is obtained.

Takizawa [*17*] reported that, in addition to the Le^b activity, a small amount of Le^a activity was detected in glycolipid or glycoprotein fractions in the Le(a−b+) erythrocytes, and Le^b activity was also detected in the Le(a+b−) erythrocytes. The coexistence of Le^a and Le^b substances was also reported [*1,2*] in saliva, and the same phenomenon is observed when typing saliva or urine samples [*18–20*]. Figure 5 shows similar results. The coexistence in our case may be partly due to the property of the monoclonal antibodies we used. Therefore, we interpret the results only for the cases where the Le^a and Le^b potencies differ greatly (over 10%).

As shown in Fig. 6, where the same materials and the same diluted antibodies were used, PMHA and PMLA were demonstrated to be reliable methods by comparison with ELISA. The equipment is less expensive than that for ELISA or radioimmunoassay (RIA). We hope that our methods will be used for the benefit of the future of forensic medical science.

Acknowledgment

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