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ORIGINAL ARTICILE

A Quantitative Immunochromatography Assay of Whole Blood Samples for Antigen-specific IgE—A New Method for Point of Care Testing for Allergens—

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ABSTRACT

Background: The development of an inexpensive point-of-care testing system for antigen-specific IgE is greatly needed. We, therefore, tried to develop a quantitative enzyme immunochromatography assay system for antigen-specific IgE in fresh whole blood.

Methods: Whole blood sample was mixed with a reagent containing detergent to lyse red blood cells, and the mixture was applied to an immunochromatography strip. The lysate was observed to migrate in the strip and was washed away by the substrate buffer. When the sample contained the specific IgE, the antigen-specific IgE line was clearly observed on the strip macroscopically.

Results: Results were obtained 20 minutes after the application of hemolysed blood sample to immunochromatography, and these results showed positive correlation with those obtained by the AlaSTAT system, which is one of the popular assay kits for specific IgE. The results were not affected significantly by the hematocrit value of the blood sample, by the kind of anticoagulant in the blood collection tube, or by the concentration of the total IgE, provided it was lower than 20000 IU/ml.

Conclusions: These results indicate that our system is applicable for point-of-care testing for antigen-specific IgE.

KEY WORDS

Cryptomeria japonica pollen, enzyme immunochromatography, quantification, specific IgE, whole blood

INTRODUCTION

The number of patients with allergic diseases has been increasing recently, and the development of a simple and inexpensive method to assay antigen-specific IgE is greatly needed. Currently, there are different kinds of assay systems used to quantitate antigen-specific IgE,[1] including CAP® (Pharmacia Diagnostics, Uppsala, Sweden) and AlaSTAT® systems (Diagnostic Products Corp., Los Angels, CA, USA). However, these systems require complicated procedures and costly instrumentation. Recently, we developed an enzyme immunochromatography assay (EICA) system for antigen-specific IgE.[2] In our system, antigen-specific IgE can be assayed quantitatively within 20 minutes without using any expensive instruments. To our knowledge, all of the assay systems mentioned above, including ours, are for specific IgE in serum or plasma, but not for use in whole blood. If specific IgE in whole blood can be assayed, it would be convenient for practitioners or doctors working in small-sized medical offices, because they would be able to save time used for separating plasma or serum from patient blood and obtain the

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results in a short period of time. This is also advantageous for patient.

Currently there are several immunochromatography kits used to assay whole blood, for example, for troponin T (Roche Diagnostics Ltd, East Sussex, UK), and human heart-type fatty acid-binding protein (Wakunaga Pharmaceutical Co., Ltd, Osaka, Japan). Most of these kits are designed to trap hematocytes in a plasma separation pad, and only plasma migrates into immunochromatography membrane.[3,4] However, in our preliminary experiments, the plasma separation pad did not work so well for trapping hematocytes as expected. On the other hand, Foxdal *et al.* reported that whole blood sample treated with a detergent buffer was comparable with plasma for the analysis of lactate,[5] although it was not comparable for the immunochromatography method. In the present experiment, therefore, we examined the conditions for the treatment of whole blood samples with a detergent, Triton X-100, in order to lyse hematocytes for the application of the sample to EICA. We, then, assayed the blood samples for Japanese cedar (*Cryptomeria japonica*, CJ) pollen-specific IgE, and these results were examined in correlation with those obtained by the AlaSTAT system.

METHODS

BLOOD SAMPLES

Blood samples were obtained from 136 volunteers (male, 107; female, 29), 22 to 65 years old, including CJ pollinosis patients. Informed consent was obtained after the nature of the studies had been fully explained. Blood was obtained from each subject using 3 blood collection tubes with different anticoagulants, heparin, EDTA, or sodium citrate. Blood was also obtained in a tube without anticoagulant in order to obtain serum.

We obtained plasma and hematocyte fractions from some blood samples by centrifugation at 2500 *g* for 20 minutes at 4℃. These fractions were also used to prepare the blood samples with different hematocrit (Hct) values from 30 to 60%. Hct readings were obtained by the microhematocrit method with a hematocrit capillary (Hirshmann Laborgeräte, Germany) and a capillary centrifuge (Tominaga Works LTD, Japan) according to the National Committee for Clinical Laboratory Standards.[6]

PRETREATMENT OF BLOOD SAMPLE WITH A DETERGENT

We prepared a pretreatment buffer by mixing Triton X-100 (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) in 50 mM phosphate buffer (pH 7.5) to a concentration of 4% (v/v). In some experiments, whole blood was mixed with various proportions of pretreatment buffer, and applied to EICA. Unless otherwise mentioned, 8 µl of whole blood was mixed with 12 µl of the pretreatment buffer.

