Allergology International. 2009;58:193-199
DOI: 10.2332/allergolint.08-OA-0023

ORIGINAL ARTICLE

Awarded Article, Annual Meeting of JSA

Antigen-Induced Expression of CD203c on Basophils Predicts IgE-mediated Wheat Allergy

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ABSTRACT

Background: For *in vitro* diagnosis of wheat allergy, specific IgE to wheat is known to be a poor predictive marker. Oral food challenge, the gold standard for the diagnosis, is accompanied by a risk of severe induced reactions. Reliable *in vitro* tests are needed to be developed for safe indication for oral challenge.

Objective: We examined the utility of a basophil activation marker, CD203c, for the diagnosis of IgE-mediated wheat allergy.

Methods: Fifty-eight children with suspected wheat allergy with positive CAP-FEIA to wheat were enrolled. On 70 occasions, the clinical distinction between patients with wheat allergy (WA) and patients tolerant to wheat (TW) was made by means of an oral food challenge test or recent history of immediate allergic reactions or tolerance after ingestion of wheat. Twelve replicate evaluations were performed in 9 patients over more than a 6-month interval. Thirty two patients on 43 occasions were diagnosed with WA and 27 were confirmed to be TW. One patient had both diagnoses 18 months apart. Peripheral blood was incubated with fractionated wheat extracts, purified native omega-5 gliadin (nOG5) and recombinant omega-5 gliadin (rOG5). Expression of CD203c on basophils was then analyzed by flow cytometry using a commercial kit.

Results: All wheat proteins induced concentration-dependent enhancement of CD203c expression in WA, but did not in TW. The analysis of receiver operating characteristics (ROC) showed that nOG5-induced $CD203c^{high}$% values provided the best power for discriminating between WA and TW, with a sensitivity of 85.0% and specificity of 77.0% at the cut-off level of 14.4%. AUC for CD203c with nOG5 were significantly higher than that for conventional CAP-FEIA, 0.89 and 0.73, respectively ($p < 0.01$).

Conclusions: Measurement of nOG-induced enhancement of CD203c on basophils is useful for the diagnosis of immediate wheat allergy in children.

KEY WORDS

basophil activation test, CD203c, omega-5 gliadin, wheat allergy

INTRODUCTION

Food allergy affects 5–10% of children under 6 years of age and 1–2% in the older population in Japan[1] and the prevalence appears to be increasing. Wheat is the third common allergen in those under 20 years of age, accounting for about 10% of all food-induced immediate reactions in Japan.[1] Wheat causes a variety of IgE-mediated symptoms including baker's asthma by inhalation of wheat flour, allergic reactions including skin, respiratory, gastrointestinal symptoms, and shock by ingestion of wheat containing food,[2] and wheat-dependent, exercise-induced anaphylaxis (WDEIA).[3] Immediate hypersensitivity reactions following ingestion of wheat is common in young children and WDEIA has been increasingly recognized

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Received 28 June 2008. Accepted for publication 11 September 2008.

Table 1 Demographic data of the subjects

	Number of patients	Number of diagnostic evaluations	Gender (F/M)	Age in months (Mean ± SD)	Total IgE (Geometric mean; 95%CI)
WA	32*	43	15/28	33.4 ± 26.2	414; 266-643
TA	27*	27	20/7	30.8 ± 20.5	738; 389-1398

WA, wheat allergy; TA, tolerant to wheat; CI, confidence interval.

*One patient was evaluated twice. He was WA at the first evaluation and TW at the second evaluation.

in all ages.[4]

Diagnosis of wheat allergy is, however, not simple. Oral food challenge tests are the gold standard for the diagnosis but are accompanied by a risk of severe induced reactions and are impractical in busy practice settings. A reliable *in vitro* test to predict the diagnosis is thus necessary. Although the usefulness of measurements of serum specific IgE has been demonstrated for the diagnosis of egg, cow's milk, and peanut allergy, measurement of specific IgE to wheat has been shown to be much less reliable.[5,6] A possible reason for poor predictability of wheat-specific IgE is that water-soluble wheat extracts used for the specific IgE assays may not contain major allergenic epitopes in wheat protein.

There are number of IgE-binding epitopes in wheat protein. According to the method for extraction, it is composed of water/salt-soluble proteins and water/salt-insoluble proteins. The former includes albumins and globulins such as α-amylase inhibitors, peroxidase, and serpin,[7,8] and have been considered to be major allergens in baker's asthma. The latter includes α-gliadin, ω-5 gliadin (or fast ω-gliadin), and low molecular weight glutenin and are reported to cause wheat allergy in children and WDEIA.[9-12] Recently, ω-5 gliadin-specific IgE measurement has been demonstrated to be highly predictive for the diagnosis of WDEIA.[13] In young children with wheat allergy, IgE-binding to both salt-soluble and salt-insoluble fractions by immunoblot has also been reported.[14] These observations indicate that a variety of wheat proteins are allergenic and major epitopes responsible for each type of wheat allergy are still to be determined.

Flow cytometry-based tests for basophil activation status have been described to diagnose or to confirm sensitization in allergic patients.[15] CD63 can discriminate resting and allergen-activated basophils.[16] Recent reports described an ectoenzyme CD203c as a more suitable basophil marker that is not only constitutively expressed on resting basophils but also upregulated at high levels on activated basophils.[17] A commercial kit for quantification of CD203c expression on basophils, Allergenicity Kit® (Beckmann Coulter, Fullerton, CA, USA), identifies basophils as CD3-negative and CRTH2-positive fractions from whole blood samples and measures fluorescent intensity of CD203c that is enhanced by cross-linking of surface-bound IgE molecules.

In order to establish a predictive *in vitro* test for wheat allergy in children, we measured basophil CD203c expression induced by various wheat protein fractions and evaluated the diagnostic efficiency of the reactions in patients whose allergic status to wheat had been defined by food challenge or a convincing history and compared them with that of conventional wheat-specific IgE measurement.

METHODS

SUBJECTS

Fifty-eight children who were suspected to have wheat allergy as measured by a positive wheat specific IgE antibody (CAP-FEIA, Phadia, Tokyo, Japan) were enrolled in the study. A diagnosis of wheat allergy was based on an open food challenge or a convincing history of wheat-induced immediate reactions in the previous month. Tolerance to wheat was diagnosed as a negative food challenge after ingestion of more than 50 g of udon (1.3 g of wheat protein), which is comparable to a half serving of it for toddlers, or negative symptoms with daily ingestion of wheat as a staple food. Although double-blind placebo-controlled food challenge (DBPCFC) is the gold standard for diagnosing food allergy, most of the subjects were infants or toddlers and unlikely to be influenced by suggestions. Patients who had only subjective symptoms were re-examined with single-blind placebo-controlled food challenge. Since immediate wheat allergy is often outgrown by the age of 6, serial evaluations were made in 12 patients within at least 6-month intervals and a total of 70 evaluations were performed.

Thirty-two patients were diagnosed with WA on 43 evaluations and 27 patients were diagnosed to be TW. Induced symptoms in WA occurred within 3 hours after challenge. One patient was WA on the first evaluation and became TW at the second evaluation 18 months later. Demographic data of the subjects are described in Table 1. Blood sampling for CD203c tests and IgE measurements was performed at each evaluation.

We excluded patients with negative CAP-FEIA to wheat by design since the definition of "suspected" wheat allergy can be ambiguous without objective data for IgE. However, to further test the diagnostic ability of the CD203c test, an additional 8 patients with negative wheat IgE (6 boys and 2 girls, mean

age of 35 months) who performed wheat challenge test were analyzed separately.

This study was performed with the approval of the ethics committee of the Mie National Hospital. Informed consent was obtained from the guardians of the subjects.

EXTRACTION OF WHEAT PROTEINS

Four grams of wheat flour were ultrasonicated in 40 mL of PBS on ice for 15 minutes and stirred at 4℃ overnight, then the supernatant was obtained after centrifugation at 20,000 g for 15 minutes (water/salt-soluble fraction; PBS fraction). After washing with PBS three times, the sediment were then dissolved in 40 mL of 70% ethanol (EtOH) with ultrasonification on ice for 15 minutes. The supernatant was obtained after centrifugation at 20,000 g for 15 minutes (water/salt insoluble, EtOH fraction). After washing with 70% EtOH three times, the sediment was further dissolved in 40 mL of 2%Na_2CO_3/0.1N NaOH and the supernatant was obtained as an alkaline soluble fraction (alkali fraction). The fractions were dialyzed in PBS overnight and protein concentrations were determined with a densitometry, then diluted to 100 μg/ml in PBS and stored at −20℃ until use.

Native wheat ω-5 gliadin (native ω-5 gliadin; nOG5) and recombinant of ω-5 gliadin were prepared as described previously.[18-20]

MEASUREMENT OF CD203c EXPRESSION ON BASOPHILS

A commercial kit (Allergenicity Kit, Beckman Coulter) was used for quantification of basophil CD203c expression as described previously.[21] Briefly, EDTA-containing whole blood was incubated with various concentrations of the five kinds of wheat proteins at concentrations indicated for 15 minutes after addition of sufficient amounts of calcium solution to override the chelating capacity of EDTA. Anti-IgE antibody at 4 μg/ml as a positive control and PBS as a negative control were also used. PC7-conjugated anti-CD3, FITC-conjugated anti-CRTH2, and PE-conjugated anti-CD203c antibodies were also added during the reaction. The samples were analyzed on a FC500 flow cytometer (Beckman Coulter). Basophils were detected on the basis of forward side scatter characteristics and expression of negative CD3 and positive CRTH2. Up-regulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control) and expressed as $CD203c^{high}$%. At least 500 basophils were analyzed at each assay.

WHEAT-SPECIFIC IgE

Serum levels of wheat-specific IgE were measured with a CAP-FEIA system (Phadia, Uppsala, Sweden).

