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A Unique and Sensitive ELISA Technique for Typing ABH Antigens in Bloodstains Using UEA-I Lectin—The Removal of Detergent with a Sephadex G-25 Mini-Column Improves Sensitivity

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ABSTRACT: A unique sandwich enzyme-linked immunosorbent assay (ELISA) for the determination of ABH antigens in bloodstains has been developed. Human anti-A and -B antisera and *Ulex europaeus* anti-H lectin were coated on the inner surface of microplate wells. The sample antigens from bloodstains, solubilized with n-octyl-β-D-glucopyranoside which was then removed by passing through a Sephadex G-25 (G-25) mini-column, were placed in the wells. After washing the wells repeatedly, peroxidase-conjugated *Ulex europaeus* lectin I was added and incubated. Antigen activities were determined by the development of colors using o-phenylenediamine/H_2O_2. This technique permitted clear detection of all ABH antigens corresponding to the antisera and lectin with high sensitivities. The A and B antigens were solubilized as aggregates with H antigen from the erythrocyte membrane. Excess detergent remaining in the sample reduced the sensitivity and accuracy of this ELISA, probably due to the removal of antibody from the wells by the effect of the surfactant. The treatment of solubilized antigens with G-25, an indispensable step, eliminated the adverse effect of the detergent on the ELISA. The ELISA method reported here was proved to be easy, economical and sensitive, and this technique should be useful in the forensic practice.

KEYWORDS: criminalistics, ELISA, ABO typing, *Ulex europaeus* lectin I, bloodstain

The technique of enzyme-linked immunosorbent assay (ELISA) has been developed for the typing of ABH (1–3), Lewis (4,5) and Rh (6) blood groups, and such assays are regarded as powerful tools for the forensic individualization. However, there are some problems involved in blood grouping of small bloodstains (4,7,8). One of the major problems is the necessity to hydrate and solubilize blood group substances located on the erythrocyte membrane surface. The erythrocyte ABH antigens exist as glycolipids and glycoproteins and are hardly extracted with aqueous solution. Many detergents have been employed to solubilize ABH antigens from the bloodstains (9,10). However, the detergent used for the extraction of erythrocyte antigens also gives adverse effects on the results of ELISA (11,12). In fact, the ELISA methods previously reported have not improved the sensitivities as compared to the conventional absorption-elution tests for ABH typing of bloodstains (7,8). There is another problem concerning the cost of the ELISA methods; these techniques usually require each antibody/enzyme conjugate corresponding the antigen and/or monoclonal antibodies other than antisera for clinical use. Here, we developed a unique sandwich ELISA for ABH typing, based on the final detection of H antigens in ABH blood group substances solubilized from bloodstain with peroxidase-conjugated *Ulex europaeus* lectin I (UEA-I/PO). We also tested the effects of the detergent on the ELISA and developed the method for removing it using a Sephadex G-25 (G-25) mini-column.

Materials and Methods

Reagents

Antisera for A and B (from human), Rh (C, c, D, E and e; from human), P_1 (from goat), MN (from rabbit), Duffy (Fy^a and Fy^b, from human) and Kidd (Jk^a and Jk^b; from human) and monoclonal antibodies for Lewis (Le^a and Le^b; from mouse) antigens were obtained from Ortho Diagnostic Systems. UEA-I/PO and H-specific lectin were purchased from Honen. Each antiserum was diluted two-fold with 0.01 M phosphate buffered saline (PBS, pH 7.2). UEA-I/PO was diluted 500 times with PBS just before use. Bovine serum albumin fraction V (BSA) was obtained from Sigma. Tween-20 was purchased from Nacalai Tesque. G-25 (fine) was obtained from Pharmacia. Microplates and other chemicals were from Corning (96 flat wells) and from Wako, respectively. n-Octyl-β-D-glucopyranoside was dissolved in 0.2 M tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 10.5) and adjusted to a 0.5% solution (extraction solution) before storing at −80°C. Ten milligrams of o-phenylenediamine, 20 mL of 0.1 M Tris/HCl (pH 7.6) and 40 μL of 3% H_2O_2 were mixed just before use as a developing reagent.