PREPARATION OF EICA STRIP

The EICA strip was prepared as described in our previous report.[2] Briefly, *Sugi* basic protein (SBP)[7] solubilized in 5 mM borate buffer (pH 8.5) at a concentration of 1 mg/ml was sprayed in a line on an immunochromatography membrane. The SBP preparation we used in the present experiments contained both Cry j 1 and Cry j 2 at a ratio of 5 : 1. The membrane was, then, assembled with an absorbent pad, a substrate reservoir pad, and a laminate card. The assembled sheet was cut into strips 5 mm in width, and a polyvinyl pad containing alkaline phosphatase-labeled anti-IgE antibody (ALP-anti-IgE) was located on the center of the membrane. The ALP-anti-IgE mouse monoclonal IgG antibody was supplied by Diagnostic Products Corp.

EICA PROCEDURES

The assay procedures are essentially the same as those described in our previous report[2] except that the treatment of the blood sample was with the pretreatment buffer. First, 20 µl of the blood sample mixed with the pretreatment buffer as described above was applied to the pad containing ALP-anti-IgE. Soon after the application, 200 µl of the enzyme substrate, a mixture of 5-bromo-4-chloro-indolyl-phosphate and nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was loaded onto the substrate reservoir pad. Twenty minutes after the sample application, the color intensity of the antigen-immobilized line on the membrane was quantified by an immunochromatography detector, QuadScan (BioDot, Irvine, CA, USA). We evaluated the color intensity of the antigen-immobilized line expressed as peak % reflectance (P%R) according to manufacturer's instruction.

ASSAY FOR TOTAL AND ANTIGEN-SPECIFIC IGE CONCENTRATION IN SERUM

Concentrations of total and CJ pollen-specific IgE in serum were assayed by IATRO ACE IgE (Mitsubishi Kagaku Iatron, Japan), a latex aggregation assay system, and AlaSTAT system, an enzyme-linked immunosorbent assay method,[8] respectively. For the AlaSTAT assay, CJ pollen antigen supplied in the kit was used. Both total and specific IgE concentrations were standardized with the WHO Second International Reference Preparation for Human Serum IgE, 75/502.

PREPARATION OF BLOOD SAMPLE WITH HIGH CONCENTRATION OF TOTAL IGE

Blood samples with various concentrations of total IgE were prepared by the addition of purified human monoclonal IgE obtained from CHEMICON International, Inc., CA, USA.

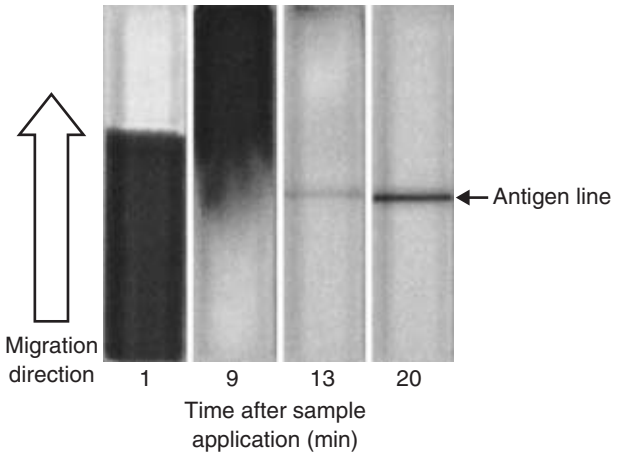

Fig. 1 Blood migration and antigen line stained on EICA strip. Whole blood sample treated with a detergent buffer migrated to the direction of the arrow. Substrate buffer migrated following the blood sample and washed away the red blood cell lysate. Twenty minutes after the blood sample application, the blue color intensity of antigen line was analyzed.