STATISTICAL ANALYSIS

Differences were analyzed with the Mann-Whitney U test for unpaired samples. For multiple comparisons, two-way ANOVA followed by Dunnett's multiple comparison test was employed. The discriminative usefulness of $CD203c^{high}$% and CAP-FEIA was evaluated by constructing ROC curves[22] where sensitivity versus 1-specificity was plotted for each possible cutoff level. For this analysis, WA was labeled as diseased compared with TW and the area under the curves (AUCs) were determined. From each ROC curve we determined the ideal cut-off levels which correspond to the closest point to the top left-hand corner and which most efficiently discriminates between the presence or absence of disease. The respective sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) were calculated. Comparison of two ROC curves was performed utilizing a method reported by Hanley *et al.*[23]

RESULTS

INDUCTION OF CD203c EXPRESSION ON BASOPHILS WITH VARIOUS WHEAT PROTEINS

We first examined induced expression of CD203c on basophils with the wheat extracts and native and recombinant ω-5 gliadin at various concentrations (Fig. 1) in representative subjects of WA and TW (n = 5). All the fractions significantly induced the enhancement of CD203c expression in a concentration-dependent manner in WA subjects, in contrast, no significant enhancement of CD203c was observed in TA subjects. Based on the results, protein concentrations at 10 μg/ml for PBS fraction, EtOH fraction, alkali fraction, and rOG5, and 1 μg/ml for nOG5, were employed for evaluation of diagnostic usefulness. In addition, nOG5 appeared to give the highest signal to noise ratio regarding discrimination of WA and TA and we focused on the nOG5-induced reactions for further analysis.

SERUM TOTAL IgE, SPECIFIC IgE TO WHEAT, AND CD203c INDUCED BY nOG5

There was no difference in serum total IgE levels between WA and TW (Fig. 2a). Wheat-specific IgE levels by CAP-FEIA in WA were significantly higher than those in TW (p = 0.002) (Fig. 2b). $CD203c^{high}$% induced by nOG5 at 1 μg/ml in WA were again significantly higher than those in TW ($p < 0.0001$) (Fig. 2 c).

DIAGNOSTIC ABILITY OF WHEAT PROTEIN-INDUCED $CD203c^{HIGH}$% AND SPECIFIC IgE TO WHEAT

To evaluate the diagnostic ability of the different *in vitro* tests for the diagnosis of wheat allergy, the ROC analyses for each test were performed. The AUC for nOG5-induced $CD203c^{high}$% test gave the highest value, 0.89, compared with the other tests (Table 2)

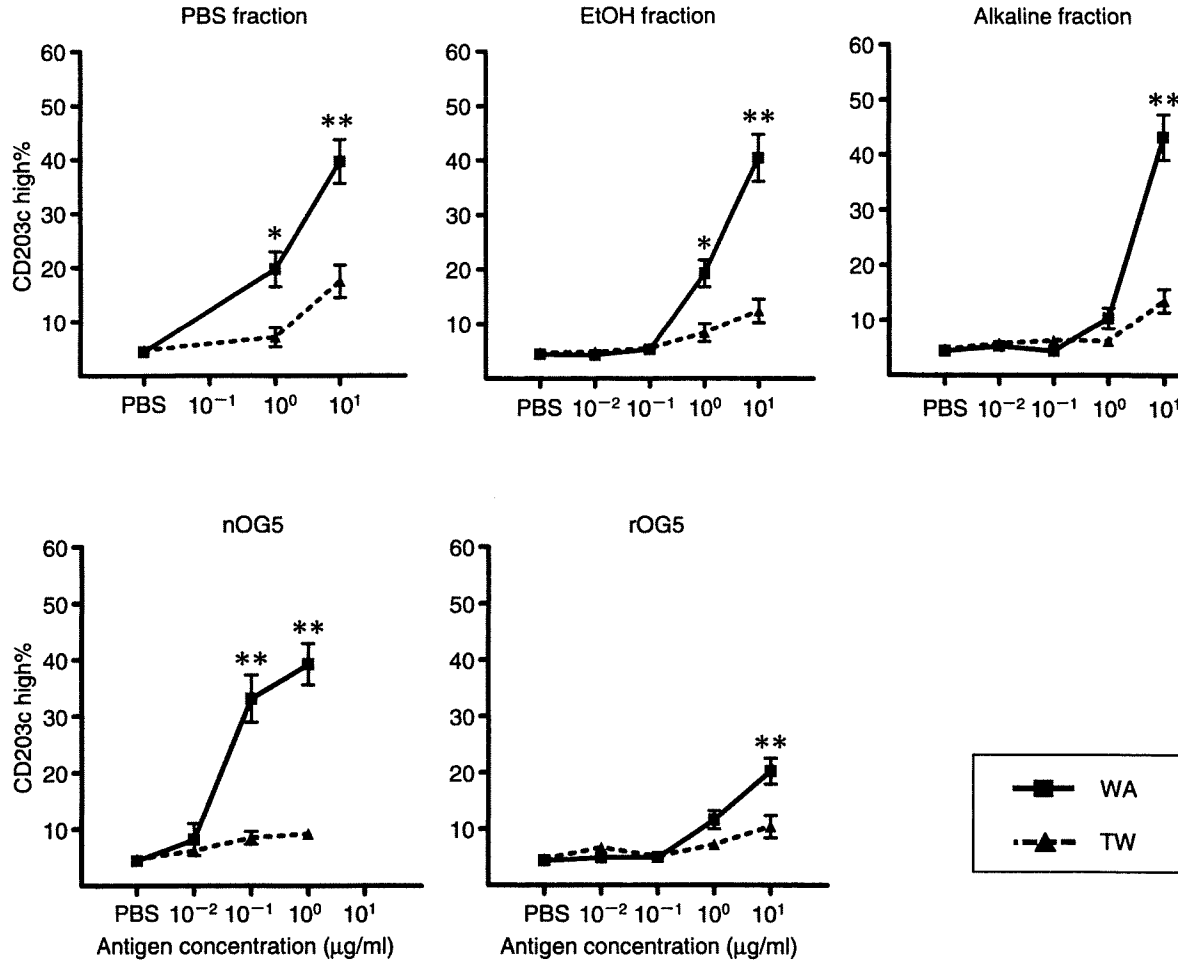

Fig. 1 Expression of CD203c on basophils induced by various wheat protein extracts and gliadins. WA, subjects with definite wheat allergy ($n = 5$); TW, subjects tolerant to wheat ($n = 5$). Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$, Dunn's multiple comparison test.

and was significantly higher than wheat-CAP-FEIA ($p < 0.001$) (Fig. 3). With the cut-off values obtained from the ROC analyses, sensitivity, specificity, PPV, and NPV were calculated. The nOG5 CD203c test showed the highest sensitivity and specificity among the tests, 85.0% and 77.2% respectively. Conventional wheat-CAP-FEIA gave comparable sensitivity and PPV, but lower specificity and NPV.

To further confirm the utility of the CD203c test, 8 patients with negative wheat-CAP-FEIA were also analyzed. By the food challenge test, 4 patients were confirmed to be WA and nOG5-induced CD203c$^{high\%}$ in 3 of them were above the cut-off level (positive). Although one patient with WA showed a negative nOG5 CD203c, significant enhancement of CD203c expression was observed with the alkaline fraction. All 3 patients with TA were negative in nOG-induced upregulation of CD203c. One patient was tolerant to udon but showed immediate symptoms after ingestion of bread. CD203c upregulation in the patient was negative with nOG5 but positive with PBS and alkaline fractions.

DISCUSSION

In the present study, we demonstrated that wheat protein-induced basophil activation test quantifying CD203c expression had efficient diagnostic ability for immediate wheat allergy in children. Since patients with wheat allergy may respond to a variety of epitopes contained in wheat, we tested different kinds of wheat extracts and purified ω-5 gliadins (native and recombinant forms) for the CD203c test and found that native ω-5 gliadin (nOG5) gave the best sensitivity and specificity for the diagnosis, 85.0% and 77.2%, respectively. The AUC received from ROC analysis for the nOG5 CD203c test was significantly higher than that for the conventional wheat-specific IgE test.

There are numerous wheat allergens which cause immediate reactions including skin, respiratory, and systemic symptoms after wheat ingestion. A study using a number of purified protein fractions in immunoblotting and RAST demonstrated that water/

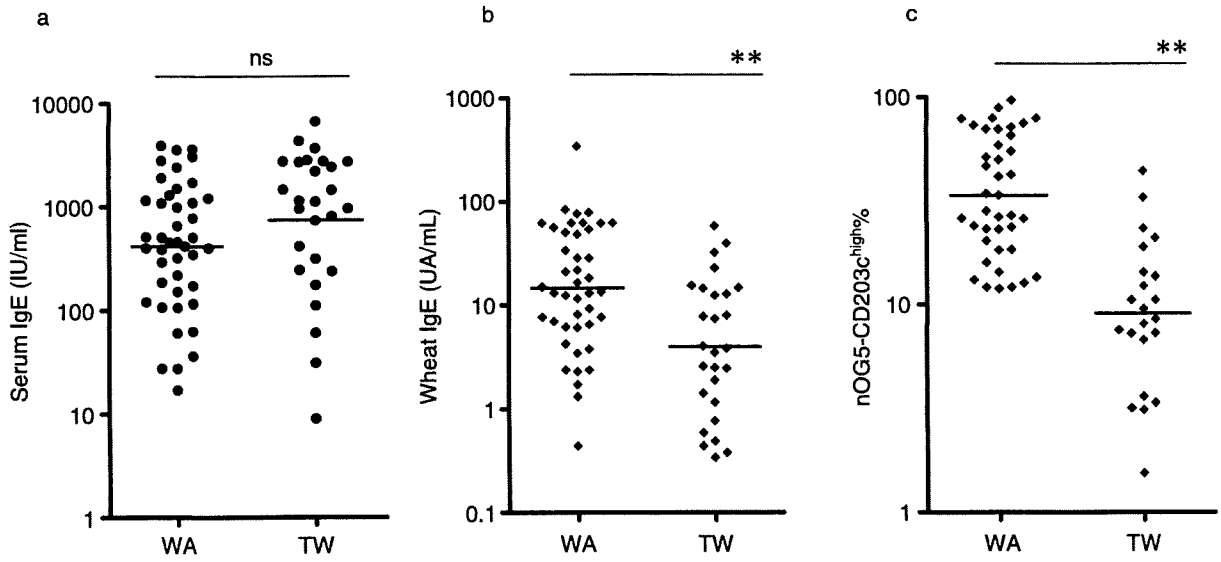

Fig. 2 Total IgE (**a**) , wheat-specific IgE (**b**) , and nOG5-induced CD203c expression on basophils (**c**) in subjects with wheat allergy (WA) and tolerance to wheat (TW) . Concentration of nOG5 used was 1 μg/ml. $**P < 0.01$, Mann-Whitney U test.

