

Quantitative Measurement of Serum Allergen-Specific IgE on Protein Chip

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Abbreviations : Dp, *Dermatophagoides pteronyssinus*

Abstract

Type I allergy is an immunoglobulin E (IgE)-mediated hypersensitivity disease inflicting more than quarter of the world population. In order to identify allergen sources, skin provocation test and IgE serology was performed using allergen extracts. Such process identifies allergen-containing sources but cannot identify the disease-eliciting allergenic molecules. Recently, microarray technology has been developed for allergen-specific IgE detection using rolling circle amplification. This study was carried out to evaluate protein chip technology for the quantitative measurement and limits of sensitivity of multiple allergen-specific IgE by an immunofluorescence assay. Significance of positive calibrators was tested using purified human IgE. *Dermatophagoides pteronyssinus* (Dp), egg white, milk, soybean, and wheat were used as allergens and human serum albumin as negative control. Sensitivity and clinical efficacy of protein chip were evaluated using allergy immune serum for Dp. The fluorescent intensities for purified human IgE as calibrator were well correlated with the concentrations of human IgE. Two-

fold dilution of serum allowed an optimal reaction with Dp (1 mg/ml) at which serum Dp-specific IgE levels by protein chip were compatible with those by UniCap®. The sensitivity of protein chip in this study was found at level of 1 IU/ml of IgE. Dp-specific IgE levels by protein chip correlated well with those of UniCap® by comparing 10 atopic dermatitis. Additional 18 sera were tested for above multiple antigens other than Dp and significant results were obtained for many antigens as well as Dp. These results indicated that spotting of heterogeneous protein mixture on protein chip and the quantitative measurement of serum allergen-specific IgE levels using immunofluorescence assay can be successfully applied in the clinical laboratory for the diagnosis of allergy and could be applied to diagnosis of autoimmune and infectious diseases

Keywords: Crude Allergen Extracts, IgE, Protein Chip, Allergy

Introduction

Allergy is a state of immune dysregulation from Th1/Th2 balance and this state leads to overproduction of IgE (Lacour, 1994) which plays a central role in allergy (Jujo *et al.*, 1992). Avoidance of allergen is the first principle for the treatment of allergic diseases (David, 1992). However, clarifying the true causative agents that provoke allergy in individual patients still remains challenging task. Several methods such as skin prick test, patch test as well as identification of serum allergen-specific IgE are performed to suspect the causative allergen (Brown *et al.*, 1979). Especially, identification of allergen-specific IgE in circulation is one of the most important methods for the diagnosis of allergic causes (Rance and Albero, 1984).

The allergen-specific antibody was the first reported by Millman (Millman *et al.*, 1964). Discovery of allergen associated IgE was reported by Isizaka (Isizaka *et al.*, 1966) and Johansson and Bennich (Johansson and Bennich *et al.*, 1967). IgE concentration is the lowest among the five antibody isotypes and the detection of antigen-specific IgE is different from those to detect other antibodies. In consequence, serum IgE concentration is too low to detect by conventional immunoassay and to enhance the sensitivity of assay, radioallergosorbent test (RAST) was developed by Wide (Wide *et al.*, 1967). However, due to the safety issues of isotope, it was replaced by fluorescence-tagged assay system (Tsay and

Halpern *et al.*, 1984). Recently, even enzyme immunoassay method has appeared to identify allergen-specific IgE (Ali *et al.*, 1980).

Protein chip based on the established DNA microarray technology (Barinaga, 1991, Kim *et al.*, 2001) was launched in 1999 (Lueking *et al.*, 1999) by covalent linking of protein ligands to the activated silica surface of microscope slides (Gavin and Stuart, 2000). Possibility of protein microarray technology for characterization of variation patterns in hundreds of thousand different proteins in clinical application was explored (Brian *et al.*, 2001). Protein chip based on the quantitative immunoassay for prostate-specific membrane antigen (PSMA) was recently developed (Xiao *et al.*, 2000). Recently, allergen-specific IgE detections on microarrays using rolling circle amplification were reported (Mullenix *et al.*, 2001, Wiltshire *et al.*, 2000).