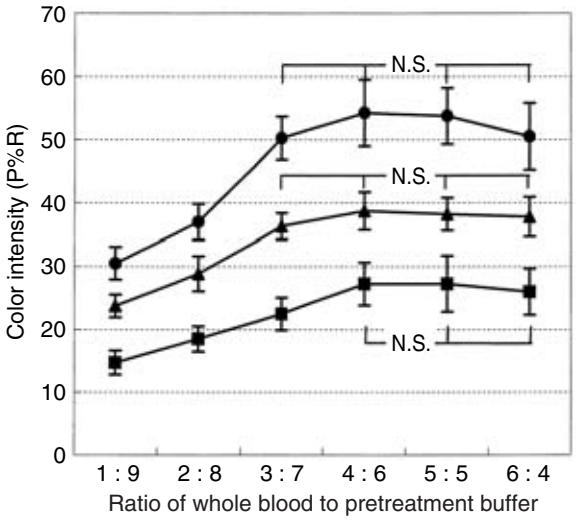

Fig. 2 Effect of the pretreatment buffer volume on color intensity of antigen line. Whole blood samples from 3 individuals (●, ▲, ■) were mixed with the pretreatment buffer in various ratios from 1 : 9 to 9 : 1. When the ratio of blood to the buffer was 7 : 3 or more, the samples did not migrate smoothly, so results were not shown. The color intensity of antigen line at 20 minutes in EICA was expressed as P%R. Data are presented as the average ± 2SD of quadruplicate assay. N.S.; not significant ($p > 0.05$)

STATISTICAL ANALYSIS

The effects of various factors in the blood sample were on the results evaluated by a one-way analysis of variance. Correlation of class scores was analyzed by concordance rate and Spearman's rank order correlation coefficient testing.

RESULTS

PRETREATMENT OF WHOLE BLOOD SAMPLE FOR EICA

First, we tried to determine the appropriate conditions to lyse hematocytes in the blood sample. Blood samples from 3 subjects confirmed to contain CJ pollen-specific IgE in AlaSTAT were mixed with the pretreatment buffer described in the METHODS at various ratios ranging from 1 : 9 to 9 : 1. After an incubation for 1 minute, the sample was applied to EICA. When the ratio of blood to the buffer was 7 : 3 or more, the samples did not migrate smoothly through the membrane and the red color was not washed away sufficiently from the antigen-immobilized membrane at 20 minutes after the application of substrate buffer. However, when the ratios of blood sample to the buffer were 6 : 4 or less in volume, the samples seemed to migrate smoothly through the membrane and the red color was washed away by the substrate buffer (Fig. 1). The smaller the ratio of blood to the buffer, the faster the sample migrated. The color intensity of the antigen line on the EICA membrane 20 minutes after the sample application was not affected significantly by the ratio of blood to the buffer from 4 : 6 to 6 : 4 (Fig. 2).

We also examined the effect of the incubation time of blood sample with the pretreatment buffer. We applied the blood samples to EICA at various time intervals after the incubation of 8 µl of whole blood with 12 µl of the buffer and found that the incubation period did not affect the results in EICA at a time period ranging from a few seconds to 60 minutes (data not shown).

In the following experiments, therefore, we applied the blood samples to EICA after the blood was incubated for 1 minute with the pretreatment buffer in the ratio of 4 : 6.

EFFECT OF HCT VALUE

We then examined the effect of Hct values of the blood samples on the result in EICA. First, blood samples with Hct readings of 30, 40, 50 and 60% were prepared by mixing plasma with hematocyte fractions from each of the 3 subjects described above. The Hct values of these samples were confirmed by the microhematocrit method. These samples were incubated with the pretreatment buffer for 1 minute and applied to the EICA membrane. The blood samples with a Hct value of 30% migrated faster through the membrane than those with a Hct value of 60%. However, 20 minutes after the sample application, antigen lines on EICA membranes were shown not to differ significantly in color intensity over the Hct values ranging from 30 to 60% ($p > 0.05$, Figure 3).