Table 2 Diagnostic abilities of *in vitro* tests for wheat allergy

	IgE (CAP-FEIA)	$CD203c^{high}$%				
	Wheat	PBS Fraction	EtOH fraction	Alkaline fraction	nOG5	rOG5
AUC	0.73	0.77	0.81	0.84	0.89	0.74
P value	0.002	0.0002	< 0.0001	< 0.0001	< 0.0001	0.0017
Cut off	4.1 UA/mL	11.1%	8.1%	11.7%	14.4%	7.9%
Sensitivity	81.4	86.1	83.3	83.7	85.0	82.5
Specificity	55.6	57.7	69.2	66.7	77.2	63.4
PPV	74.5	77.1	81.4	80.0	86.8	80.5
NPV	65.2	71.4	69.2	72.0	70.8	66.7

AUC, area under the ROC curve; PPV, positive predictive value; NPV, negative predictive value.

salt insoluble gliadins and glutenins as well as water/salt soluble albumins and globulins were IgE-binding allergens in patients with wheat allergy.[24] Another study employing confirmed wheat-allergic patients with DBPCFCs in Italy stated that lipid transfer protein in the albumin/globulin fraction and low molecular glutenins were major allergens in these patients.[25] Recently, IgE antibodies to ω-5 gliadin have been reported to be present in sera from wheat-induced anaphylaxis[26] and WDEIA[13]. These findings are in agreement with our present study showing that ω-5 gliadin-induced basophil activation most efficiently predicted wheat allergy in young children. In addition, the fact that water/salt soluble and alkali soluble fractions, which contain allergen proteins other than gliadins, also induced significant basophil activation indicates that many epitopes are involved in immediate wheat allergy.

Although we found that the nOG5 CD203c test showed high sensitivity and specificity, the rOG5 CD203c test had lesser discriminating power for wheat allergy with AUCs of 0.89 and 0.74, respectively. In addition, a higher concentration of the recombinant form of the protein in inducing CD203c expression were needed, compared to the native form (Fig. 1), indicating lesser IgE binding ability of rOG5 in this group of patients. A possible explanation for the discordance may be the difference in allergenic epitopes contained in the proteins. Several IgE-binding epitopes in WDEIA have been identified and sequenced.[9] The recombinant protein used in the present study was the C-terminal half of the omega-5 gliadin protein[20] and was confirmed to contain the identified 11 IgE epitope sequences related to WDEIA. However, it may not contain some of the major allergenic epitopes in wheat allergy in young children. There is a possibility that distinct epitopes are involved in WDEIA and immediate wheat allergy. In

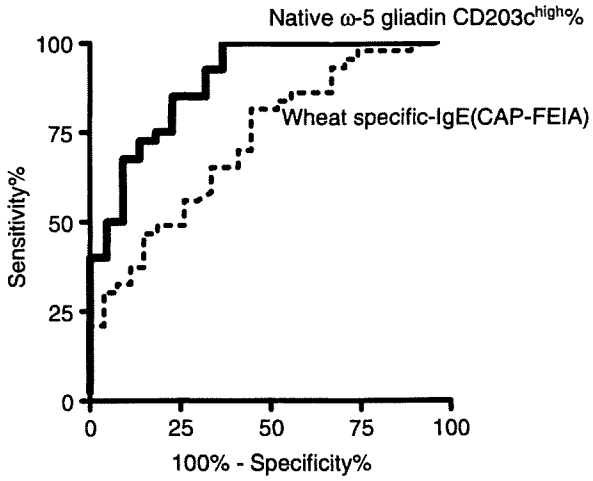

Fig. 3 ROC curves for native ω-5 gliadin-induced CD203c expression test and wheat specific IgE test. Horizontal bars indicate geometric means.

fact, we recently observed profound CD203c expression by rOG5 and less expression by nOG5 in young adults with WDEIA (data not shown), which is the opposite reaction profile we often observed in children with immediate allergy to wheat. Both immediate wheat allergy and WDEIA are caused by IgE antibodies to wheat proteins, however, there are a number of differences between the two ailments in terms of age,[4] symptoms, and pathogenic mechanisms.[3] These findings warrant further studies to identify major epitopes for these similar but distinct diseases.

There are limitations to the present study, which are noteworthy. We did not compare diagnostic abilities of the CD203c test and specific IgE quantification by using identical antigens. Although not presented in the results, we measured CAP-FEIA titer to rOG in a limited number of the subjects. The AUC received from ROC analysis for rOG-specific IgE was 0.78, which was comparable to the value in the corresponding CD203c test. For nOG-specific IgE, we could not perform an ELISA since a large amount of antigens, about 100-fold than those for the basophil activation test, are necessary to establish a regular ELISA and we did not have an ample amount of the antigen to assay a large number of samples. An additional study to solve the problem may be necessary. The fact that the basophil activation test needs only a small amount of antigens, however, can be an advantage of the test since it is possible to utilize the test for rare antigens.

The CD203c assay system employs whole blood during incubation with wheat allergens, which allows not only surface-bound IgE on basophils but possibly serum factors or other circulating cells to affect activation status of basophils. We recently found that induced expression of CD203c by Japanese cedar pollen (JCP) extract significantly decreased after rush immunotherapy (RIT) in patients with JCP pollinosis without decrease in specific IgE levels to JCP.[21] In passive sensitization experiments, the patients' sera obtained both before and after RIT showed essentially similar sensitizing capacity for basophils. In contrast, basophil degranulation in response to the pollen extract was effectively suppressed by addition of post-RIT serum samples, indicating the presence of blocking antibody in the serum[27] and that the CD203c test reflected not only the presence of IgE antibodies but serum factors such as blocking IgG antibodies. Even though IgE is the key molecule in food allergy, it is not a single factor to determine severity of the disease or development of tolerance. In this sense, the CD203c test may have an advantage over specific IgE measurements since it may possibly detect factors modifying the food allergy.

In conclusion, the findings in this study suggest that measurement of basophil CD203c expression by ω-5 gliadin is highly useful in predicting a positive food challenge in children who are suspected of wheat allergy with positive CAP-FEIA to wheat. This test may help to determine a safe indication for the challenge.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid for scientific research from the Ministry of Health, Labour, and Welfare, Japan. We thank Ms. Yuiko Ezaki, Manami Negoro, and Kumiko Ota for their excellent technical assistance and Professor Naomi Kondo of Department of Pediatrics, Gifu University Graduate School of Medicine for his support and critical comments regarding the project.

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Allergology International. 2009;58:163-170
DOI: 10.2332/allergolint.09-RAI-0097

REVIEW ARTICLE

Biomarkers for Allergen Immunotherapy in Cedar Pollinosis

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ABSTRACT

To initiate, monitor, and complete effective immunotherapy, biomarkers to predict and visualize the immune responses are needed. First, we need to identify the right candidate for immunotherapy. Secondly, the immune responses induced by immunotherapy should be monitored. For the first objective, analysis of polymorphisms of candidate genes may be helpful, but still be in development. Regarding biomarkers for immune responsese, there are numerous reports that evaluate immunotherapy-induced immune changes such as suppression of effector cells, deviation to Th1 cytokine production, and induction of regulatory T cells. No standardized methods, however, have been established. Among them, a functional assay of blocking IgG activity, the IgE-facilitated allergen binding assay, may be useful. We quantitated induced expression of an activation marker, CD203c, on basophils and found that the assay efficiently predicts sensitivity to particular allergen and severity of the allergen-induced symptoms. In patients who received rush immunotherapy for Japanese cedar pollinosis, reduction in CD203c expression after the therapy was observed, suggesting the utility of the test for monitoring immunotherapy.

KEY WORDS

basophils, CD203c, cedar pollinosis, IgG4, immunotherapy

INTRODUCTION

The incidence of Japanese cedar pollinosis (JCP) is increasing at an astonishing pace, which was first recognized in early 1960s and now affects around one fourth of the population in Japan.[1-3] Effective pharmacotherapy including non-sedating antihistamines, leukotriene receptor antagonists, and topical corticosteroids, has evolved and quality of life of the patients has been improving.[4,5] Yet, the remedies merely control symptoms and do not change natural history of the disease. Further, social burden of the disease is still significant.[6] On the other hand, allergen immunotherapy generally not only alleviate allergic symptoms but has potential to modify the disease since clinical benefits are reported to be maintained at least for 3 years, even for 12 years after discontinuation.[7,8] In children, immunotherapy prevents new sensitizations[9,10] and reduces progression of rhinitis to asthma for up to 10 years.[11] Long-term efficacy of immunotherapy in Japanese cedar pollinosis has also been reported.[12]

Although immunotherapy confers a multitude of benefits, there still exist issues to be addressed; the present form of immunotherapy is still bound to IgE-mediated side effects, some patients may not benefit from the treatment, long periods for treatment are required and the timing of stopping therapy is not well defined. Along with various efforts to improve the therapy, effective biomarkers have to be developed to tailor the existing therapy and to evaluate new forms of the therapy. The markers should identify right patients with favorable therapeutic responses without adverse events, monitor the efficacy based on immunological responses to particular allergen, and identify the right timing of discontinuation. Although "ideal" biomarkers are yet to be established, prospects for the biomarkers in allergen immunotherapy will be discussed in this article. We also describe quantification of allergen-induced CD203c expression

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Received 17 February 2009.