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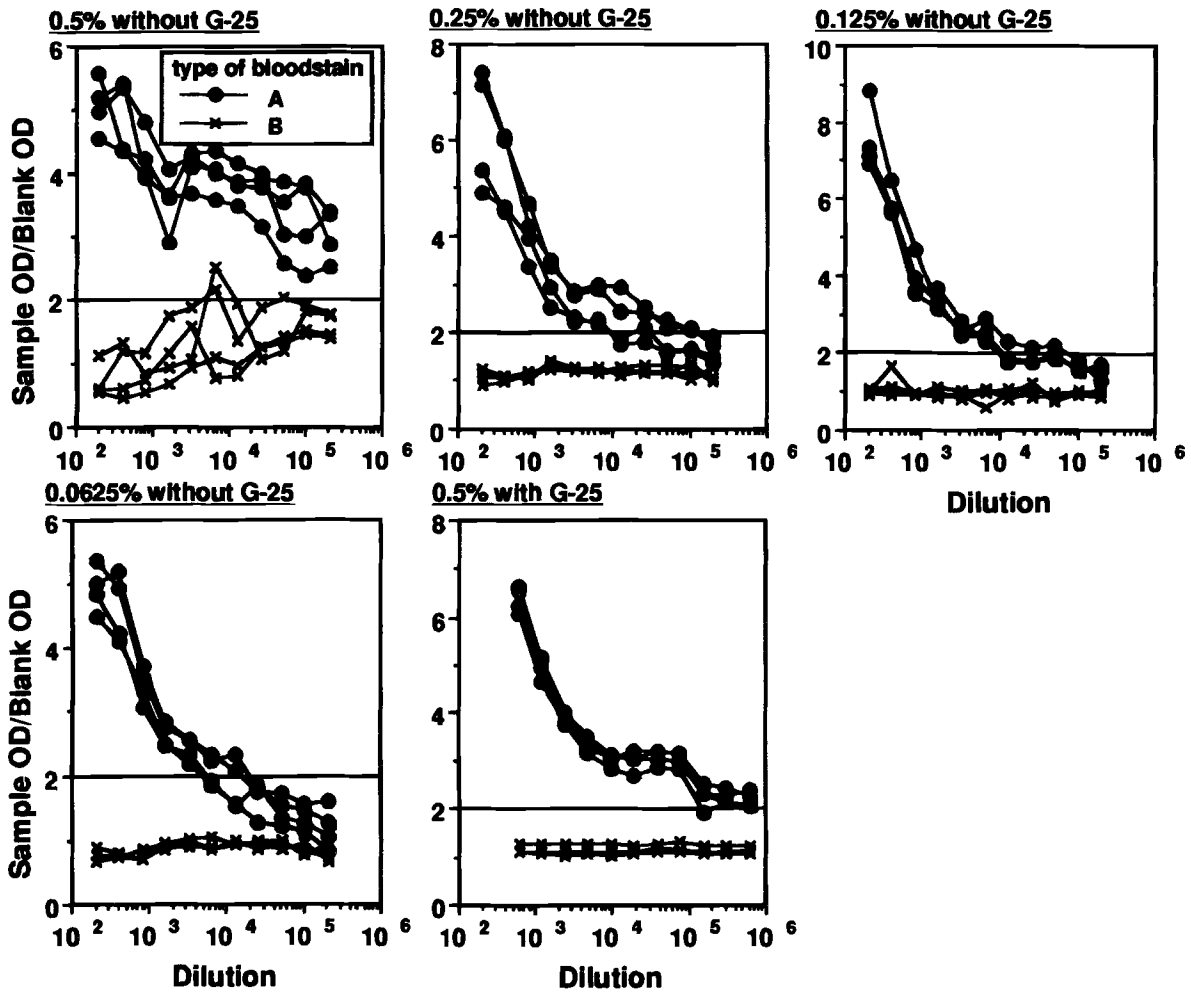