We also prepared the blood samples with various Hct values as mentioned above using blood from

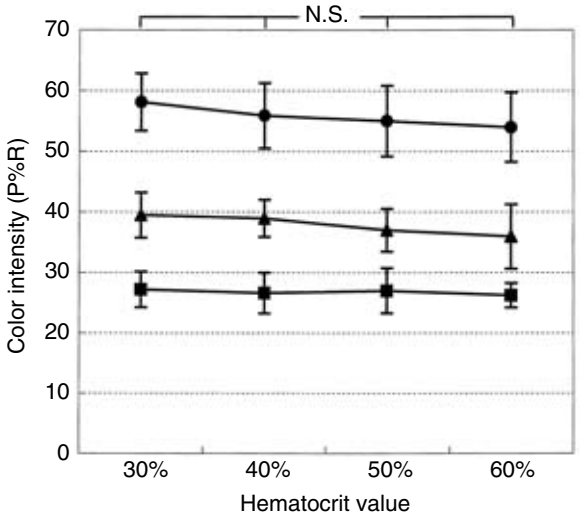

Fig. 3 Effect of hematocrit value on the color intensity of antigen line. Blood samples from 3 individuals (●, ▲, ■) prepared with hematocrit values from 30 to 60% were assayed for CJ pollen-specific IgE in EICA. The color intensity at 20 minutes after the sample application was expressed as P%R. Data are presented as the average ± 2SD of quadruplicate assay. N.S.; not significant ($p > 0.05$)

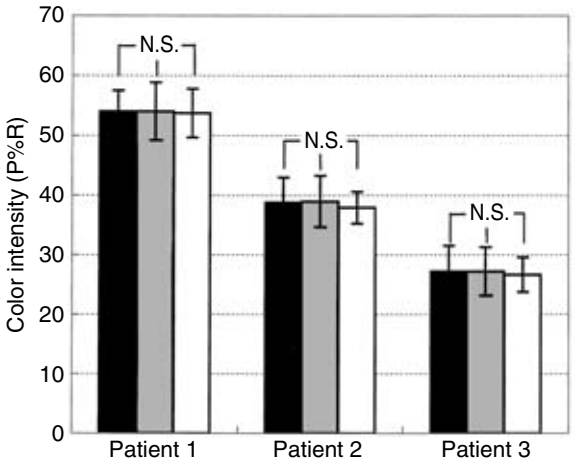

Fig. 4 Effect of various kinds of anticoagulant on the color intensity of antigen line. Whole blood obtained from 3 individuals each with 3 different kinds of anticoagulant (heparin, black: EDTA, gray: Citrate, white) were assayed in EICA. The color intensity of antigen line at 20 minutes was expressed as P%R. Data are presented as the average ± 2SD of quadruplicate assay. There was no significant difference among blood samples from each individual. N.S.; not significant ($p > 0.05$)

each of the 5 other subjects, and repeated the experiment to examine the effect of the Hct values of the sample on EICA. The results also showed that the Hct values from 30 to 60% did not affect the results in EICA (data not shown). These results indicate that Hct values of the blood from 30 to 60% do not practically interfere the results in EICA.

EFFECT OF KINDS OF ANTICOAGULANT

We examined the effects of 3 different kinds of anticoagulants, heparin, EDTA-2Na, and sodium citrate on EICA, to see if any of these anticoagulants in blood interfere the reaction in EICA. Blood samples obtained from each of the 3 subjects mentioned above using 3 tubes with different anticoagulants were pretreated with buffer as described above, and assayed for CJ pollen-specific IgE. There was no apparent difference in the migration of blood samples through the membrane and in the blue color intensity of the antigen lines among blood samples obtained with different anticoagulant from any of these 3 subjects ($p > 0.05$, Figure 4). We also mixed the blood sample with each of these anticoagulants at a volume 5 times more than the volume described in manufacturer's instructions. However, the results in EICA were not affected by any of these anticoagulants (data not shown).

EFFECT OF TOTAL IGE CONCENTRATION

Because ALP-anti-IgE reacts first with IgE in the blood sample in our EICA system, a high concentration of nonspecific IgE in the sample may interfere with the result of EICA. Therefore, we prepared the blood samples containing 2000, 6000, 20000, and 60000 IU/ml of IgE by adding purified human IgE to each blood from the 3 subjects mentioned above. The original concentrations of total IgE in sera from these 3 subjects were 63, 216, and 232 IU/ml, respectively.

When the blood sample contained 60000 IU/ml of IgE, the antigen line on the EICA membrane became significantly lower in color intensity than that obtained by using original blood ($p < 0.05$, Figure 5). However, there was no significant change in the color intensity when the blood contained up to 20000 IU/ml of nonspecific IgE ($p > 0.05$). To confirm the results mentioned above, we repeated the experiments using blood samples prepared by adding various concentrations of nonspecific IgE up to 20000 IU/ml to blood samples from 2 other subjects. The results confirmed that these concentrations of nonspecific IgE did not affect the assay for the specific IgE (data not shown). These results suggest that the pad on our EICA strip holds enough ALP-anti-IgE antibody to assay for antigen-specific IgE in the blood sample with up to 20000 IU/ml of total IgE.