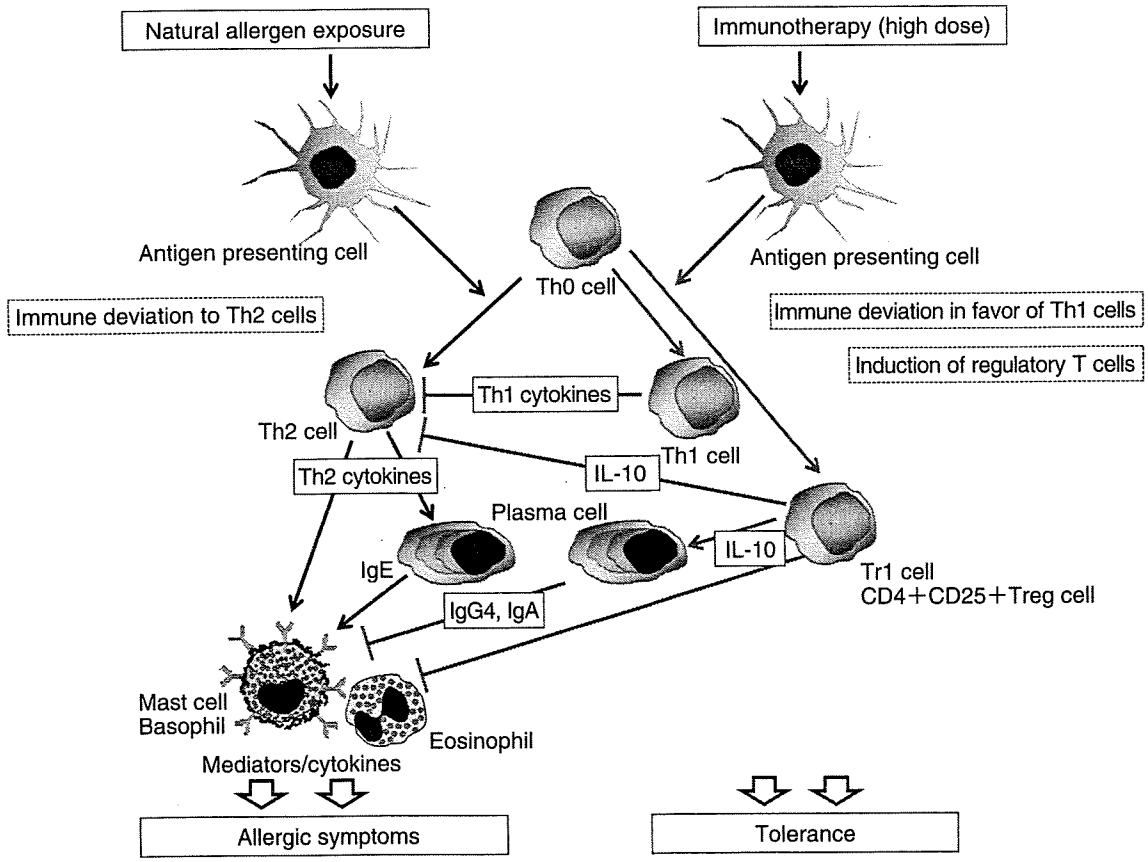

Fig. 1 Mechanisms of allergen immunotherapy.

on basophils as a possible biomarker for Japanese cedar pollinosis. Basophils are important effector cells in the pathogenesis of allergic diseases[13] because they infiltrate in the nasal mucosa of patients with allergic rhinitis[14] and produce a number of mediators and cytokines involved in immediate and late allergic responses.[15] In addition, the fact being circulating cells easily enables us to test the cells *ex vivo* by utilizing a flowcytometry. Here, we show that the basophil activation test utilizing CD203c expression may measure "blocking" activity induced by immunotherapy.

IMMUNOLOGICAL MECHANISMS IN ALLERGEN IMMUNOTHERAPY

THE ALLERGIC RESPONSE

Before discussing biomarkers in allergen immunotherapy, the putative immunological mechanisms are summarized (Fig. 1). The exposure of cedar allergen in the nose, eyes, or bronchi of genetically susceptible individuals causes Th2-deviated immune responses. Cytokines such as IL-4, IL-5, IL-9, and IL-13 derived from Th2 cells are responsible for specific IgE production, differentiation and activation of effector cells such as mast cells, basophils, and eosinophils, and direct stimulation of responder organs including mucus glands and vascular cells in the affected organ. Upon re-exposure to the allergen in the season, IgE-dependent activation of mast cells and basophils results in release of numerous mediators including histamine, cysteinyl leukotrienes, prostaglandins, and platelet activating factor, leading to sneeze, pruritus, waterly discharge, stuffy nose, and sometimes bronchospasm. In addition, mast cells and basophils, are large producers of Th2 and proinflammatory cytokines including IL-4 and TNF-α to potentiate chronic Th2-deviated inflammation in the tissue.

Allergen immunotherapy has potential to inhibit or reverse each step of the above allergic responses and to confer tolerance to the allergen (Fig. 1). Significantly higher amount of allergen is administered in immunotherapy compared to natural exposure. Because it has been shown that deviation to Th2 as expressed by IgE production depends on the allergen dose used to prime the corresponding experimental systems,[16-18] where low allergen doses favor and high allergen dose suppress IgE production. In fact, clinical efficacy is related to the allergen dose,[19,20] higher doses results in better protection.

MECHANISMS OF IMMUNOTHERAPY IN THE EFFECTOR PHASE

Recently, time course analysis of clinical and immunologic measurements during the first year of grass

pollen immunotherapy[21] has been reported, which could substantiate a number of partial information previously observed. The first change was reduction of late phase responses (LPR) to intradermal challenge testing that was observed as early as after the first 2 weeks during up-dosing stage of the conventional injection immunotherapy. Then, elevation of specific IgG4, inhibition of basophil histamine release, and inhibition of binding of allergen-IgE complex to B cells were observed during 6 to 8 weeks at maintenance allergen doses. Reduction of early skin responses, which usually associates with clinical efficacy, was accompanied with these later immunological changes. The investigators also found that allergen-induced IL-10 production from peripheral blood mononuclear cells was a very early event accompanied with LPR suppression. They concluded that IgG responses may be necessary for clinical protection, inhibition of histamine release and allergen/IgE binding to B cells, but that the preceding IL-10 production could contribute to this process.

MECHANISM OF IMMUNOTHERAPY IN T CELL DIFFERENTIATION

The important upstream events that immunotherapy bring about in immune responses to allergen is T cell differentiation, a critical step in regulating downstream effector mechanisms. Cumulative evidence revealed that Th1 cells and T regulatory cells are the key cells in this context.

First, in patients who received grass pollen immunotherapy, increase in cells expressing IFN-γ mRNA were found in the nasal mucosa during allergen-induced late responses and the number of the cells and symptoms scores were inversely correlated.[22] IL-12 is known to be a major cytokine to induce IFN-γ-producing Th1 cells and significant increases in allergen-induced IL-12 mRNA+ cells in cutaneous biopsy specimens was observed in the immunotherapy-treated patients and IL-12+ cells correlated positively with IFN-γ+ cells, inversely with IL-4+ cells.[23] In terms of Th2 cells, seasonal increases in IL-5 and IL-9-expressing cells in the nasal mucosa were significantly inhibited in immunotherapy patients.[24,25] Collectively, Th1 cells are induced and Th1/Th2 balance is altered in favor of Th1 cells by immunotherapy.

There are several subsets of T regulatory cells[26] and there exists inappropriate balance between allergen activation of regulatory T cells and effector Th2 cells in allergy. It was reported that CD4+CD25+ T cells, so-called naturally occurring regulatory T cells (nTreg), from non-allergic donors suppressed proliferation and IL-5 production by their own allergen-stimulated CD4+CD25- cells while the inhibition by CD4+CD25+ T cells from allergy patients were significantly reduced.[27] For these conditions, immunotherapy induces regulatory T cells in the treated patients, so called inducible regulatory T cells (Tr1 cells) and many studies have constantly identified induced expression of IL-10.[21,28-30] One report demonstrated that local increases in IL-10 mRNA and protein-positive cells were observed in the nasal mucosa from patients after 2 years of grass pollen immunotherapy. The changes were observed in treated patients only during the pollen season, not during off-season, nor in placebo-treated subjects and healthy controls.[30] These results suggest that IL-10 responses are allergen-specific, inducible phenomenon. IL-10 acts on B cells to induce production of IgG4.[31] IL-10-induced "blocking" IgG4 inhibits mast cell histamine release and IgE-facilitated allergen-binding to B cells. IL-10 also directly blocks IgE-mediated mast cell activation.[32] Further, IL-10 blocks T cell activation by inhibiting costimulatory molecule CD28 signaling pathway,[33] leading to reduction in cytokines such as IL-5[34] and reduction in inflammatory cell recruitment such as eosinophils.[24]