FIG. 1—*Effects of detergent on the ELISA for A antigen. The sample bloodstains were treated with various concentrations of n-octyl-β-D-glycopyranoside. Each percent value indicates the concentration of the detergent used. The desalting procedure was done using the G-25 column (bed volume, 9 mL). A and B represent phenotypes of bloodstains.*

Extraction of ABH Antigens from Bloodstains

Fresh blood samples were obtained from volunteers. Bloodstains were made on gauze with fresh blood samples following the determination of ABO allotypes by the hemagglutination method according to the supplier's instruction manual, and air-dried at room temperature. Blood stains were stored in a refrigerator during the experimental periods for several months. These stains were cut into small pieces (5 × 5 mm^2; ca 1 mg of dried blood) prior to placing in tubes containing 200 μL of extraction solution. Following incubation at 37°C for 2 h, the supernatant solution was isolated and applied onto a column (Bio-Rad Poly-Pre chromatography column) filled with G-25 (bed volume, 750 μL; G-25 mini-column) pre-suspended in PBS. An aliquot of 350 μL of PBS was passed through the column and the last 300 μL eluate was collected. The eluted sample was diluted repeatedly with PBS before use.

To remove the detergent well, a larger size of G-25 column (bed voluve, 9.0 mL; G-25 column) was also tested. The sample (4 pieces of bloodstains) was solubilized with 800 μL of extraction solution was applied to the G-25 column and an aliquot of 4.2 mL PBS was passed through the column. The last 2.4 mL eluate was collected and assayed by the present ELISA method. The amount of residual detergent in the sample after G-25 treatment was determined using a spectrophotometric method. n-Octyl-β-D-glucopyranoside in the sample was hydrolyzed with 6 N HCl in boiling water for 40 min. The hydrolyzed product was reacted with o-toluidine/acetic acid solution in boiling water for 8 min, and then the optical density (OD) of the solution was measured at 635 nm (Hitachi model 220A spectrophotometer).

Assay Procedure

An aliquot of 100 μL of diluted anti-A, -B antiserum or H-specific lectin was added to each well of the microplate prior to incubation at 4°C overnight. The antiserum or the lectin was then collected from the wells and re-used several times thereafter. The wells of the microplate were washed 3 times with 0.05% Tween-20/PBS and treated once with 0.1% BSA/PBS for 2 h. The wells were subsequently washed with PBS. Aliquots of the samples (100 μL volumes) were placed into the antibody- or lectin-coated wells prior to overnight incubation at 4°C. After washing the wells 5 times with 0.05% Tween-20/PBS, aliquots of 100 μL UEA-I/PO were added to the wells before further incubation at 37°C for 3 h. Excess UEA-I/PO solution was then removed and the wells washed 5 times with 0.05% Tween-20/PBS. Following the addition of 100 μL of o-phenylenediamine solution to each well, the microplate was stored in a dark place for 30 min. UEA-I/PO bound to the H antigen was detected following color development with o-phenylenediamine/H_2O_2 (catalyzed by conjugated peroxidase). After terminating the reaction with 100 μL of 1 M H_2SO_4, the