The allergen protein microarray technology allows the determination and monitoring of allergic patients' IgE reactivity profiles to large numbers of disease-causing allergens by using single measurements and minute amounts of serum.

In this study we have evaluated protein chip for the quantitative measurement of multiple allergen-specific IgE by immunofluorescence detection methods and assessed sensitivity limitations and test conditions in parallel with commercially available test kit (UniCap®) using well known allergens; *Dermatophagoides pteronyssinus* (Dp), egg white, milk, soybean, and wheat. Our finding showed that this method may change established practice in allergy diagnosis, efficacy test, prevention, and therapy.

Materials and Methods

Patients and immune sera

This study included a total of 57 atopic dermatitis patients who visited Seoul Allergy Clinic, Seoul, Korea from November 1, 2001 to March 31, 2002. They fulfilled the criteria of Hanifin and Rajka (Hanifin and Rajka, 1980). Venous blood samples were drawn from the antecubital vein and sera were stored at -80°C until analysis. A parts of all sera were pooled as one for conditioning and calibration of protein chip. The blood levels of specific IgE for *Dermatophagoides pteronyssinus* (Dp), egg white, soybean, milk, and wheat were also measured by UniCap® method as described previously (Yunginger *et al.*, 2000). Consent form was given to all subjects whose blood were tested.

Protein preparation

Fresh wheat grains and soy beans materials were washed, lyophilized, and milled into powder. The prepared powder, milk, and egg white were delipidated

by extraction with 20% hexane solution. The delipidated powders were suspended in phosphate buffered saline (PBS: 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4 containing 137 mM NaCl and 2.7 mM KCl) at a 1:3 weight ratio, stirred for several hours at 4°C, and insoluble residues were removed by centrifugation at 20,000 *g*. Most of the proteins in supernatant precipitated with 70% saturation of ammonium sulfate were collected by centrifugation, then precipitates were dissolved in PBS, dialyzed against distilled water, and lyophilized. These proteins were kept at -80°C till use. Delipidated egg white and milk proteins were prepared in a similar manner. Allergen extracts of Dp were kindly donated from Institute of Allergy and Department of Parasitology, Yonsei University, College of Medicine. Purified human IgE (Chemicon, Temecula, CA, USA) and human serum albumin was obtained from Sigma, (St. Louis, MO, USA)

Preparation of protein chip by spotting crude allergen extracts

For spotting, protein extracts were dissolved in PBS containing 40% glycerol at a concentration of 1 mg/ml and 10 µl aliquots were plated into each 384-well plates. Microsys 4100 (Cartesian, Irvine, CA, USA), a robotic microarrayer of spray type spotted 10 nl of the protein solutions in an ordered array onto nitrocellulose membrane on slides at a 1500 µm spacing. FAST™ slides (Schleicher & Schull, Dassel, Germany) to which nitrocellulose membrane binds were used for spotting. The resulting protein chips were sealed in a slide box and stored at 4°C.

Reaction and detection

The arrays were briefly rinsed in a 3% bovine serum albumin (BSA) solution containing PBS and 0.1% Tween-20 to remove unbound proteins. Protein chips were immersed into a 3% BSA solution containing PBS and 0.02% sodium azide for blocking unreacted sites for overnight at 4°C. Protein chips were then washed three times with PBS containing 0.1% Tween-20 (washing buffer). These prepared protein chips were incubated with patients sera for 1 h in a chamber with shaking rotor, washed three times with washing buffer, and incubated with biotin-conjugated anti-human IgE (Vector, Burlingame, CA, USA) for 1 h at room temperature, washed three times with the washing buffer, and incubated with Cy3-conjugated streptavidin (Sigma, St. Louis, MS) for one hour, and washed three times with washing buffer. The reacted chips were spin-dried and scanned in Axon Laboratories (Palo Alto, CA, USA) scanner (Palo Alto, CA, USA) using 532 nm laser. The fluorescence intensities were measured in the Cy3-specific channels at each spot using GenePix Pro (Axon Laboratories).

