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Basophil Activation Marker CD203c Is Useful in the Diagnosis of Hen's Egg and Cow's Milk Allergies in Children

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Key Words
Basophil activation test · CD203c · Egg allergy · Food allergy · Milk allergy · Oral food challenge test

Abstract
Background: The diagnosis of food allergy (FA) is usually based on oral food challenge tests (OFC). However, OFCs occasionally induce severe adverse reactions. CD203c expression on basophils is emerging as a potential diagnostic index. We evaluated whether CD203c expression on basophils would be a useful marker of OFC-associated symptoms in hen's egg and cow's milk allergies in children. ***Methods:*** Seventy-one patients who had been diagnosed with FA based on OFCs or a convincing history of FA symptoms in the Department of Pediatrics, Sagamihara National Hospital, were recruited. CD203c expression was assessed after stimulation with antigens (egg white, ovomucoid, milk or casein) using allergenicity kits. The CD203c stimulation index (SI = the allergen-induced CD203c expression level divided by the baseline expression level) and the threshold of CD203c expression (the minimum concentration of antigen to induce CD203c SI ≥2) were analyzed in association with tolerance acquisition. ***Results:*** For the CD203c SI, the areas under the receiver-operating characteristic curve were 0.72 for egg white, 0.82 for ovomucoid, 0.84 for milk and 0.67 for casein. The positive predictive value for the threshold of CD203c expression was 94.7% for egg white, 100% for ovomucoid, 85.7% for milk and 75.0% for casein. ***Conclusions:*** Assessment of food antigen-induced CD203c expression on basophils is useful to determine if children will outgrow FA as well as in decision making regarding whether or not to perform OFCs.

Introduction

The definitive diagnosis of food allergy (FA) is generally based on oral food challenge tests (OFC). Since OFCs occasionally induce severe reactions, whether or not to perform OFCs should be carefully considered. Antigen-specific IgE levels, a useful predictor of OFC-associated symptoms, have been widely used in clinical practice [1–4]. However, antigen-specific IgE is highly sensitive but less specific, and the development of more reliable tests for FA diagnosis has been sought.

When a causative antigen is bound to specific IgE on the surface of the cell membrane of a basophil, cross-linking of the high-affinity IgE receptor (FcεRI) occurs, inducing the release of histamine and the production of leukotrienes and cytokines. Because of this mechanism,

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peripheral blood basophils have been employed in tests, such as the histamine release test, to identify specific antigens that induce type I hypersensitivity [5].

A basophil activation test (BAT) determines CD63 and CD203c expression on basophils using flow cytometry. Recently, BATs have been reported to be useful in the diagnosis of IgE-dependent allergies [6, 7] and have been evaluated in association with a variety of different allergies [8–12].

CD203c (also called ectonucleotide pyrophosphatase/phosphodiesterase 3; E-NPP3) is an ecto-enzyme consisting of 875 amino acids belonging to a family of ectonucleotide pyrophosphatases and phosphodiesterases [13, 14]. It is expressed on the cell membrane of human peripheral basophils and mast cells [14, 15], and cross-linking of the high-affinity IgE receptor up-regulates CD203c expression on the cell membrane [11, 12]. In a previous study, CD203c expression was up-regulated following antigen stimulation in patients with bee venom [12] and grass pollen allergies [11], as well as after immunotherapy in patients with a history of systemic reactions to bee venom [9]. The sensitivity and specificity of antigen-induced CD203c expression were reported to be 75 and 100%, respectively, in the diagnosis of latex allergy [8]. More recently, Tokuda et al. [16] and Ocmant et al. [17] published papers on the utility of food antigen-induced CD203c expression in the diagnosis of FA. Ocmant et al. [17] reported that sensitivity and specificity of ovalbumin (OVA)-induced BATs applied to diagnose egg allergy were 62.5 and 96.4, respectively. Consequently, BATs may be beneficial in the diagnosis of FA. In the study by Tokuda et al. [16], measurement of purified native ω-5 gliadin-induced enhancement of CD203c on basophils is useful for the diagnosis of immediate wheat allergy in children. In the present study, we evaluated whether CD203c expression on basophils induced by food antigen would be useful in the prediction of OFC-associated symptoms in hen's egg and milk allergies.