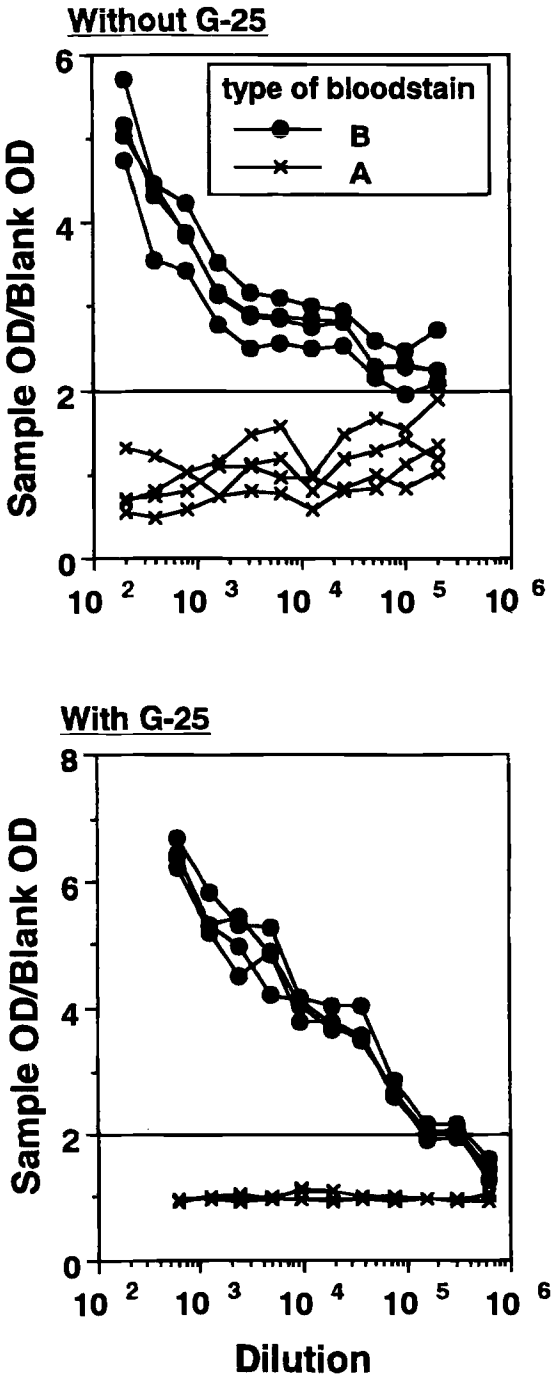

FIG. 2—*Effect of G-25 on the ELISA for B antigen. The sample bloodstains were treated with 0.5% n-octyl-β-D-glycopyranoside (extraction olution). The desalting procedure was done using the G-25 column (bed olume, 9 mL). A and B represent phenotypes of bloodstains.*

OD of the solution was measured at 490 nm (Bio-Rad Model 3550 microplate-reader). As a blank, an aliquot of 0.2 mL of extraction solution without bloodstain was treated through the whole procedure. The detection limits, expressed by sample dilution (diluent volume/dried blood weight in bloodstain), were defined by the points at which the OD values of diluted samples were twice higher than those of blank samples.

Reactions of UEA-I with Other Erythrocyte Antigens

The extracts from fresh bloodstains by the procedure mentioned above were tested using the microplate wells coated with the corresponding antibodies for Rh, MN, P_1, Kidd, Duffy and Lewis blood group systems. The phenotype of each blood sample was prior determined by the hemagglutination method according to the supplier's instruction manual.

Results and Discussion

Solubilization of Antigens and Effect of Detergent

n-Octyl-β-D-glucopyranoside, a surfactant which solubilizes proteins of the cell membrane, has been proven to be a useful reagent for extracting ABH, Lewis and Rh system antigens from