Significance of protein chip for the identification of allergen-specific IgE

For calibration, purified human IgE solutions serially diluted to 500, 250, 125, 62.5, 31.3, and 15.6 IU/ml were spotted. Human serum albumin was used as negative control and evaluated as 0 IU/ml. The significance of positive calibrator was also tested using 29 patients sera by plotting the absolute fluorescence values for all spots.

To determine optimal antigen and serum level, Dp antigen serially diluted to 2,000, 1,000, 500, 250, 125, and 62.5 $\mu\text{g/ml}$ was spotted and tested with the pooled serum whose Dp-specific IgE level had been measured with UniCap® (Pharmacia & Upjohn Diagnostics AB, uppsala, Sweden) method and serially diluted to 1-, 2-, 4-, 8-, 16-, and 32-fold, respectively. All tests were done in triplicate. Various protein mixture of four antigens spotted were also tested. Ten immune sera for Dp, 4 for egg white and wheat, and 5 for soybean and milk were evaluated by both protein chip and UniCap® methods and estimation of IgE levels for each allergens using UniCap® as reference were compared with the results obtained with protein chip method and the statistical significances were evaluated.

Statistical analysis of data was performed using the SPSS version 7.0. Data is shown as mean \pm standard deviation. The correlation and linear regression was used in this study. Differences associated with a probability of $p < 0.05$ were considered significant.

Results

Significance of calibrator

To determine optimal reaction conditions of the biotin-streptavidin coupled immune assay on a microarray, purified human IgE serially diluted from 500 to 15.6 IU/ml was used as a reference standard and human serum albumin as a negative control. Fluorescent Intensity of spots increased linearly with the rise of IgE concentration. (Figure 1a) ($y = 1.5x + 711$, $R^2 = 0.84$, $P < 0.05$, Correlation Coefficient = 0.93) (Figure 1b). Some non-specific fluorescence detected on the negative control was always below positive control and also constituted a part of the calibration curve.

Optimal Reaction Conditions for Antigen Concentration and Serum Dilution

To determine the optimal reaction conditions for allergen and serum levels, varying levels of Dp antigens were spotted on the protein chip and reacted with two-fold diluted pooled sera that are again serially diluted 1- to 32-fold respectively. Results showed that 2-fold diluted sera gave optimal yield (Figure 2a). Data showed a corresponding fluorescence intensity decrease correlated