Patients and Methods

Patients

Seventy-one patients (49 males and 22 females aged 6.6 ± 3.2 years, mean ± SD) who had previously been diagnosed with FA and had been followed up in our hospital (Division of Pediatrics, Sagamihara National Hospital) between July 2006 and October 2007 were recruited to this study. Acquisition of food tolerance was diagnosed based on the results of OFCs or a convincing history of symptoms after food ingestion during the 6 months before or after sample collection.

Grouping of Subjects Based on the Acquisition of Food Tolerance

Of all subjects, those who were tested for simultaneous measurement of CD203c expression and antigen-specific IgE were grouped as follows: for egg allergy, subjects with heated- and raw-egg allergy who reacted after ingestion of heated and raw eggs (HEA+/REA+), those without heated-egg allergy and raw-egg allergy who did not develop symptoms after ingestion of heated eggs but reacted after ingestion of raw eggs (HEA–/REA+), and those without heated- and raw-egg allergy who did not develop symptoms after ingestion of heated and raw eggs (HEA–/REA–); for milk allergy, subjects with milk allergy (MA+) who developed symptoms after drinking milk and those without milk allergy (MA–) who did not develop symptoms after drinking milk. The number of subjects tested depended on the antigens used as follows: for stimulation by egg white, 38 subjects with HEA+/REA+ versus 5 with (HEA–/REA+) versus 8 with (HEA–/REA–); for stimulation by ovomucoid, 35 versus 5 versus 6; for stimulation with milk, 27 with MA+ versus 23 with MA–, and for stimulation by casein, 21 versus 17. Areas under the curve (AUCs) were determined by receiver-operating characteristic (ROC) analysis to evaluate the utility of CD203c on basophils in determining the outgrowing of FA. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of the CD203 stimulation index (SI) and the threshold of CD203c were also determined.

Measurement of CD203c Expression on Peripheral Blood Basophils

CD203c expression on peripheral blood basophils was assessed using the Allergenicity Kit (Beckman Coulter, Fullerton, Calif., USA) on the day of sample collection. First, whole blood was drawn into plastic tubes containing EDTA 2Na (100 μl), 20 μl of antigen, and 20 μl of tricolored antibodies (CRTH2-FITC, CD203c-PE and CD3-PC7) and 100 μl of activation buffer were mixed in a plastic tube. The mixture was then incubated at 37°C for 15 min. After incubation, 100 μl of stop solution was added to stop the reaction, as well as lysis-fixing solution, and the mixture was incubated for 10 min at room temperature. The mixture was centrifuged at 200 *g* for 5 min, and the supernatant was aspirated. The sample was washed with phosphate-buffered saline, fixed with 0.1% formaldehyde, and then assayed by flow cytometry. The following antigens were used: anti-IgE antibody (Beckman Coulter), egg white, milk (Greer Laboratories, Lenoir, N.C., USA), ovomucoid (trypsin inhibitor from purified hen egg white ovomucoid type III-0, No. T-2011, Sigma-Aldrich, St. Louis, Mo., USA) and casein (α-casein; Sigma-Aldrich). The antigen concentrations used were as follows: 1,000 ng/ml for anti-IgE antibody, and 0.1, 10 and 1,000 ng/ml were employed for egg white, milk, ovomucoid and casein. CD203c expression on basophils was assessed using geometric mean fluorescence intensity. Antigen-induced CD203c expression was calculated using the formula of 'the antigen-induced CD203c expression – the baseline CD203c expression,' and the maximum CD203c expression was defined as the highest expression. The CD203c SI was defined as the antigen-induced CD203c expression divided by the baseline CD203c expression [11], and the threshold of CD203c expression was defined as the minimum concentration of an antigen that produces CD203c SI ≥2.