Table 1 Development of biomarkers for allergen immunotherapy

- Patient selection
  - ✧ Prediction of therapeutic responses
  - ✧ Prediction of serious adverse reactions
  - ✧ Identification of candidates for secondary prevention
- Maintenance
  - ✧ Monitoring of "protective" immune responses
    - "Blocking" antibodies
    - Regulatory T cells, IL-10 and other inhibitory cytokines
    - Suppression of effector cells: mast cells, basophils, eosinophils
  - ✧ Prediction of serious adverse reactions
- Completion
  - ✧ Identification of "normalized" immune responses to allergen
  - ✧ Prediction of recurrence after discontinuation

BIOMARKERS TO MONITOR ALLERGEN IMMUNOTHERAPY

To initiate, monitor, and complete effective immunotherapy, biomarkers to predict and visualize the immune responses are needed (Table 1). First, we need to identify the right candidate for immunotherapy. Although the present form of immunotherapy is effective, some patients may not respond to well the therapy and some may suffer from serious adverse events. We have to select ones who will benefit most. It has been shown that immunotherapy for children with rhinitis prevented "atopic march" from advancing to asthma.[11] We have to select the right child for the intervention since not all children with rhinitis develop asthma. Recent progress in genetics has led to the identification of several candidate genes that are associated with various phenotypes of allergic diseases.[35] It is hopeful in the future that novel genetic

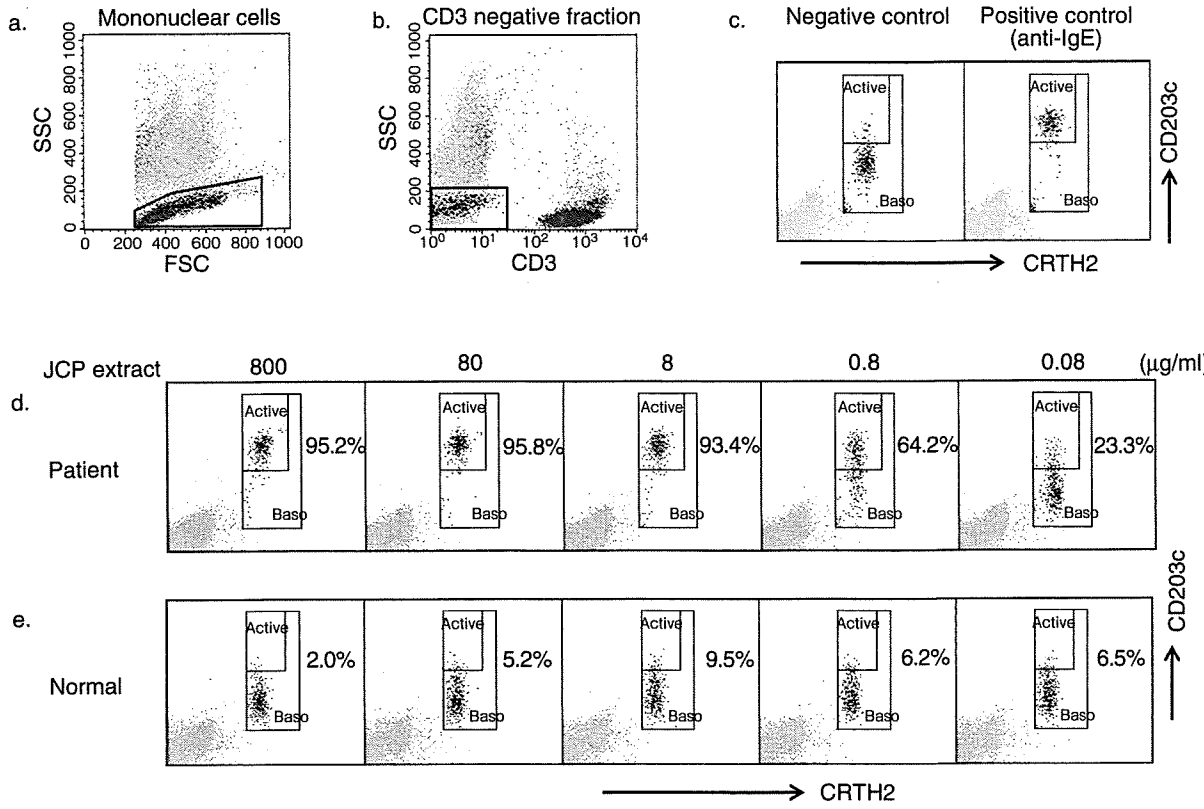

Fig. 2 Flowcytometric analysis of allergen-induced expression of CD203c. EDTA-containing whole blood was incubated with various concentrations of the Japanese cedar pollen (JCP) extract (Torii Pharmaceutical, Tokyo, Japan) for 15 min after addition of sufficient amount of calcium solution to override chelating capacity of EDTA. Anti-IgE antibody as a positive control and PBS as a negative control were also used for stimulation (**c**). PC7-conjugated anti-CD3, FITC-conjugated anti-CRTH2, and PE-conjugated anti-CD203c antibodies were also added during the reaction. The samples were analyzed on a FC500 flow cytometer (Beckman Coulter, CA, USA). Basophils were detected on the basis of forward side scatter characteristics (**a**) and expression of negative CD3 (**b**) and positive CRTH2 (**c**). Up-regulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control) and expressed as $CD203c^{high}$% (**c**). JCP extract induced concentration-dependent enhancement of CD203c expression in a patient with JCP pollinosis (**d**) and no enhancement was observed in a normal control (**e**).

biomarkers identify patients who respond to the therapy without risk of developing side effects.[36]

Secondly, the immune responses induced by immunotherapy need to be evaluated. Based on the knowledge of the mechanisms of immunotherapy, several assays have been reported. Studies of peripheral blood mononuclear cells from patients receiving immunotherapy have identified reductions in proliferative responses to allergen, shifts from Th2 to Th2 cytokine production, and enhanced inhibitory IL-10 production.[25,28,31,37] Some investigators, however, did not reproduce these findings in assays using peripheral blood although changes in the local tissue were demonstrated.[38] Variations in methodology in the peripheral T cell assays may be responsible for the discrepancies and standardization is necessary. Elevation of serum allergen-specific IgG or IgG4 antibodies after immunotherapy have been clearly demonstrated but again correlation between IgG or IgG4 titers and clinical responses to immunotherapy still to be established. Instead, functional assay of blocking IgG activity have been developed. Among them, the IgE-facilitated allergen binding (IgE-FAB) assay is reported to be a validated assay for monitoring allergen immunotherapy.[39] Receptors for IgE, expressed on the surface of antigen presenting cells, B cells in this assay system, facilitate the presentation of allergens in the presence of specific IgE resulting in effective T cell activation at low concentrations of allergen. "Blocking" IgG antibodies interfere with the interaction and the assay simulates the process *in vitro*. Allergen-IgE complexes are incubated with an EBV-transformed B-cell line and complexes bound to CD23 on the surface of cells are detected by flow cytometry. Inhibition of allergen-IgE complex binding to CD23 on B cells by addition of serum from patients who have received allergen-specific immunotherapy is then quantitated. They have demonstrated that the

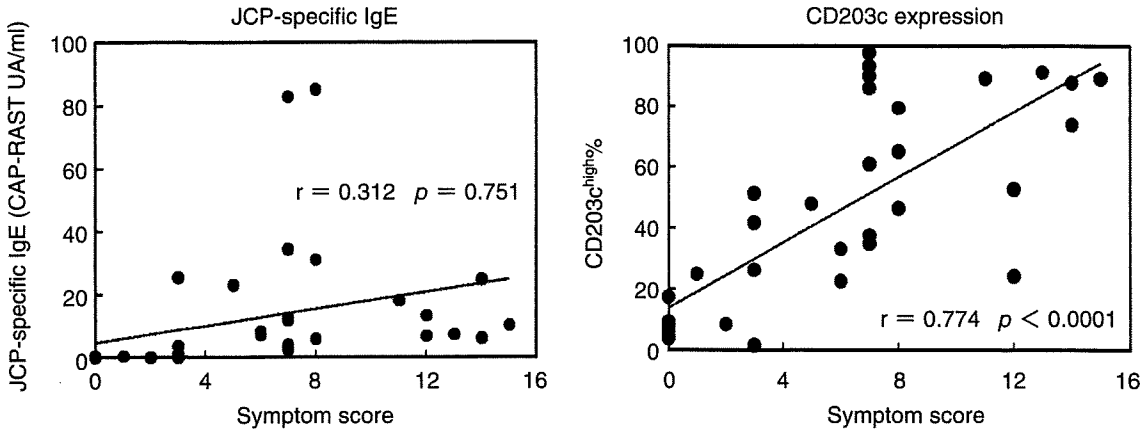

Fig. 3 Correlation of symptom score and JCP-specific IgE levels, CD203c expression by JCP extract. Thirty patients with JCP pollinosis were evaluated. Relationships between symptom score[54] and CAP-RAST titer to JCP, symptom score and JCP allergen-induced CD203c^{high}% were analyzed. Significant correlation was found in the latter.