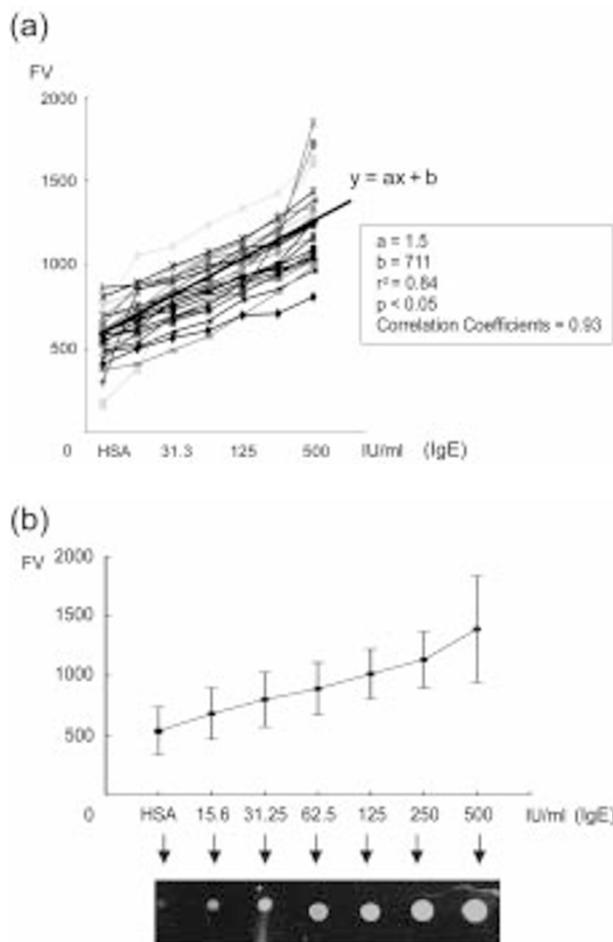


Figure 1. a) Calibration curve of protein chip which were spotted with purified human IgE of variable IgE concentrations as positive calibrator using 29 immune sera ($y = 1.5x + 711$, $R^2 = 0.85$, $P < 0.05$, Correlation Coefficient = 0.93), b) Calibration curve plotted with mean values of spots of variable human IgE concentrations showed a significant linearity. FV, fluorescent intensity values of spots; HAS, human serum albumin as a negative control. conc., concentration.

with the dilution. Spots containing Dp levels above 500 $\mu\text{g/ml}$ reacted with appropriate sera IgE levels measured with the UniCap® method. To determine the antigen level on the chip spot whose reaction with IgE sera compatible with the UniCap® measurement, serially diluted Dp antigens were spotted from 2.0 mg/ml to 62.5 $\mu\text{g/ml}$ and tested 7 times using pooled serum. Based on IgE calibration chart, Dp level of 1 mg/ml showed optimal response where protein chip spotted Dp-specific IgE levels became compatible with those levels measured by UniCap® method (Figure 2b).

Antibody Dilution and Sensitivity

Protein chips spotted with 1 mg/ml of Dp antigen were reacted with serially diluted sera to examine the sensitivity. Three sera with different Dp-specific IgE levels of 24.5, 39.8, and 67.3 IU/ml, respectively were

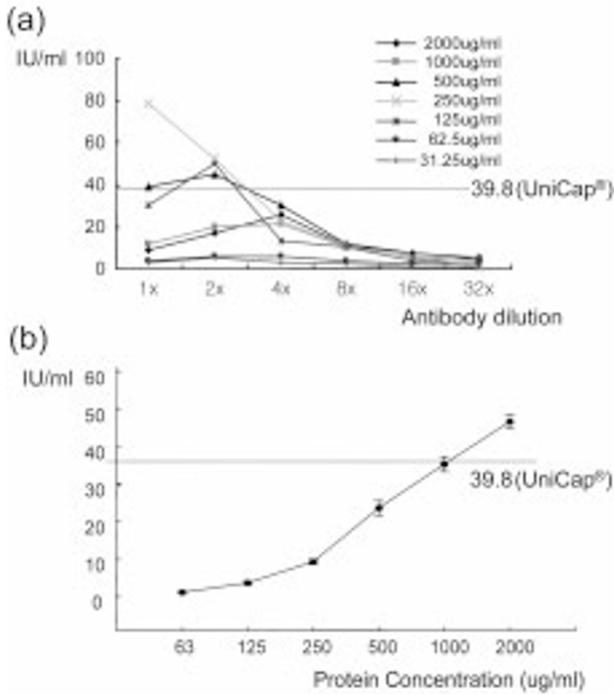


Figure 2. Optimal antigen concentration and proper serum dilution factor. a) *Dermatophagoides pteronyssinus* (Dp) antigens were serially diluted from 2.0 mg/ml, 1.0 mg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, and 31.3 µg/ml. Pooled sera was diluted to 1-, 2-, 4-, 8-, 16-, and 32-fold. Dotted line indicated the serum value of Dp-specific IgE. Proper dilution factor was 2-fold. Spots of antigen concentration above 500 mg/ml showed significant IgE concentration which were confirmed by UniCap®. b) For optimal conditions for antigen concentration and serum dilution, serially diluted Dp antigen was spotted as 2.0 mg/ml, 1.0 mg/ml, 500 µg/ml, 250 µg/ml, and 125 µg/ml, 62.5 µg/ml, and 31.3 µg/ml. Optimal concentration which resulted in Dp-specific IgE levels that were compatible with those by UniCap® was 1 mg/ml.

