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Optimization of Critical Factors Affecting the Performance of an Allergen Chip for the Analysis of an Allergen-specific Human IgE in Serum

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A sensitive and multiplexed assay of allergen-specific human immunoglobulin E (IgE) is of great significance in the precise diagnosis of allergies. We report on the optimization of critical factors for chip-based analysis of IgE in human serum with a high reliability. Extracts of two mite species were used as model allergens, and were spotted onto a glass slide for the construction of an allergen chip. Respective allergen-specific IgE in human serum was analyzed by using biotinylated anti-human IgE and a streptavidin-Cy3 conjugate. Factors affecting the performance of the allergen chip were investigated and optimized. Especially, the effect of additives, the concentrations of biotinylated anti-human IgE and the streptavidin-Cy3 conjugate, the serum dilution factor, and the concentration of allergen extract as a capturing agent were examined in detail. Under the optimized conditions, a chip-based analysis for sera from 43 patients revealed a reliable and reproducible diagnosis of respective allergies, showing a good correlation with a conventional CAP assay.

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Introduction

About one-fourth of the world population is known to suffer from immunoglobulin E (IgE)-mediated hypersensitivity disease, Type-I allergy, which is mainly caused by various allergens, and is considered to be a major health problem in industrialized countries.[1] Although allergens are not the sources of a disease by themselves, the binding of an allergen to IgE on mast cells induces the release of inflammatory mediators, like histamine and leukotrienes, from the cells of a sensitive person. Released mediators resulted in various clinical symptoms, like allergic rhinitis, allergic asthma, anaphylaxis, urticaria, and angioedema.[2,3] Chronic manifestation by repeated exposure to an allergen causes an elevation of the IgE concentration in serum. For the efficient treatment and ease of allergy, a precise diagnosis of allergen-specific IgE is a prerequisite, and various assay methods have been reported, including a skin-test,[1] radio allegro sorbent assay, and a chromogenic or fluorescence enzyme immunoassay.[4] A CAP assay system (Pharmacia Diagnostics AB, Uppsala, Sweden), which employs an allergen-coated cellulose disc and anti-IgE conjugated β-galactosidase, is most widely used. However, these methods are time-consuming and costly, requiring large amounts of sample and relevant reagents. When considering the increasing number of allergens and very low abundance of IgE in serum (10^{-3} – 10^{-4} fold of immunoglobulin G),[5] the development of a more multiplexed and sensitive assay method is highly required.[6]

Recently, chip-based analyses have been attempted in conjunction with various detection methods, such as rolling circle amplification,[7] chemiluminescence,[4] and fluorescence[6,8,9] as a promising approach, because it enables a sensitive and high-throughput assay with tiny amounts of reagents.[10] As capturing agents of the chip-based analysis of an allergy, allergen extracts[6] or purified allergen molecules[8] were of preferred use. Of them, purified allergens produced by recombinant DNA techniques often resulted in incorrect folding or missing glycosylation required for IgE recognition,[11,12] which causes a loss of some IgE-binding epitopes.

Despite numerous attempts to create an allergen chip, its practical application still remains a challenge. To develop a viable allergen chip, its performance, like reproducibility, sensitivity, and specificity, should be satisfied. Especially, the conditions for fabricating an allergen chip and signal detection as well as the preparation of clinical samples for assays should be optimized. Although there have been reported some approaches[3,4,8] regarding optimization of the analytical conditions, most of them have dealt with only a limited number of parameters. In addition, comparative analysis between chip-based and conventional CAP assays has not been considered for optimization.

In this work, we report on the optimization of the crucial factors affecting the chip-based analysis of allergen-specific human IgE in serum. Extracts of two mite species, *Dermatophagoides farinae* and *D. pteronyssinus*, were used for constructing of an allergen chip. Allergen-specific IgE in human serum was detected by a subsequent treatment with biotinylated anti-human IgE and streptavidin-Cy3 conjugate. Factors affecting the performance of the developed allergen chip were investigated and optimized. Sera from 43 patients were analyzed using the allergen chip and compared with a conventional method. The details are reported herein.