CORRELATION BETWEEN RESULTS IN EICA AND THOSE IN ALASTAT

Blood samples treated with the detergent buffer and also sera from 136 subjects were assayed for CJ pollen-specific IgE by EICA and AlaSTAT, respec-

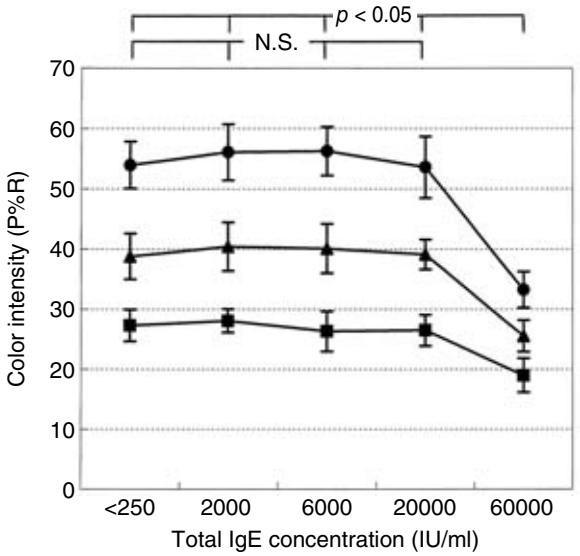

Fig. 5 Effect of total IgE concentration on the color intensity of antigen line. Whole blood from 3 individuals (●, ▲, ■) with various concentrations of total IgE were assayed in EICA. Data are presented as the average ± 2SD of quadruplicate assay. Total IgE concentration did not affect the results in EICA up to 20000 IU/ml. N.S.; not significant ($p > 0.05$)

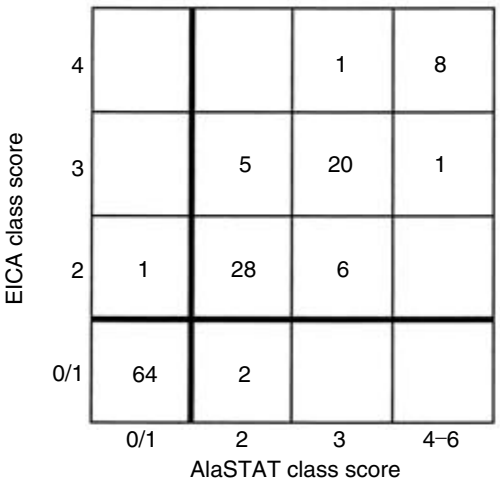

Fig. 6 Correlation between class scores in EICA and AlaSTAT ($N = 136$). Classification of the results in EICA is defined as described in RESULTS. Class 0/1, and class 4/5/6 in AlaSTAT were treated as one class score, respectively. Spearman's rank correlation coefficient was 0.956.

tively. Because data on antigen-specific IgE are commonly evaluated in terms of class score in clinical examinations, we classified the results obtained by EICA into 4 grades (class 0/1, 2, 3, and 4) : class 0/1, P%R < 10 ; class 2, 10 ≦ P%R < 20 ; class 3, 20 ≦ P% R < 50 ; class 4, 50 ≦ P%R. Serum samples obtained from these blood collections were also assayed by AlaSTAT system, and the results were classified into 7 grades (class 0–6) according to the manufacturer's instruction.

Class scores of 136 sera for CJ pollen-specific IgE in EICA corresponded well to those in AlaSTAT system (Fig. 6). The concordance rate was 97.8% when class 0 and 1 were categorized as negative and others were categorized as positive. The Spearman's rank correlation coefficient was 0.956 when class scores 0–1 and 4–6 in AlaSTAT assay were treated as one class score, respectively, and the numbers of class score for both assay systems were arranged similarly. Average ± 2SD of Hct values and total IgE concentration of these blood samples were 46 ± 8% and 190 ± 668 IU/ml, respectively. In these samples, the maximum Hct value was 57%, and the maximum IgE concentration was 1912 IU/ml.