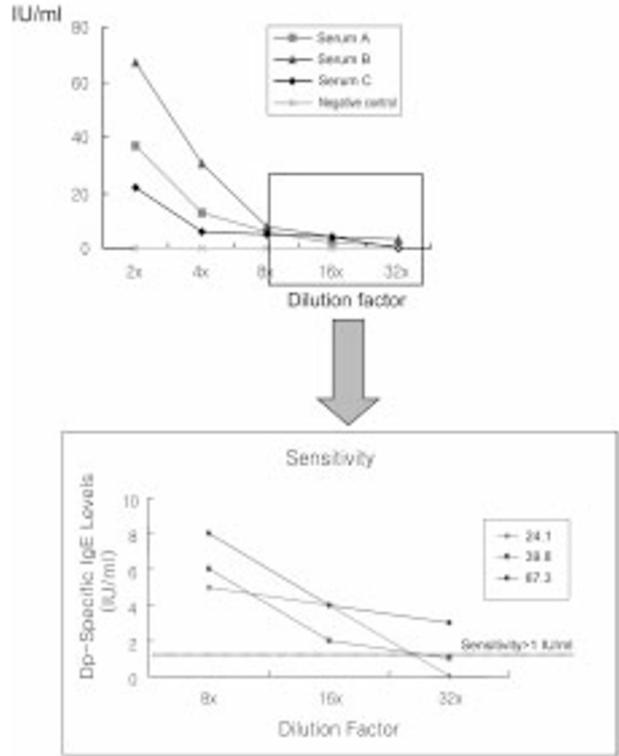


Figure 3. Antibody dilution effects and sensitivity of protein chip. Three sera with known *Dermatophagoides pteronyssinus* (Dp)-specific IgE levels were tested by diluting serially in this study. The sensitivity of protein chip in this study is over 0.5 IU/ml. Actual sensitivity for clinical use is over 1 IU/ml because sera.

examined (Figure 3). The results showed sera dilution dependent near linear decrease response where 32-fold dilution of 39.8 and 67.3 IU/ml samples gave reactions within a linear reaction curve above the negative control. However, 32-fold dilution of the 24.5 IU/ml sample showed reacted spot below the negative control level. Based these results, the sensitivity limit of protein chip can reach to 1 IU/ml IgE level.

Clinical significance of protein chip

The experimental and clinical relevance for the quantitative measurement of Dp-specific IgE using protein chip was evaluated using 10 immune sera containing Dp antigen. Reaction was carried out with Dp antigen level at 1 mg/ml and two-fold diluted sera. The results were compared with those measured by UniCap® method. The results by protein chip well correlated with those of UniCap® ($y = 1.11 \times -0.23$, $R^2 = 0.98$, $P < 0.05$, Correlation Coefficient = 0.99)