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Experimental

Construction and assay of an allergen chip

An allergen chip was constructed by spotting allergens onto an epoxy-coated glass slide (Slide E, Schott Nexterion, Jena, Germany) using a robotic arrayer (Microsys, Cartesian Technologies, Irvine, CA, USA) and CMP 3 spotting pin (Telechem International, Sunnyvale, CA, USA). As model allergens, two lyophilized mite species, *D. farinae* and *D. pteronyssinus*, were purchased from Allergopharma (Reinbek, Germany), solubilized with phosphate-buffered saline (PBS, pH 7.4), and used. Anti-bovine serum albumin (Anti-BSA, Sigma-Aldrich, St. Louis, MO, USA) and anti-human IgE (Chemicon International, Temecula, CA, USA) were used at a final concentration of 0.5 mg ml^{-1} as negative and positive controls, respectively. All spotting solutions were supplemented with an additive and spotted in quadruplicate at 70% relative humidity. The distance between spots was about 400 μm.

Following spotting and incubation, the resulting slide was blocked with 3% BSA in PBS at 4°C overnight, washed with PBS, and incubated with 15 μl of PBS-diluted human serum at room temperature for 1 h, as described elsewhere.[3,4,6-8] Subsequently, the slide was washed with PBS, incubated with a solution of biotinylated anti-human IgE (Vector Laboratories, Burlingame, CA, USA) and 0.1% BSA in PBST (PBS containing 0.1% Tween-20) for 1 h, followed by a reaction with streptavidin-Cy3 conjugate (Sigma-Aldrich) for 1 h in the dark at room temperature. The slide was then washed three times with PBST and distilled water, and dried with N_2 gas before scanning.

Analysis

The Cy3 fluorescence intensity was measured by using a fluorescence scanner (GenePix Personal 4100A; Axon Instruments, Union City, CA, USA) at a photomultiplier tube (PMT) value of 630, and scanned data were analyzed using the GenePix Pro 4.1 program provided by the manufacturer. The median intensity of the background region was subtracted from those of the spot and the subtracted values were collected to calculate the average fluorescence intensity. Spots showing either an obvious defect or a signal-to-noise ratio less than 2 were discarded prior to the analysis.

Calibration of IgE concentration with fluorescence intensity

In order to calibrate the IgE concentrations with Cy3 intensities, 0.5 mg ml^{-1} anti-human IgE was spotted onto an epoxy-coated glass slide, followed by blocking with 3% BSA and incubation with different concentrations of purified human IgE (Chemicon International) containing 0.1% BSA/PBST. The concentrations of human IgE were 484, 242, 121, 48.4, 12.1, 4.84, and 2.42 U ml^{-1} (1 U = 2.42 ng of IgE).[13] As a negative control, 0.1% BSA/PBST without IgE was applied in the same manner. After washing and scanning, the fluorescence intensity of the negative control was subtracted from those of spots in IgE concentration, and the resulting values were plotted as a function of the IgE concentrations. The saturated signal was excluded from the linear regression. Detection limit was defined as the concentration corresponding the sum of fluorescence intensity of the negative control and standard deviation multiplied by 2.5 (F.I. + 2.5σ).

Optimization of analytical condition

Sera from patients were pooled into 5 or 6 groups to offset individual diversity, as shown in Table 1 (*D. pteronyssinus*, 1 – 5; *D. farinae*, 1 – 6), and used for further study. The resulting pools were subjected to analysis using an allergen chip under different conditions. Among various factors affecting the chip performance, additives in a spotting solution, the serum dilution factor, the concentrations of allergen extracts, biotinylated anti-human IgE, and streptavidin-Cy3 conjugate were investigated in detail. The concentration of the allergen extract for spotting changed from 0.3 to 10 mg ml^{-1}, and the serum dilution factor ranged from 1× to 32×. Under the specified conditions, the IgE concentration of the respective pool was determined from the fluorescence intensity using the calibration curve. The resulting data were correlated with those by a CAP assay, as shown in Fig. 2B. The slopes of a plotted linear graph at respective condition are represented in Table 2. As the slope approaches one, the chip-based assay shows a better accordance with the results by a CAP assay. The conditions under which the chip data showed the best coincidence and a linear correlation with a CAP assay over the whole dynamic range as well as good spot morphology were considered to be optimal.

Table 1 List of pooled sera

		Average IgE conc./U ml^{-1}	Number of included serum
Pool *D. pteronyssinus*	1	0.56	4
	2	1.71	3
	3	9.98	4
	4	37.5	5
	5	75.7	5
Pool *D. farinae*	1	0.45	4
	2	1.97	3
	3	5.09	5
	4	22.9	4
	5	78.5	5
	6	81.1	5

For *D. farinae* and *D. pteronyssinus*, 5 and 6 pools were used, respectively. The IgE concentrations were the average of included sera's IgE concentrations that were determined by the CAP assay.