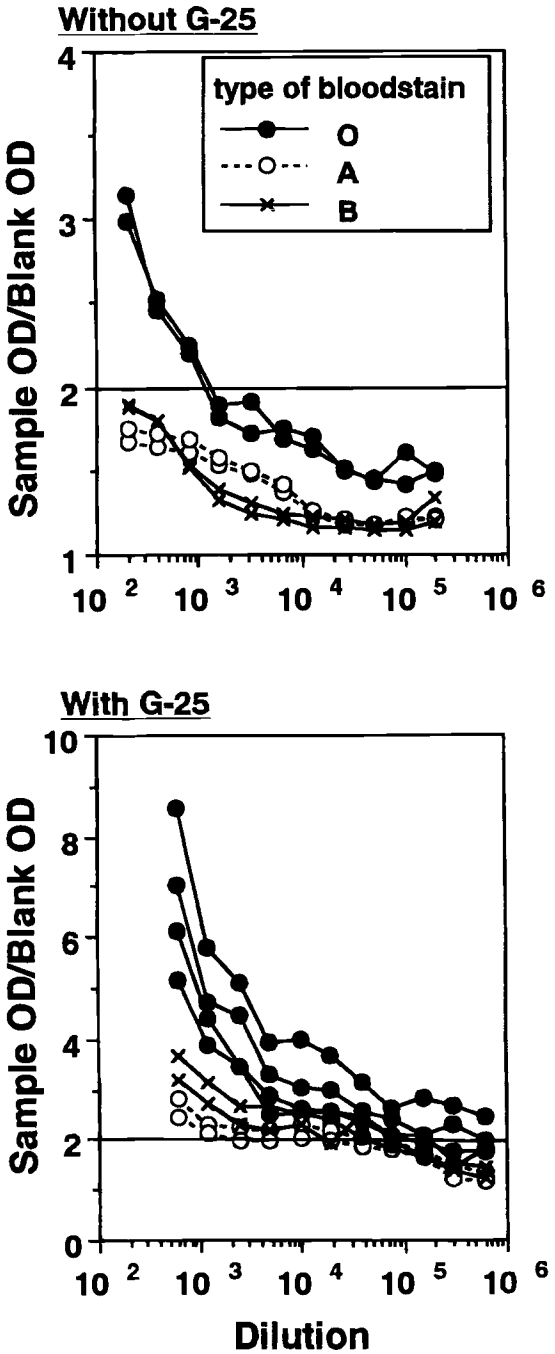

FIG. 3—*Effect of G-25 on the ELISA for H antigen. The sample bloodstains were treated with 0.5% n-octyl-β-D-glycopyranoside (extraction solution). The desalting procedure was done using the G-25 column (bed volume, 9 mL). A, B and O represent phenotypes of bloodstains.*

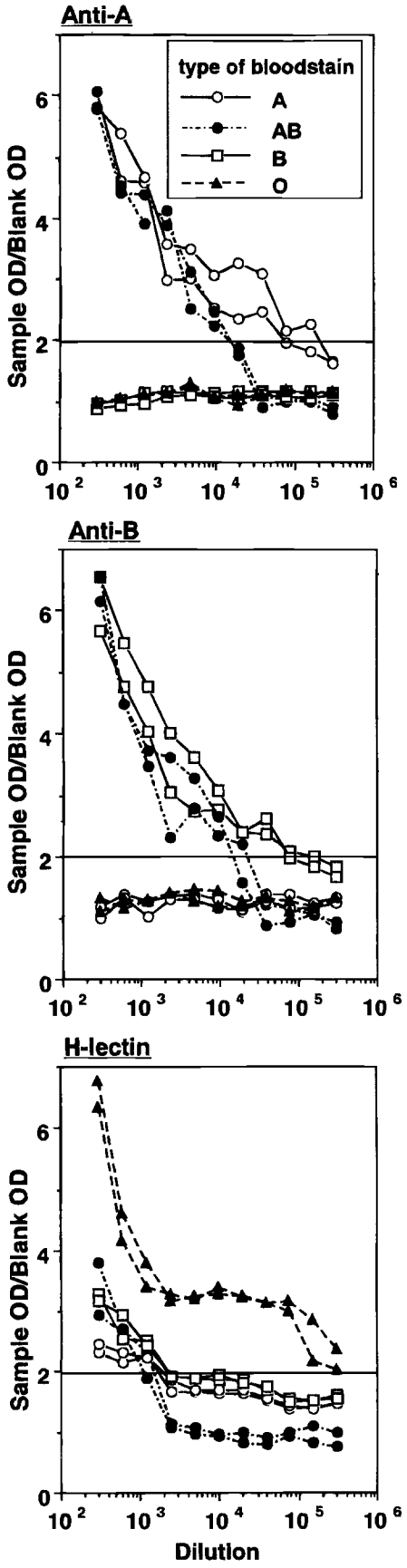

FIG. 4—*Dilution curves for ABH antigens extracted from bloodstains. The ABH antigens were extracted with 0.5%-n-octyl-β-D-glycopyranoside (extraction solution) from the bloodstains and then passed through the G-25 mini column (bed volume, 750 μL). A, B, AB and O represent phenotypes of bloodstains.*