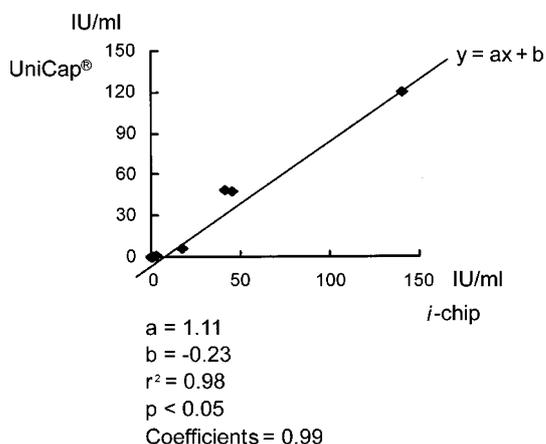
(Figure 4a). The results were classified according to grading system. Especially, grading of Dp-specific IgE levels by protein chip in this study matched well with those by UniCap® ($y = 1.02 \times -0.12$, $R^2 = 0.98$, $P < 0.05$, Correlation Coefficient = 0.99) (Figure 4b). Multiple allergen-specific IgE levels by protein chip and by UniCap® were compared (Figure 5a) and well correlated with each other by statistical analysis ($y = 0.99 \times -0.56$, $R^2 = 0.95$, $P < 0.05$, Correlation Coefficient = 0.97) (Figure 5b).

Discussion

Protein chip array method was applied for the quantitation of multiple allergen-specific IgE in this study by immunofluorescence assay. Examination of protein chips spotted with varying concentrations of purified human IgE as a reference gave fluorescent intensities values closely parallel with its concentrations (Figure 1). Such results suggested that an application of protein chip by immunofluorescence assay for the quantitative

(a)

Test sample	D.P	
	i-chip (IU/ml)	UniCAP (IU/ml)
Total (11)		
Sample 1	140	120
Sample 2	41	48.5
Sample 3	0	0.31
Sample 4	3	1.41
Sample 5	17	6.48
Sample 6	0	0.2
Sample 7	0.5	0.38
Sample 8	45	47.4
Sample 9	0	0
Sample 10	0.34	0.3
Sample 11	0	0.2



(b)

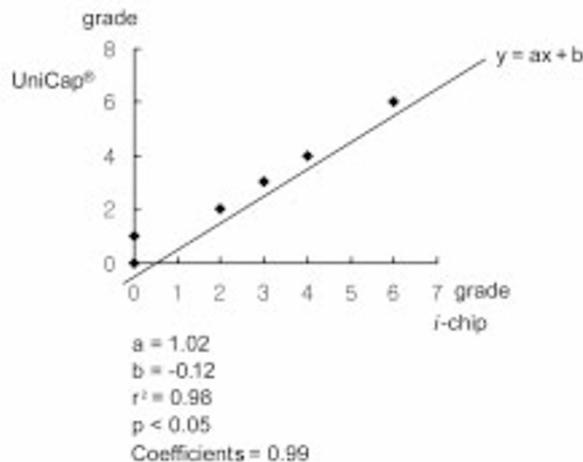
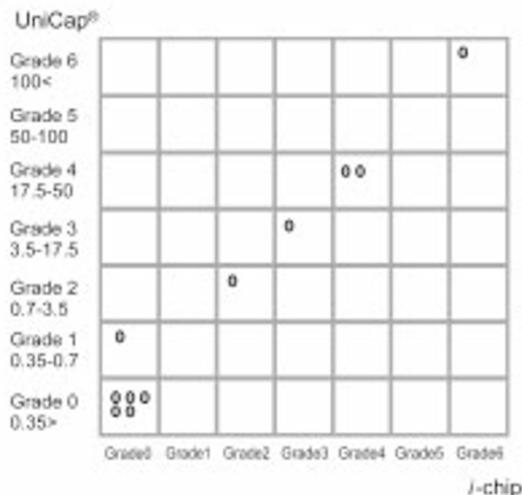


Figure 4. Correlation of the serum *Dermatophagoides pteronyssinus* (Dp)-specific IgE levels with those by UniCap®. a) Serum Dp-specific IgE levels by protein chip in this study were significantly correlated with those by UniCap® ($p < 0.05$) using 10 Dp-allergy immune sera ($y = 1.11x - 0.23$, $R^2 = 0.98$, $P < 0.05$, Correlation Coefficient = 0.99). b) Correlation between grades of serum Dp-specific IgE levels by protein chip and those by UniCap® was more significant statistically ($y = 1.02x - 0.12$, $R^2 = 0.98$, $P < 0.05$, Correlation Coefficient = 0.99).