CAP assay

The IgE concentration was determined by a CAP assay according to the manufacture's instructions. Briefly, serum was added to allergen-coated cellulose disc, followed by incubation with β-galactosidase-conjugated anti-human IgE and methyl umbelliferyl-β-galactoside as a substrate. The IgE concentration was determined from the fluorescence intensity of the enzyme-reaction product, methyl umbelliferone, which have a correlation with the IgE concentration in serum.

Sera

Forty-three human sera were obtained from Ajou University School of Medicine, and stored at –70°C until use.

Results and Discussion

Calibration of the IgE concentration with the Cy3 intensity

In order to test whether the IgE concentration shows a linear correlation with the Cy3 fluorescence intensity from the chip, an anti-human IgE chip was constructed and subjected to a treatment with different concentrations of purified human IgE. As shown in Fig. 1, the fluorescence intensity linearly

(R^2 = 0.9995) increased in proportion to the IgE concentration up to 50 U ml^{-1}, and reached a plateau. The observed dynamic range of the calibration curve was somewhat narrow to measure the IgE concentration in serum of a high-class allergic patient (> level 5).[4] However, according to the definition in Experimental section, the detection limit was estimated to be 0.09 U ml^{-1}, which was sufficiently lower than the IgE concentration in serum of a low-class allergic patient. This result supports the idea that the allergen chip can be used for the sensitive detection of IgE and reliable allergy diagnosis after a proper dilution of serum. Lowering the PMT value below 630, which might alleviate the signal saturation, and subsequently avoid the plateau, was not attempted because the signal intensity from sample sera with a low IgE concentration resulted in much lower fluorescence intensities, and subsequently large deviations, causing an unreliable analysis.

Optimization of critical factors

In order to optimize the chip-based analysis conditions, the critical factors that appear to affect the performance of the allergen chip were examined. For this, pooled sera were analyzed by using an allergen chip under various assay conditions, and compared with those by a conventional CAP assay. In clinical diagnosis, the severity of an allergy is determined based on the IgE concentration by using the conventional CAP assay. In this context, we optimized the conditions for a chip-based assay compared with the CAP assay in terms of the dynamic range, sensitivity, and spot morphology, as well as coincidence with the results from a CAP assay.

We first tested the effect of an additive in a spotting solution. An additive is used to prevent the dehydration of a spotted solution.[14,15] We tested 40% glycerol and 0.5% trehalose as a typical additive. As a result, both 0.5% trehalose and 40% glycerol showed a similar result in terms of spot morphology and the stability of capture molecules. In this work, 0.5% trehalose was selected as the additive of marginal dilution of the spotting solution by the addition of 0.5% trehalose and ease of handling. The serum dilution factor and the spotting concentration of the allergen extract were simultaneously investigated. As shown in Table 2, sera diluted by 3× or 5× gave rise to the best results. With low dilution (1× or 2×), the measured signals from high-class allergic sera were beyond the linear dynamic range (Fig. 1). Serious discrepancy between the chip and a CAP assay was observed with dilution factors ranging from 8× to 32×, which seems to be mainly due to the low IgE concentration. Moreover, spot morphology was undesirable (Fig. 2A), and the measured signals from low-class (< level 2)[4] allergic sera were below the detection limit (Fig. 1). As for the allergen concentration for spotting, 1 mg ml^{-1} of allergen showed the best correlation. If the allergen concentration is too high, spotted molecules might hinder the binding of IgE, and too many allergen extracts are required. On the other hand, too a low allergen concentration would not provide enough allergen molecules on a glass slide to capture the analyte from serum, resulting in an undesirable spot

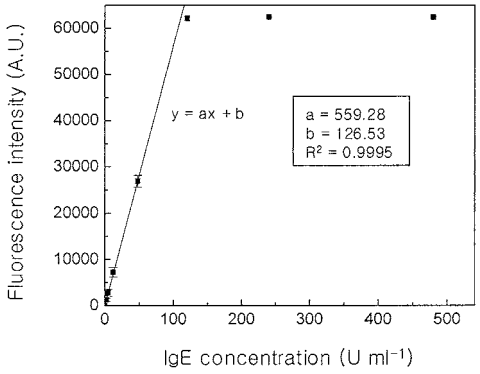

Fig. 1 Calibration of the IgE concentration with the Cy3 fluorescence intensity using an anti-human IgE chip. The fluorescence intensities were plotted as a function of the IgE concentration, determined by a CAP assay. The error bar indicates the standard deviations in triplicate experiments. The coefficients of the curve are shown in the inset.