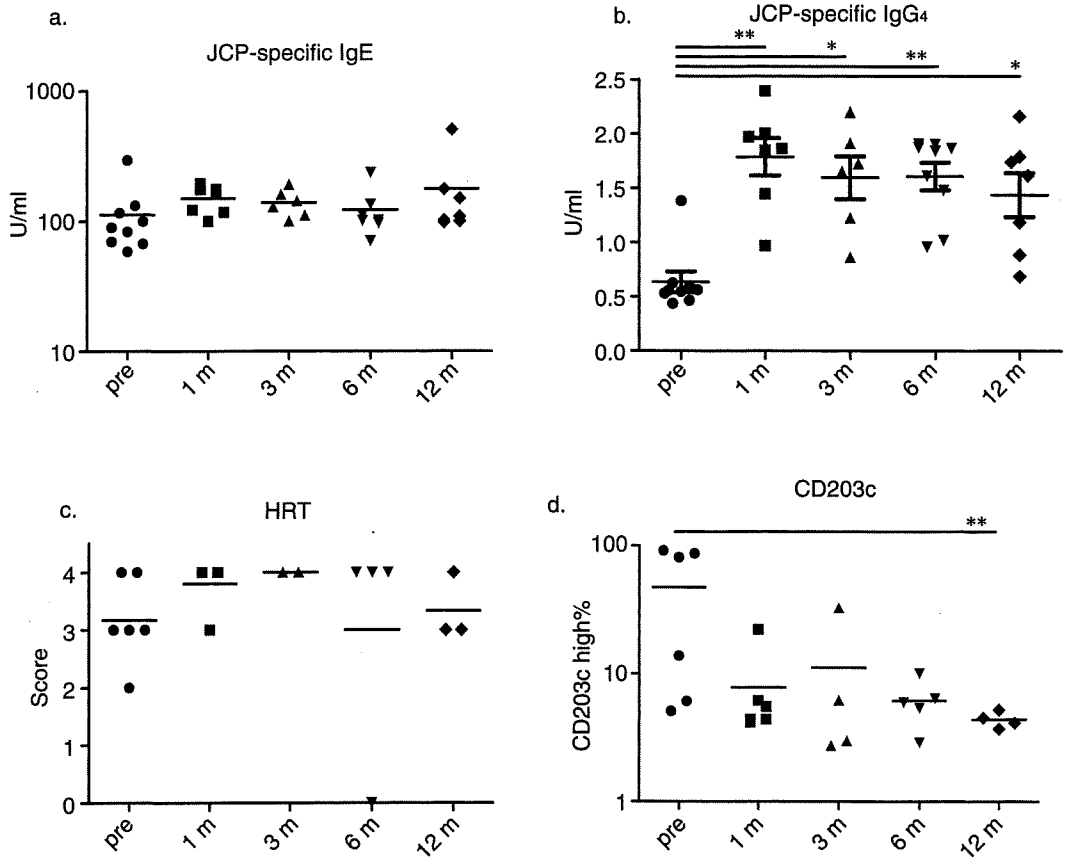

Fig. 4 Changes in JCP-specific IgE levels (**a**), JCP-specific IgG$_4$ levels (**b**), JCP-induced basophil histamine release score in HRT (**c**), and JCP-induced CD203c^{high}% in basophils (**d**) after rush immunotherapy in patients with JCP pollinosis. * $P < 0.05$, ** $P < 0.01$, Dunn's multiple comparison test (adapted from reference 49 with permission). Two subjects in whom basophils did not respond to stimulation with an anti-IgE antibody (non-responders) were excluded from the analysis for HRT and CD203c.

IgE-FAB assay have high specificity and sensitivity to diagnose clinical responses to immunotherapy. Recently, several other studies utilize the method to monitor efficacy of immunotherapy.[21,40]

ALLERGEN-INDUCED EXPRESSION OF CD203c ON BASOPHILS

Basophils play important roles in allergic diseases in effector phase by liberating mediators like histamine as well as in induction phase by producing Th2 cytokines, IL-4 and IL-13.[41] Upon activation through cross-linking of FcεRI by allergen, basophils rapidly express surface molecules such as CD63 and CD203c prior to the mediator and cytokine release. Flowcytometry-based tests for peripheral blood basophils can easily quantify these *in vitro* reactions, which presumably represent their *in vivo* activity. We utilized a commercial kit, Allergenicity Kit (Beckman Coulter, Fullerton, CA, USA), to detect expression of a basophil activation marker, CD203c. CD203c belongs to a family of ecto-nucleotide pyrophoshatase/phosphodiesterases (E-NPPs)[42,43] and has been described as being selectively expressed on basophils, mast cells and their $CD34^+$ progenitors.[44,45] Since CRTH2, a prostaglandinD2 receptor, is selectively expressed on basophils, Th2 cells, and eosinophils,[46,47] the kit identifies basophils as CD3-negative and CRTH2-positive fractions from whole blood samples and measures fluorescent intensity of CD203c that is enhanced by cross-linking of surface-bound IgE molecules (Fig. 2). As CD203c is rapidly up-regulated after allergen challenge in sensitized patients and the levels of up-regulation are well correlated with their symptoms (Fig. 2, 3), it has been proposed as a new tool for allergy diagnosis.[44,48] An important characteristic of the kit is that it employs whole blood during incubation with allergen, which not only detects specific IgE antibodies on basophils but also allows serum and other factors, possibly "inhibitory" factors induced by immunotherapy, in the blood to modify the reaction.

We recently found that induced expression of CD203c by Japanese cedar pollen (JCP) extract decreased after rush immunotherapy (RIT) in patients with JCP pollinosis without decrease in specific IgE levels to JCP.[49] We also found that significant elevation in JCP-specific IgG4 titers after RIT. There was no changes in JCP-induced histamine release from purified basophils[50] after RIT (Fig. 4). In passive sensitization experiments, the patients' sera obtained both before and after RIT showed essentially similar sensitizing capacity for basophils, corroborating the fact that specific IgE did not change. In contrast, basophil degranulation in response to the pollen extract was effectively suppressed by addition of post-RIT serum samples, which correspond with the elevation of specific IgG4 in the serum.[51] These results suggest that the CD203c test can detect blocking activity of IgG antibodies and other factors induced by immunotherapy. We also extend application of the assay to diagnosis of food allergy, especially of tolerance. Although specific IgE levels roughly predict sensitivity to food allergens,[52] markers that represent tolerance levels during outgrow phase of food allergy in childhood are not well-known. We found that the CD203c test effectively predicts sensitivity as well as tolerance to egg, milk (manuscript in preparation), and wheat[53] in children with food allergy.

CONCLUSIONS

Allergen immunotherapy is a promising disease-modifying therapy for allergic diseases including Japanese cedar pollinosis. To successfully initiate, maintain, and complete immunotherapy, predictive biomarkers have to be developed. Some prospects of biomarkers in the mechanisms of immunotherapy were reviewed in this article. Measurement of "blocking" activity of IgG such as IgE-facillitated allergen binding assay may efficiently monitor treatment effect of immunotherapy. Quantification of enhanced expression of CD203c on basophils employing whole blood during reaction with allergen may represent not only sensitization status but also tolerance levels in immunotherapy-treated patients. Larger scale studies are needed to standardize the CD203c assay for general laboratory use.

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Pediatr Allergy Immunol 2009: 20: 633–641
DOI: 10.1111/j.1399-3038.2009.00851.x

PEDIATRIC ALLERGY AND IMMUNOLOGY

Serum measurement of thymus and activation-regulated chemokine/CCL17 in children with atopic dermatitis: elevated normal levels in infancy and age-specific analysis in atopic dermatitis

Fujisawa T, Nagao M, Hiraguchi Y, Katsumata H, Nishimori H, Iguchi K, Kato Y, Higashiura M, Ogawauchi I, Tamaki K. Serum measurement of thymus and activation-regulated chemokine/CCL17 in children with atopic dermatitis: elevated normal levels in infancy and age-specific analysis in atopic dermatitis.
Pediatr Allergy Immunol 2009: 20: 633–641.

Elevated blood levels of thymus and activation-regulated chemokine (TARC)/CCL17 have been observed in atopic dermatitis (AD) and may serve as a new biomarker for AD. However, the normal levels, especially in children, have not been well determined. We sought to establish an efficient enzyme-linked immunosorbent assay (ELISA) with a wide range of detection that would be suitable for measurement of serum TARC/CCL17 and to determine the normal ranges of this chemokine in different age groups and its diagnostic usefulness for AD. A sensitive specific ELISA for TARC/CCL17, which we previously reported, was modified to accommodate the wide range of TARC/CCL17 values often found in sera. Twenty-seven children with AD under 6 yr of age and 25 age-matched normal non-atopic controls, and 18 patients with AD and 27 controls who were 6 yr and older were enrolled. The severity of AD was evaluated using the SCORAD index. The serum levels of TARC/CCL17 were measured with the ELISA, and the serum levels of IP-10/CXCL10 were also measured. With the novel ELISA system, the assayable range of TARC/CCL17 was 14–8000 pg/ml, and the coefficient of variation at various concentrations ranged from 2.3% to 5.0%. The serum levels of TARC/CCL17 in normal individuals were significantly higher in young children, especially in the age group of 0–1 yr. The cut-off values of TARC/CCL17 for the diagnosis of AD were 1431 pg/ml for 0–1 yr group, 803 pg/ml for 2–5 yr group and 510 pg/ml for the 6 yr and older group, with high sensitivity and specificity of 0.83 and 0.93, 0.83 and 0.92, 0.85 and 0.96, respectively. The magnitude of the decrease in the SCORAD index after treatment with topical steroids correlated significantly with the decrease in serum TARC/CCL17. There was no difference in the serum levels of IP-10/CXCL10 between AD and the controls. The TARC/CCL17:IP-10/CXCL10 ratio tended to be higher in the control children aged 0–1 yr than in those aged 2–5 yr. The serum level of TARC/CCL17 reflects the severity and therapeutic response in AD. The high normal levels in infants should be taken into account when assaying TARC/CCL17.