measurement of allergen-specific IgE was possible. The spotted antigen level at 1 mg/ml was found to yield result compatible with the serum *Dermatophagoides pteronyssinus* (Dp)-specific IgE levels by UniCap® method (Figure 2). The level appeared to be much higher in comparison with concentrations used in enzyme-linked immunosorbent assay (ELISA) or in other protein chip (Mullenix et al., 2001). The higher IgE levels were detected at the higher protein concentration of spotting suggest that actual antigen level might be much less in the crude antigen extracts used for spotting. The high concentration (1 mg/ml) was selected for spotting. The two-fold dilution of sera reduces the serum volume requirement for analysis. Sensitivity of protein chip for serum Dp-specific IgE was found to be at 1 IU/ml for clinical use (Figure 3)

comparing with 0.35 IU/ml of UniCap®. Dp-specific IgE levels of less than 1 IU/ml were classified as Grade 1 (Tsay and Halpern, 1984). For milk allergy, 7.5 IU/ml is the minimum allergen-specific IgE level for clinical application (Sampson, 2001). The sensitivity of protein chip is not a single issue for clinical use. Especially, in clinical fields, grading rather than absolute value of allergen-specific IgE is more useful. Not only the serum Dp-specific IgE concentration but also the grades of IgE levels by protein chip well-correlated with those of UniCap® (Figure 4). Moreover, the multiple allergen-specific IgE detection other than Dp was also significant. To enhance the sensitivity of protein chip, either selective enrichment of serum IgE concentration or more sensitive IgE coupled detection methods need to be developed.