Table 2 Correlation between the IgE concentrations determined by the allergen chip and the CAP assay against *D. farinae* under various conditions

Serum dilution factor	Allergen conc./mg ml^{-1}						
	10	7	4	2	1	0.5	0.3
1×	0.560	0.590	0.669	0.771	0.821	0.731	0.437
2×	0.659	0.658	0.724	0.849	0.957	0.893	0.571
3×	0.912	0.924	0.886	1.234	1.055	1.153	0.660
5×	1.145	1.228	1.256	1.476	1.622	1.327	0.761
8×	1.563	1.110	1.288	1.671	1.834	1.943	1.124
16×	1.071	1.057	1.117	1.586	1.943	1.792	0.777
32×	2.796	2.658	2.929	3.504	3.993	2.991	2.152

Each value represents the slope from linear regression between the IgE concentrations determined by the allergen chip and the CAP assay, as shown in Fig. 2B. Similar result obtained for *D. pteronyssinus*.

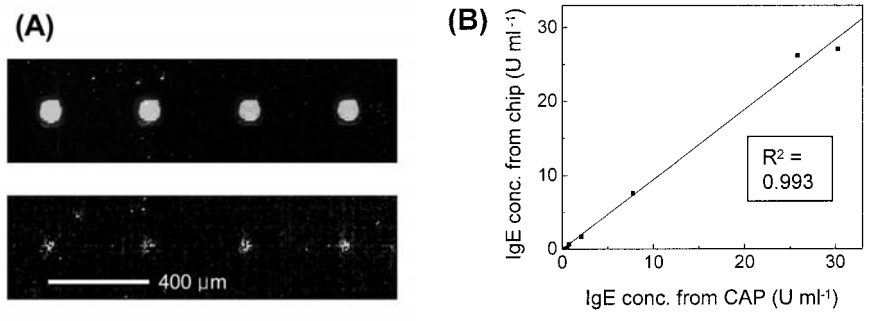

Fig. 2 (A) Fluorescent spot images when 1 mg ml^{-1} allergen and 3×-diluted serum (top) or 0.3 mg ml^{-1} allergen and 32×-diluted serum (bottom) were used. The distance between the spots is 400 μm. (B) Correlation between the IgE concentrations determined by the allergen chip and a CAP assay under the optimized conditions.

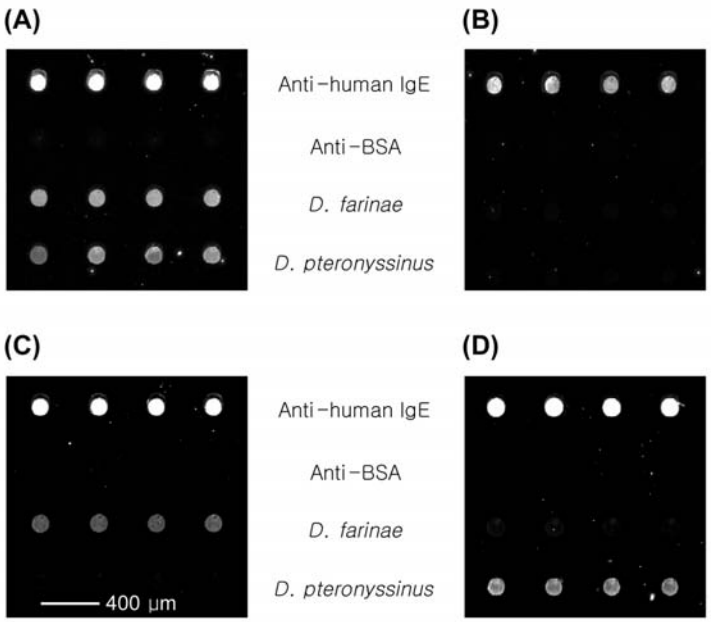

Fig. 3 Fluorescence images of the allergen chip after a treatment with different sera. Sera from patients allergic to both *D. farinae* and *D. pteronyssinus* (A), to none of them (B), to *D. farinae* only (C), and to *D. pteronyssinus* only (D). Anti-BSA was used as a negative control, and anti-human IgE as a positive one. Distance between spots is 400 μm.