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Key words: atopic dermatitis; infants; TARC/CCL17; IP-10/CXCL10; Th_1/Th_2 balance

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Accepted 12 January 2009

Thymus and activation-regulated chemokine (TARC)/CCL17 is a ligand for CC chemokine receptor 4 (CCR4) that is selectively expressed on Th_2 cells and may play an important role in the pathogenesis of allergic diseases such as atopic dermatitis (AD). TARC/CCL17 has been detected in the inflamed skin of an animal model of AD and in patients with AD and has been shown to recruit Th_2 cells into inflammatory sites (1, 2). The serum and plasma levels of this chemokine are also reported to be elevated in patients with AD, and the levels correlate closely with the disease severity, suggesting that TARC/CCL17 may be a good clinical marker for AD (3–5). To further characterize the clinical utility of TARC/CCL17, we previously showed that the serum level of TARC/CCL17 is 10- to 50-fold higher than its plasma level and that the serum/plasma ratio of TARC/CCL17 was significantly higher in AD patients than their normal counterparts (4). We also demonstrated that platelets contained and released TARC/CCL17 and that the TARC/CCL17 content in platelets from patients with AD was significantly higher than in those from normal individuals, suggesting that the serum level of TARC/CCL17 represents the circulating or plasma level of TARC/CCL17 plus additional protein released *ex vivo*, partly from activated platelets in AD. These observations indicate that the TARC/CCL17 level in serum may better reflect the disease severity of AD than its plasma level. In addition, as serum samples are easier to handle than plasma samples in ordinary laboratories, serum TARC/CCL17 would be a better clinical marker for AD.

In order to further establish the clinical utility of TARC/CCL17 measurement for diagnosis of AD, it is important to determine the normal range of TARC/CCL17. However, serum TARC/CCL17 levels in children, especially those at a young age, have not been well studied. It was reported that Th_2-skewed responses to common environmental allergens are seen in all newborns, suggesting default Th_2 deviation at a young age (6). This fact may have an impact on serum levels of Th_2-type chemokines such as TARC/CCL17.

Previously, we developed a sensitive enzyme-linked immunosorbent assay (ELISA) for TARC/CCL17 with a lower limit of detection of 1.4 pg/ml (7). Because the serum levels of TARC/CCL17 are very high, the assay has to be refined to be applicable to the range of TARC/CCL17 levels ordinarily found in serum from patients with AD. In this study, we have established a novel ELISA with a broad detection range for measurement of serum TARC/CCL17 and investigated the chemokine levels in normal children and patients with AD. We found that the serum TARC/CCL17 level was significantly elevated in normal infants compared with older subjects.

Table 1. Subjects

	Age (yr) group	n	Mean ± s.d.	Male /female
Atopic dermatitis	0–5	27 (56)*	1.9 ± 1.8	17/10
	6 and above	18 (36)*	17.2 ± 8.2	9/9
Control	0–5	25	2.0 ± 1.7	10/15
	6 and above	27	24.9 ± 11.5	11/8

*Total number of replicate measurements of TARC/CCL17.

Materials and methods

Subjects

Ninety-seven subjects were enrolled in the study. Diagnosis of AD was based on the published clinical criteria for the disease (8): an elevated serum immunoglobulin E (IgE) level and sensitivity to more than one inhalant or food allergen, demonstrated by a positive test for specific IgE antibodies performed with a UniCAP® system (Phadia, Uppsala, Sweden). As we focused on subjects of a young age, 27 patients with AD under 6 yr of age and 25 age-matched normal controls were included. Eighteen patients with AD and 27 controls who were 6 yr and over were also included (Table 1). In some patients with AD, blood samples for TARC/CCL17 were obtained more than two times during the course of treatment. Table 1 shows the total number of blood samples. The control subjects had no history of allergic diseases, with IgE serum levels of < 160 IU/ml for those aged 2 yr and above and 60 IU/ml for those < 2 yr old. They also had no detectable specific IgE antibodies to common inhalant and food allergens, namely, house dust mites, Japanese cedar pollen, cat dander, dog dander, egg white, milk and wheat. Subjects were recruited via the hospital web page and posters in the hospital. Many of the young volunteers, especially the infants, had an inguinal hernia and were scheduled for operation, but were otherwise healthy. Blood samples were drawn from these infants as part of the routine pre-operative blood testing. The severity of AD was evaluated using the SCORAD (severity scoring of atopic dermatitis) index (9). None of the patients were administered systemic corticosteroids.

This study was performed with the approval of the Ethics Committee of Mie National Hospital. Informed consent was obtained from all the subjects or the guardians of child subjects (< 16 yr old).

Blood sampling

Venous blood was drawn into vacuum tubes (Venoject II; Terumo, Tokyo, Japan) and allowed to stand for 1 h at room temperature. Then the serum was separated by centrifugation at 2000 *g* for 10 min. The obtained serum samples were stored below −20°C until chemokine assay.

ELISA for TARC/CCL17 (SD-8864)

Recombinant human TARC/CCL17 (hTARC/CCL17; PeproTech, Rocky Hill, NJ, USA) was used as a calibrator for the ELISA and an in-house antigen (7) was used for the experiment to test the assay precision. The in-house antigen was calibrated with the recombinant human TARC/CCL17, and both proteins had similar immunoreactivity in our ELISA (data not shown). Anti-hTARC/CCL17 monoclonal antibodies (mAb; 4A3 and 5F12) were purified from BALB/c mouse ascitic fluid. The preparation procedures for the in-house antigen and antibody have been described in detail elsewhere (7). The Fab' fragment of the 5F12 mAb was conjugated with horseradish peroxidase (HRP, EC 1.11.1.7; Boehringer Mannheim, Germany) as described elsewhere (10).

Each well of a 96-well microplate (Immuno Module F8 Maxisorp; Nunc, Roskilde, Denmark) was filled with a solution (200 µl) of anti-hTARC/CCL17 mAb 4A3 [10 mg/l in 10 mmol/l phosphate-buffered saline (PBS), pH 9.4] and incubated for 18 h at 4°C. After removal of the antibody solution, the wells were washed three times with 300 µl/well of PBS-T (PBS containing 0.5 ml/l Tween 20), followed by aspiration. PBS containing 10 g/l bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) and 100 g/l saccharose was added to each well of the microplate, which was then incubated for 1 h at 25°C. After aspiration, the microplate was dried in a desiccator under a vacuum for 4 h at 25°C and then stored at 4°C until use.

At the time of use, 100 µl of assay buffer (50 mmol/l phosphate buffer containing 0.3 mol/l NaCl, 0.5 ml/l Tween 20, 1 g/l BSA and 0.2 g/l NaN_3, pH 7.4) and then aliquots of hTARC/CCL17 standard (0–8000 pg/ml in assay buffer) or sample (25 µl each), were added to the wells of the immunoplate, which was then incubated for 2 h at 25°C. Each well was washed three times with PBS-T, and then 100 µl of Fab'-HRP (75 ng) in PBS containing 4 g/l BlockAce (Dainippon Pharmaceutical, Osaka, Japan) and 1 g/l Kathon CG (Rohm and Haas, Philadelphia, PA, USA) were added to each well, followed by incubation for 1 h at 25°C. The assay plates were washed five times with PBS-T, and then the immunoreactivity was visualized by addition of 100 µl/well of substrate solution (ABTS solution; Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at 25°C. The reaction was stopped by addition of 50 µl of 1 g/l sodium dodecyl sulphate to each well, and the absorbance was measured at 405 nm using an Immunoreader NJ-2000 (Nalge Nunc International K.K., Tokyo, Japan). The hTARC/CCL17 levels were calculated based on the standard curve for each assay plate, and experiments were performed in duplicate except when noted otherwise.

IP-10/CXCL10 assay

The serum levels of IP-10/CXCL10 were measured in subjects under 5 yr of age using a commercial kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The data on the serum levels of TARC/CCL17 and IP-10/CXCL10 were expressed as geometric means because the logarithmically transformed values of the data followed a normal distribution. Differences were analysed with the Mann–Whitney *U*-test for unpaired samples and with the Wilcoxon's signed rank test for paired samples. For multiple comparisons, ANOVA was followed by Dunnett's multiple comparison test. Spearman's correlation coefficients between two parameters were calculated. The discriminative usefulness of TARC/CCL17 was evaluated by constructing receiver operating characteristic(ROC) curves (11) where sensitivity vs. 1 − specificity was plotted for each possible cut-off level. For this analysis, the group with AD was labelled as diseased compared with the control group. The area under the curve was determined for each age subgroup. From each ROC curve, we determined the ideal cut-off level which corresponds to the closest point to the top left-hand corner and which most efficiently discriminates between the presence or absence of disease. The respective sensitivity, specificity and predictive values were calculated.

Results

Assay characteristics

A representative standard curve of this ELISA (SD-8864) is shown in Fig. 1. The assayable

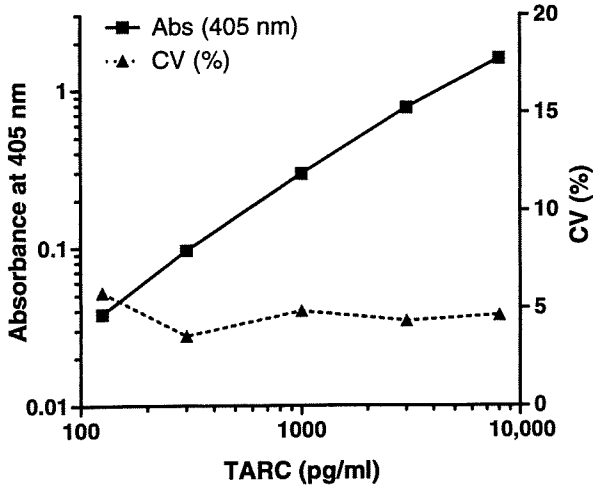

Fig. 1. A typical standard curve for the present ELISA for CCL17 and the precision profile (CV, %), calculated from five determinations for each point in one assay.