DISCUSSION

Currently there are several immunochromatography systems available for assaying analytes in whole blood. In most of these systems, hematocytes are trapped by a plasma separation pad and released plasma migrates through an immunochromatography membrane. However, in our preliminary experiments, hematocytes could not be trapped completely and a part of hematocytes moved to immunochromatography membrane interfered with the immunochromatography assay. We, therefore, tried to treat whole blood samples with the detergent to lyse hematocytes before the application of the sample to the membrane. A similar method to lyse hematocytes in blood sample has been reported in the analysis of lactate in blood,[5] and has been applied to assay for C-reactive protein by a latex turbidometric assay (CRP-2100, Nihon Kohden Corporation, Tokyo, Japan). To our knowledge, however, there is no report on the application of the hemolyzed blood sample to immunochromatography system. The consecutive application of the substrate buffer to the substrate reservoir pad makes it possible to migrate the hemolyzed whole blood through the immunochromatography membrane.

The results in EICA to which hemolyzed blood was applied were not affected significantly by Hct values from 30% to 60%. The sample with higher Hct values appeared to migrate slower on the membrane. The slower migration results in taking longer time for antigen-antibody reaction. The longer reaction time of the blood sample with high Hct value could compensate for the disadvantage of the lower volume of plasma in the blood sample with high Hct value. Actually, all of our whole blood samples with various Hct values from 37% to 57% migrated smoothly through the membrane, and the results correlated well to those in AlaSTAT. Ninety five percent of males and females were reported to be 40–53% and 36–48%, re-

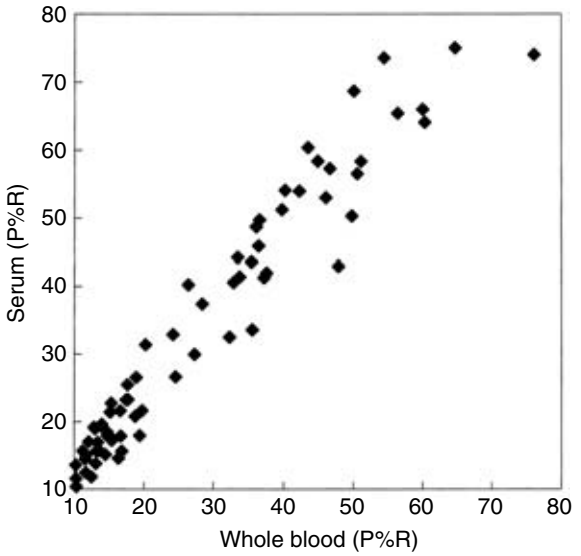

Fig. 7 Comparison of EICA results obtained using whole blood to those using serum. (N = 70, the results of the samples which were classified class 0/1 were not included.) The correlation coefficient was 0.968.

spectively, with the Hct value.[9] Therefore, our EICA system would be practically applicable to the blood samples irrespective of Hct value.

None of the anticoagulants we examined, heparin, EDTA or sodium citrate, in blood collecting-tube affected the results in EICA, when an appropriate volume of blood was obtained. However, the staining of antigen line on the membrane was a little pale, when only a very small amount of blood sample, such as less than 0.5 ml, was collected in the tube with sodium citrate, although blood samples obtained by the tube with heparin or EDTA was not affected in staining of the line even when the sample volume was 0.3 ml per tube. This could be caused by the dilution of the blood sample with sodium citrate solution and also by the interference of alkaline phosphatase activity by the sodium citrate. Therefore, it is desirable to use tubes with heparin or EDTA for our system.

There were 6 cases in 136 blood samples in which total IgE concentrations were more than 1000 IU/ml, and the maximum IgE concentration was 1912 IU/ml. Results in EICA of these samples correlated well with those in AlaSTAT. We also confirmed that the results in EICA were not affected by the concentration of total IgE up to 20000 IU/ml. There are several diseases with high IgE concentration in serum such as allergic bronchopulmonary aspergillosis,[10] hyper IgE syndrome,[11] and IgE myeloma.[12,13] When Barbee *et al.* examined the distribution of total IgE concentration in sera from 2743 subjects, the highest concentration of total IgE was 7273 IU/ml and the concentration of 95% subjects was below 553 IU/ml.[14] Therefore, most of the blood samples from subjects without particular diseases with very high total IgE could be examined by our EICA system.