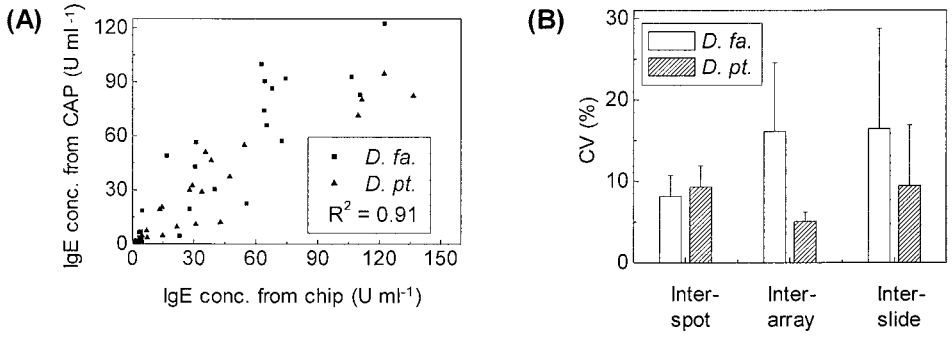

Fig. 4 (A) IgE concentrations measured from the allergen chip plotted against those from the CAP assay. The correlation coefficient is shown in the inset. (B) CVs of data from the allergen chip. CVs were calculated from 36 spots, 12 arrays and 3 slides.

morphology and serious discrepancy of the IgE concentrations between the allergen chip and CAP assays (Table 2 and Fig. 2A). Based on the above results and a comparison with CAP assays, a 1 mg ml^{-1} of allergen concentration and 3-fold serum dilution were selected as the optimal conditions at which a good spot morphology and excellent correlation with a CAP assay were observed (the slope of linear regression = 1.055) (Fig. 2). There was no difference between the $10^{-2}\times$ and $10^{-3}\times$ dilution of biotinylated anti-human IgE (original concentration = 0.5 mg ml^{-1}) and the streptavidin-Cy3 conjugate (original concentration = 1 mg ml^{-1}), thus, $10^{-2}\times$ was chosen in order to minimize the dilution error.

Chip-based analysis of allergen-specific IgE in sera

Under the optimized conditions, IgE concentrations against *D. farinae* and *D. pteronyssinus* in 43 sera were analyzed with the developed allergen chip. As shown in Fig. 3, IgE in serum was specifically bound to the respective allergen. A comparison of the results from the allergen chip with those from a CAP assay revealed a high correlation for two allergens (R^2 = 0.91 for two mites; 0.85 for *D. farinae*; 0.93 for *D. pteronyssinus*), which is comparable to a previous report,[2,6,8] as can be seen in Fig. 4A. This result clearly demonstrates that an allergen chip can be used as a diagnosis tool of an allergy. In addition, crude allergen extracts can be used effectively as a capturing agent of the allergen chip. This also implies that even though causative allergenic molecules are not identified, whole extracts might be employed instead. The use of extracts from an allergenic source is more cost-effective than that of purified allergenic molecules. Since conventional diagnostic methods also rely on complex allergen extracts as an IgE-capturing reagent, the performance of a chip constructed with allergen extracts can be easily evaluated. We tested the reproducibility of the chip assay by measuring the inter-spot, inter-array, and inter-slide variances using sera with high concentrations of IgE. As shown in Fig. 4B, the coefficient of variation (CV) for the inter-spots was lower than 10%, and CVs for the inter-array and the inter-slide were about 15%. These values are relatively lower than the

previously reported ones (around 30%),[2,8] indicating an improved reproducibility.

Based on the results, it is likely that under the optimized conditions, the allergen chip provides general advantages of a chip-based assay, such as tiny amounts of sample and reagents, cost-effectiveness, and multiplexed analysis, opening up the potential of allergy diagnosis.

Conclusions

We have optimized the critical factors affecting the performance of the allergen-chip for the analysis of specific IgE by using a biotinylated anti-human IgE and a streptavidin-Cy3 conjugate. An allergen chip was constructed by spotting extracts of two mites, *D. pteronyssinus* and *D. farinae*, onto a glass slide. The critical factors were investigated and optimized in terms of the reliability and the sensitivity. Under the optimized conditions, the chip-based analysis for sera from 43 patients revealed a reliable diagnosis of respective allergy, showing a good correlation with a conventional CAP assay. This result strongly supports the utility of the allergen chip for a sensitive, reproducible and multiplexed diagnosis of allergy.

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