Oral Food Challenges

Oral food challenges were performed in an open challenge or single-blinded challenge [18]. In the open-challenge test, the hen's egg challenge test was performed using heated whole egg (about 30 g), and 35 g of yogurt or 200 ml of milk was used for the milk challenge test. In single-blinded challenges, freeze-dried powder of raw egg or milk (QP Institute, Tokyo, Japan) was used [19]. In an OFC, the patient initially ingested 1/16 of the total challenge dose of allergenic food and, subsequently, ingested increasing portions at an interval of 15 min until finishing the total challenge dose in 1 h. The patient was monitored until 24 h after food ingestion. If symptoms developed during the OFC, the test was terminated and the patient was carefully monitored. If the patient tested negative or slightly positive, ingestion of causative food at home was checked at every hospital visit to determine the patient was outgrowing the FA.

Antigen-Specific IgE Antibodies

Antigen-specific IgE antibodies against egg white, ovomucoid, milk and casein were determined by the CAP System FEIA (Phadia, Uppsala, Sweden) on the day of CD203c measurement or within 6 months before or after. The detection limit of the assay was 0.35 kUa/l.

Statistical Analysis

Results were presented as means ± SD or means ± SEM. Comparisons of groups were performed using the Mann-Whitney U test and the χ^2 test. For multiple comparisons, one-way analysis of variance followed by Dunn's multiple-comparison test was employed. $p < 0.05$ was considered statistically significant. ROC curve analyses were performed to analyze CD203c SI induced by food antigens, and the highest levels of sensitivity and specificity were used as cutoff values. They were calculated using GraphPad Prism (version 5).

Table 1. Patient profiles

	HEA+/ REA+	HEA–/ REA+	HEA–/ REA–
Egg white (n = 51)			
Patients, n	38	5	8
Average age, years	6.5 ± 3.1	5.5 ± 2.3	7.7 ± 4.4
Antigen-specific IgE, Ua/ml	21.8 ± 24.8*	5.0 ± 6.5	3.3 ± 3.7
Ovomucoid (n = 46)			
Patients, n	35	5	6
Average age, years	6.6 ± 3.2	5.5 ± 2.3	8.3 ± 4.9
Antigen-specific IgE, Ua/ml	16.3 ± 25.0*	2.5 ± 2.4	3.4 ± 3.5
	MA+	MA–	
Milk (n = 50)			
Patients, n	27	23	
Average age, years	6.5 ± 3.4	6.3 ± 2.9	
Antigen-specific IgE, Ua/ml	22.5 ± 32.5*	6.3 ± 11.1	
Casein (n = 38)			
Patients, n	21	17	
Average age, years	7.6 ± 3.5	6.4 ± 3.0	
Antigen-specific IgE, Ua/ml	17.8 ± 24.9*	7.6 ± 13.3	

* $p < 0.05$, Mann-Whitney U test.

Results

Characteristics of the patients are summarized in table 1. The average age did not significantly differ between patients with egg allergy and those with milk allergy. The average antigen-specific IgE level was significantly higher in the HEA+/REA+ group than in other egg allergy groups and in the MA+ group than in the other milk group.

The baseline CD203c expression level was 331.1 ± 46.0 (mean ± SEM) for the HEA+/REA+ group, 385.6 ± 137.8 for the HEA–/REA+ group and 245.7 ± 41.9 for the HEA–/REA– group (nonsignificant). The baseline CD203c expression was 302.1 ± 33.9 for the MA+ group and 332.5 ± 62.2 for the MA– group (nonsignificant). No significant difference was observed between groups with egg and milk allergies.