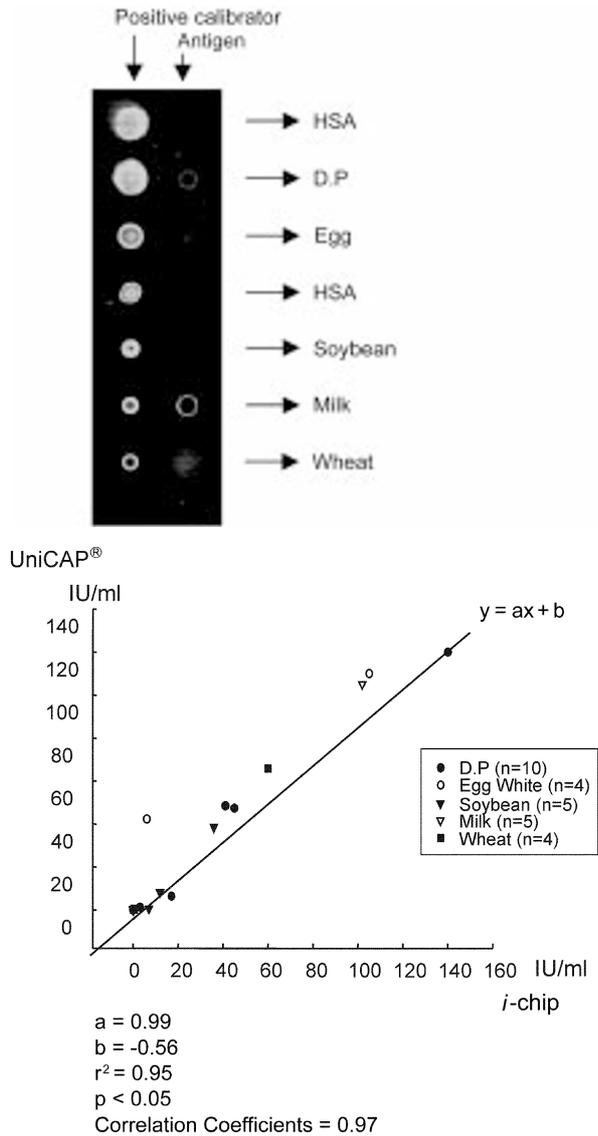


Figure 5. The quantitation of multiple serum allergen-specific IgE. a) Scan of protein chip for the analysis of multiple allergen-specific IgE. Multiple allergen of *Dermatophagoides pteronyssinus* (Dp), egg white, milk, soybean, and wheat were spotted at a concentration of 1mg/ml and reacted with sera of 2x dilution. Cal, calibrator, b) Correlation of the serum allergen-specific IgE levels by protein chip with those by UniCap[®] ($y=0.99x-0.56$, $R^2=0.95$, $P<0.05$, Correlation Coefficiency= 0.97).

The avoidance of allergen is the primary principle of treatment and the causative controls are the most important for treatment of allergy (David, 1992). For this purpose, wider array of allergens from foods to environmental sources to which patients are exposed have to be tested for the diagnosis and avoidance therapy of allergy. However, such wide spectrum of allergens have not been tested because of the needs for a large quantity of blood samples and high cost by the available detection methods.

Protein chip may solve the limitations of conventional methods such as RAST or FAST with its several essential advantages such as efficiency, low cost and a little sample quantity for tests regardless of items to be tested as well as capability of simultaneously testing many kinds of items (Nelson, 2000). It may change established practice in allergy diagnosis, efficacy test, prevention, and therapy. Using protein chip, the tests for newborn or cord blood will be possible for many kinds of items without limitations in sample quantity.

Until now, protein chip was cautiously applied to identify antibodies or antigens using purified protein or purified antibody (Xiao *et al.*, 2000). The heterogeneous protein mixture was used for spotting in this study. Heterogeneous protein mixture of crude antigen extracts rather than several purified allergens are more proper for the diagnostic purpose in allergy because all protein components in protein extracts possibly played a role as allergens. Use of heterogeneous protein mixture and detection of reacting antibody to components of the heterogeneous protein mixture is a marked advance in protein chip technology. Although human serum was used in pre-clinical tests (Schneider, 2000), human serum was used for the clinical diagnostic purpose for the analysis of allergen-specific IgE levels in this study. Spotting of heterogeneous protein for protein chip and use of serum directly for tests might be the most important factors for the clinical application of protein chip in other clinical fields. Serum total IgE is too low in concentration to detect by conventional methods in sera. Moreover, allergen-specific IgE is extremely low. The possible quantitative measurement of serum allergen-specific IgE is an important progress in protein chip technology. Recently, allergen-specific IgE detection on microarrays using rolling circle amplification was tried to solve this problem of sensitivity for IgE detection (Wiltshire *et al.*, 2000, Mullenix *et al.*, 2001). In the report, pin-tool type microarrayer was used for spotting. Pin-type microarrayer is a semi-quantitative machine for the control of spotting volume. Protein concentration was the only factor to control for spotting and conditioning. From these reasons, they might use the detection method of rolling circle amplification. However, spray-type microarrayer was used in this study. Both volume and concentration were controllable for conditioning of protein chip for spotting. For the exact conditioning of immunoassay, volume is an important factor to control as well as concentration of solution. Resultant conventional immunofluorescent assay was successful for the quantitative measurement of allergen-specific IgE in this study. Lastly we have demonstrated that the quantitative measurement of multiple allergen-specific IgE on a protein chip simultaneously. Before the appearance of protein chip, it was impossible to test multiple measurement in a well or in a test tube. However, in this study, the quantitation of multiple

allergen-specific IgE on a protein chip was successfully achieved with the statistically significant results of every items (Figure 5).

In conclusion, protein chip using immunofluorescence assay is an useful and effective method for the quantitative measurement of multiple serum allergen-specific IgE in allergy filed. Further investigation might be needed and more application to other clinical fields and other diseases might be possible.

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