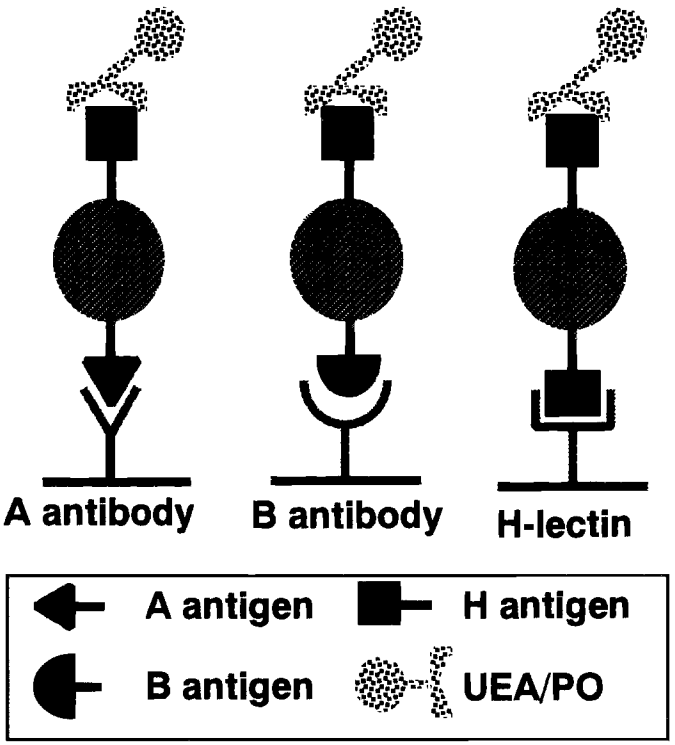

FIG. 5—*The scheme for the present ELISA and possible ABH antigens aggregates from erythrocyte membrane.*

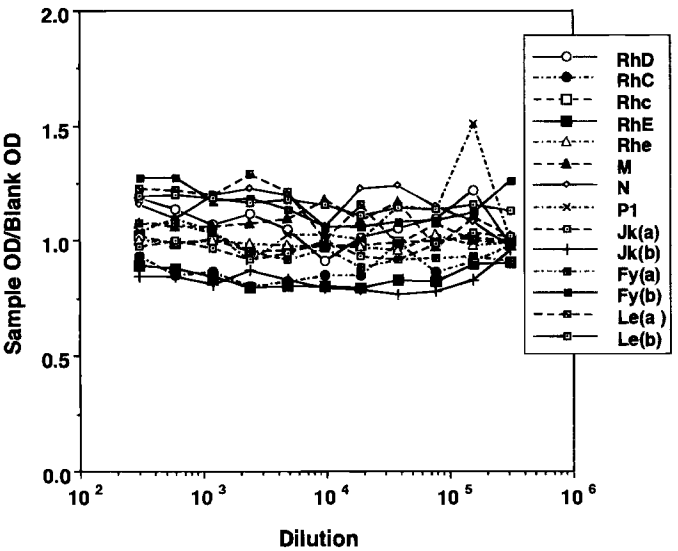

FIG. 6—*Reactions of UEA-I with erythrocyte antigens of Rh, MN, P_1, Kidd Duffy and Lewis blood group systems. Each antigen of the c, C, D, e, E, M, N, P_1 Jk^a, Jk^b, Fy^a, Fy^b, Le^a and Le^b positive samples was added to the microplate wells coated with the corresponding antiserum. Each point represents the mean of duplicate analyses.*

red cells (6,9,10). To test the effect of the detergent on the ELISA detection, various concentrations of it were used for solubilization of blood antigens without subsequent use of G-25. The ELISA results for A antigens in the presence of the detergent are shown in Fig. 1. The concentration of the detergent lower than 0.125% was not adequate to solubilize antigens from the bloodstains. With the detergent higher than 0.125% concentration, reproducible results were not obtained. The excess detergent in the sample had adverse effects on sample binding. The same results were obtained