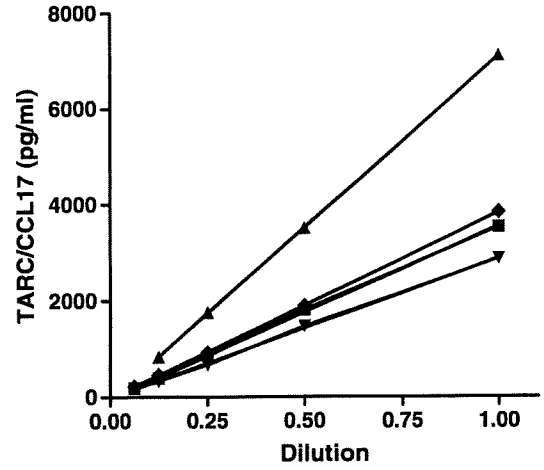

Fig. 2. Serial dilution linearity of the present ELISA. Four serum samples having different CCL17 concentrations were serially diluted with assay buffer.

range was 125–8000 pg/ml, and the coefficient of variation (CV) for the TARC/CCL17 concentration at each standard point ranged from 3.6% to 5.8% (n = 5). The reproducibility of this ELISA was estimated using clinically obtained sera having different TARC/CCL17 concentrations. The ranges of the intra- and inter-assay CVs were from 2.3% to 5.0% (n = 5) and 4.4% to 4.8% (n = 5), respectively, as shown in Table 2. Dilution curves of four sera showed preferable linearity (Fig. 2). The analytical recoveries were estimated for recombinant TARC/CCL17 added to serum samples containing three different concentrations of endogenous TARC/CCL17 (Table 3). The recovery ranged from 89.9% to 106.8%. Furthermore, we examined the cross-reactivity with other chemokines having similar structures and/or chemotactic activities for Th_2 cells leading to binding with the CC chemokine receptor 4 (CCR4) receptor. No cross-reactivity was found for TARC/CCL17 with 80 ng/ml preparations of the following chemokines: human MCP-1/CCL2 (PeproTech), eotaxin/CCL11 (12), MDC/CCL22 and mouse TARC/CCL17 (R&D Systems).

Table 2. Assay precision of the ELISA

	n	Mean (pg/ml)	s.d. (pg/ml)	CV (%)
Intra-assay				
Serum 1	5	263	13	5.0
Serum 2	5	1424	63	4.4
Serum 3	5	5817	133	2.3
Inter-assay				
Serum 4	5	239	11	4.8
Serum 5	5	844	37	4.4
Serum 6	5	3749	163	4.4

Table 3. Analytical recovery of standard CCL17 added to human serum

Sample	Endogenous (pg/ml)	Added (pg/ml)	Measured (pg/ml)	Found* (pg/ml)	Recovery (%)
Serum 7	214	500	694	480	96.1
	214	1000	1219	1006	100.6
	214	2000	2230	2017	100.8
Serum 8	1167	500	1701	534	106.8
	1167	1000	2167	1000	100
Serum 9	2883	500	3389	506	101.2
	2883	1000	3796	912	91.2
	2883	2000	4983	2100	105
Serum 10	3148	500	3664	516	103.2
	3148	1000	4047	899	89.9
	3148	2000	5138	1990	99.5

*Increase over endogenous CCL17.

Serum TARC/CCL17 levels in normal individuals

First, we examined the serum TARC/CCL17 level in normal non-atopic individuals of different ages. We found that the level in the age group of 0–5 yr was significantly higher than in the older age group of 6 yr and above ($p < 0.001$). We analysed that tendency by further dividing the subjects into four subgroups by age: <2, 2–5, 6–15 and 16 yr and older. As shown in Fig. 3, the serum TARC/CCL17 level was significantly higher in the age group of 0–1 yr than in each of the other, older age groups (Fig. 3, geometric mean values are shown in the legend). As described in Materials and methods, the control subjects had normal serum IgE levels and were not sensitized to common inhalant and food allergens. In particular, the median serum IgE value in the age group of 0–1 yr was 6 IU/ml, and except for in one subject all IgE values were <20 IU/ml. The serum TARC/CCL17 level of

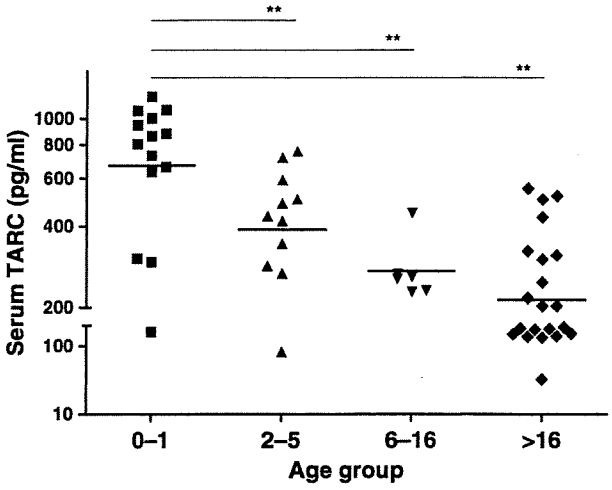

Fig. 3. Serum levels of TARC/CCL17 in normal individuals of various age groups. Horizontal lines indicate the geometric mean values, 707, 365, 273 and 213 pg/ml, in age groups of 0–1, 2–5, 6–16 and >16 yr, respectively. The serum TARC/CCL17 level was significantly higher in the age group of 0–1 yr than in the other age groups ($p < 0.01$; ANOVA and Bonferroni's multiple comparison test).

the subjects with the highest serum IgE, i.e. 56 IU/ml, was 512 pg/ml, which was below the median value in the group of 0–1 yr. There was no correlation between serum IgE and TARC/CCL17 in each group (data not shown).

Serum TARC/CCL17 levels in AD

Next, we compared the serum TARC/CCL17 level in AD with the normal controls in the corresponding age groups. In all age groups, the serum TARC/CCL17 level in AD was significantly elevated compared with the control subjects of corresponding age (Fig. 4; geometric mean values are shown in the legend). The values for the area under the ROC curves were 0.93, 0.89 and 0.96 in the respective age groups ($p < 0.001$) (Fig. 4). The cut-off values of TARC/CCL17 for the diagnosis of AD were 1431 pg/ml for the 0–1 yr old group, 803 pg/ml for the 2–5 yr old group and 510 pg/ml for 6 yr and older. With these cut-off values, the sensitivity and specificity of serum TARC/CCL17 for

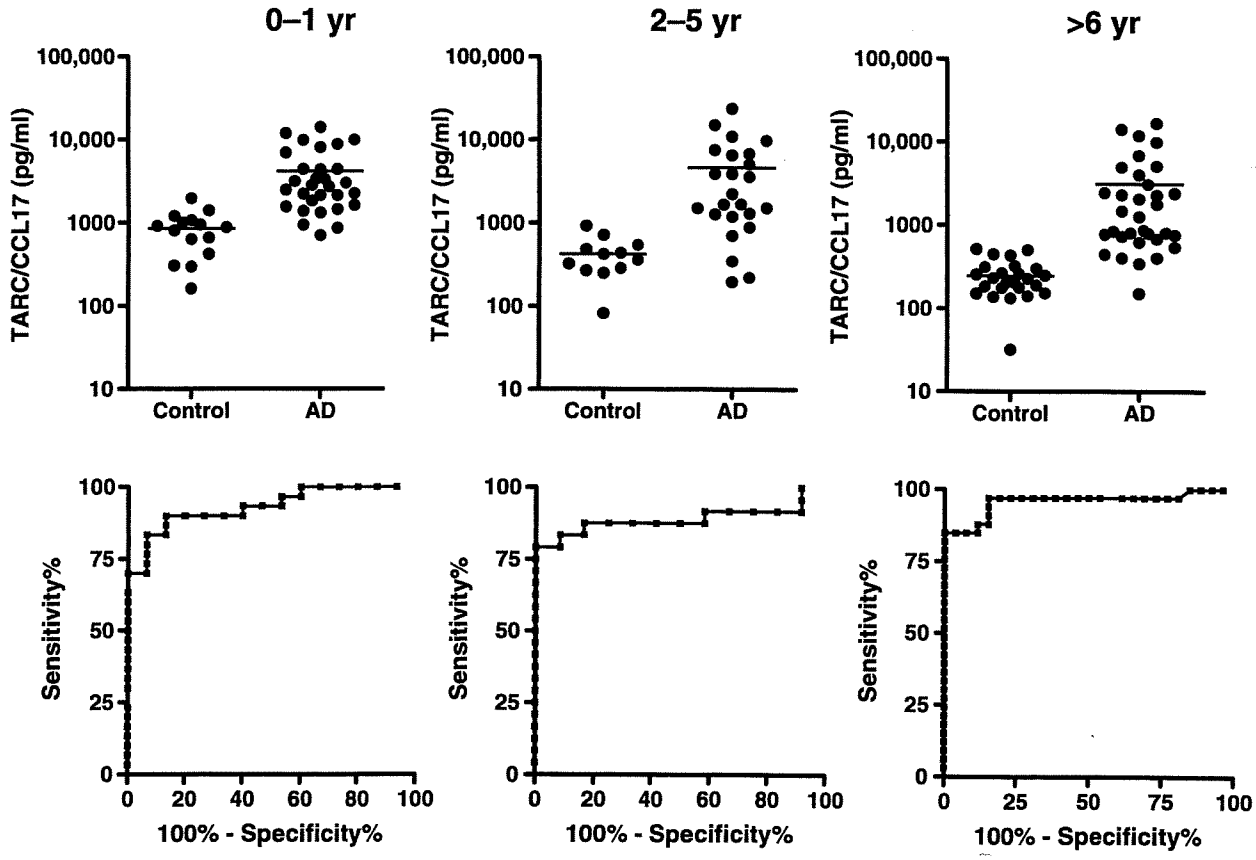

Fig. 4. Comparison of TARC/CCL17 levels between the controls and AD in age groups of 0–1, 2–5 and 6 yr and older (upper 3 graphs). The geometric mean values for the controls and AD in each age group were 707 and 3017, 365 and 2374 and 218 and 1550 pg/ml, respectively. The values for the area under the curve in ROC analysis (lower 3 graphs) in each age group were 0.931, 0.889 and 0.958, respectively.