The results obtained by our EICA and AlaSTAT on CJ pollen-specific IgE correlated well to each other. The concordance rate was 97.8% and the Spearman's rank correlation coefficient was 0.956. We also assayed the sera from these 136 persons by CAP-system for CJ pollen-specific IgE. These results also correlated quite well to those obtained by the EICA. The concordance rate was 94.1% and the Spearman's rank correlation coefficient was 0.928. These results show that the results obtained by EICA correlated well to those obtained by both AlaSTAT and CAP, which are commonly used for quantitation of antigen-specific IgE in serum.

In our previous report,[2] the results obtained by the serum assay for specific IgE by EICA were confirmed to correlate well to those by AlaSTAT. We also confirmed the reliability of the whole blood assay in the present experiment by comparing the EICA results obtained using whole blood to those obtained in EICA using sera as shown in Figure 7. Although the color intensity of the antigen line of whole blood sample was a little lower than that of the serum obtained from the same blood, results obtained using whole blood correlated well to the results obtained using serum. These results indicate that whole blood assay for antigen-specific IgE in EICA is reliable.

In our system, we used SBP for CJ pollen antigen. Cry j 1 and Cry j 2 were reported to be major allergens of the cedar pollen.[15] Therefore, antigen preparation used for our assay system has to contain both Cry j 1 and Cry j 2. The SBP we prepared for the present experiment actually contained both proteins as described in "METHODS". In our second preparation of SBP, both proteins were also contained in a similar ratio, suggesting that our SBP preparation is suitable to detect the CJ pollen-specific IgE. The good correlation of the results in our EICA with those in AlaSTAT in which we used the antigen included in the kit also indicates that our SBP preparation is appropriate to assay for CJ pollen-specific IgE.

In our previous report, we could detect CJ pollen-specific IgE as low as 0.2 U/ml.[2] However, in the present experiment, the detection limit was class 2 or 0.7 U/ml. The reasons for the difference may be because of the dilution of whole blood with pretreatment buffer and the reduction of the sample volume from 25 µl to 20 µl.

In our system, we could assay the allergen specific IgE within 30 minutes including the time for blood collection using only 8 µl of whole blood without any blood separation procedure. This would be a big advantage for patient, because specific allergen could be identified by a first visit to a clinic. A possible disadvantage of our system is the underestimation of the specific IgE concentration, especially by the effects of high Hct values and high total IgE concentration.

However, blood samples quite rarely exceed 60% Hct value or 20000 IU/ml IgE as discussed above.

Another possibility we have to consider on the assay for antigen-specific IgE is the competitive inhibition of the binding of the specific IgE to antigen by the specific IgG as previously described.[16] To examine this possibility, we screened our 136 serum samples and selected 3 samples containing the highest concentration of CJ pollen-specific IgG (#1, 2.22 μg/ml ; #2, 0.99 μg/ml ; #3, 0.89 μg/ml) but no detectable CJ pollen-specific IgE (<0.35 U/ml). We also selected a sample (#4) containing a low concentration of the specific IgG (0.13 μg/ml) with relatively high concentration of the specific IgE (5.65 U/ml). We mixed the sample #4 with an equal volume of sample #1, #2, #3, or PBS, and assayed for the specific IgE in our EICA system in quadruplicate. Results were as follows : mixed with #1, 16.8 ± 1.9 P%R (average ±2 SD) ; with #2, 17.3 ± 1.7 P%R ; with #3, 17.7 ± 3.0 P %R ; with PBS, 16.7 ± 1.8P%R. There were no significant differences in these results including #4 diluted with PBS ($p > 0.05$). We also examined the staining of antigen line by mixing the serum (#5) containing high concentrations of specific IgG (3.68 μg/ml) and IgE (3.00 U/ml) with an equal volume of sample #4 serum. There was no significant difference in P%R between the results of the sample #4 alone and those obtained using the mixture. In these combinations, there was no observed effect of the specific IgG on the antigen line staining. These results suggest that the concentrations of antigen-specific IgG in most of the samples do not affect the assay for the specific IgE in our EICA system, although an extremely high concentration of the specific IgG may inhibit the reaction of the specific IgE to the antigen line.

To our knowledge, there is no quantitative assay kit for antigen-specific IgE in whole blood. Although results of our EICA system require further evaluation in correlation with data on clinical symptom, it is possible that the present EICA system will contribute to medical service as a point of care test in near future.

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