in the typing of B and H antigens (Figs. 2 and 3, data from detergent concentrations with 0.25, 0.125 and 0.0625% are not shown). The reagent would remove antibody and lectin from the wells and then the most of antigen/antibody and antigen/lectin complexes would be washed out during a series of washes resulting in lower sensitivity and accuracy. The use of G-25 column (bed volume, 9 mL) extensively improved the ELISA results as compared to those when the sample contained the surfactant (Figs. 1, 2 and 3). After G-25 column (bed volume, 9 mL) treatment, the detergent remained in the sample was 5.75 ± 2.47% of that in the extraction solution (n = 5, mean ± SEM). However, the use of large amount of G-25 was not suitable for the small bloodstains, because the considerable elution volume was also required. Thus, we reduced the size of G-25 column (bed volume, 9 mL → 750 μL) described in Materials and Methods. This small column (G-25 mini-column) had capacity enough to remove the detergent and gave good results in the assay (Fig. 4). The residual detergent in the sample was 11.68 ± 2.71% of that in the extraction solution, after G-25 mini-column treatment.

ELISA of Various Bloodstains

Dilution curves for the ABH antigens extracted from various bloodstains measured by the present ELISA are illustrated in Fig. 4. The A and B antigens were detected from samples of blood stains diluted more than 1×10^4 fold. The H antigen was detected well in the sample diluted more than 1×10^5 fold from O type bloodstain, and was also recognized at somewhat lower levels in the extract diluted less than 2×10^3 fold from A-, B- and AB-type bloodstains. Because UEA-I does not recognize and bind L-fucose in A and B antigens (13), the fact that UEA-I reacted with the both samples extracted from A- and B-type bloodstains, indicated that n-octyl-β-D-glucopyranoside would solubilize these antigens as aggregates with antigen-H as schemed in Fig. 5. This ELISA procedure allowed to determine not only H but also A and B antigens using the same enzyme conjugated lectin. The sensitivities of the present assay technique are extensively improved compared to the methods previously reported (7,8). The improved sensitivity would increase the reliability of results and allow work with smaller bloodstains in the ABH blood group typing. In fact, ABH antigens were easily determined from the 10-month-old bloodstain on a 1 mm-length gauze fiber using this technique (data not shown). Furthermore, this method was more cost-effective and easier, since it required the only one type of enzyme-conjugated lectin for typing all of ABH antigens.

Other Antigenity of the Extracts

The extract was tested by the present ELISA using antibodies for Rh (c, C, D, e and E), MN, P_1, Kidd (Jk^a and Jk^b), Duffy (Fy^a and Fy^b) and Lewis (Le^a and Le^b) blood groups procedure using their corresponding antisera and antibody. UEA-I/PO did not react with any substances (Fig. 6), indicating that the ELISA using UEA-I was apparently specific to the ABH blood group system. UEA-I recognizes L-fucose (13), especially the L-Fucoseα1→2DGalctoseβ1→4 side chains of glycoproteins and gycolipids, which is a oligosaccharide with type 2 chain of H antigen (14). Antigens of Lewis blood group system possess similar structures to H antigen. However, UEA-I lectin does not react with Le^a and Le^b antigens (Fig. 6) as pointed out by Pereira et al. (13). These results also suggested that these solubilized substances from the erythrocyte membrane did not associate with H antigens. On the other hand, A and B antigens were solubilized as aggregates with H antigen from the erythrocyte membrane (Fig. 5).

Conclusions

We have established a unique sandwich ELISA technique for typing the erythrocyte ABH systems using UEA-I/PO conjugate. This method combined with the step for the removal of detergent by the use of G-25 min column had high sensitivity even for ABH typing of small bloodstains. This method is also easy and economical. It should be useful not only for forensic purposes but also for clinical tests.

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