As shown in figure 1a, the maximum CD203c expression induced by egg white was 755.5 ± 86.1 (mean ± SEM) for the HEA+/REA+ group, 422.6 ± 218.2 for the HEA–/REA+ group and 293.7 ± 100.2 for the HEA–/REA– group (nonsignificant). The CD203c SI was 4.2 ± 0.4 for the HEA+/REA+ group, 2.3 ± 0.4 for the HEA–/REA+ group and 2.3 ± 0.5 for the HEA–/REA– group (nonsignificant). Although average CD203c expression was increased in the HEA+/REA+ group, the difference was not significant between egg allergy groups. On the other hand, the maximum CD203c expression level induced by ovomucoid was 727.7 ± 102.0 for the HEA+/REA+ group, 325.9 ± 204.1 for the HEA–/REA+ group and 108.5 ± 32.1 for the HEA–/REA– group, i.e. CD203c expression was significantly increased in the HEA+/REA+ group ($p < 0.05$). The CD203c SI was 3.8 ± 0.4 for the HEA+/REA+ group, 2.0 ± 0.4 for the HEA–/REA+ group and 1.4 ± 0.1 for the HEA–/REA– group, demonstrating that CD203c SI was significantly higher in the HEA+/REA+ group ($p < 0.05$).

As shown in figure 1b, the maximum CD203c expression induced by milk was 944.4 ± 136.2 for the MA+ group and 273.4 ± 105.1 for the MA– group, and the CD203c SI was 4.5 ± 0.5 for the MA+ group and 1.9 ± 0.4 for the MA– group, i.e. both parameters were significantly higher in the MA+ group ($p < 0.05$). The maxi-

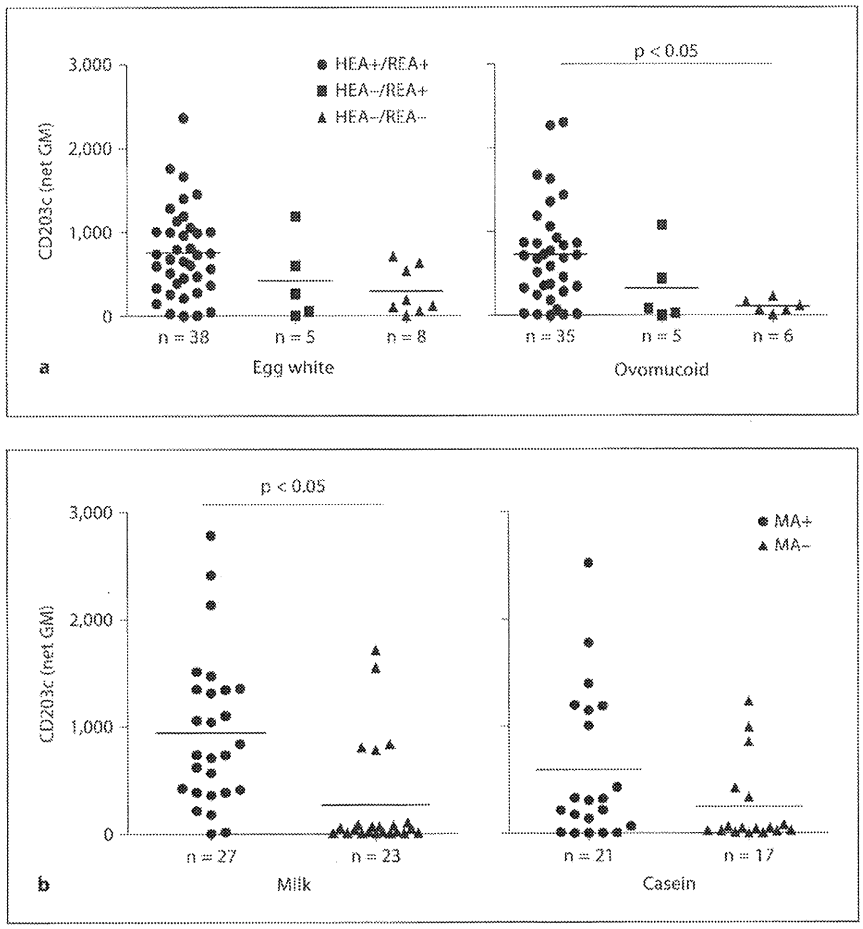


Fig. 1. Relationships between FA diagnosis and CD203c expression on basophils in patients with hen's egg allergy (**a**) and cow's milk allergy (**b**). * $p < 0.05$, Dunn's multiple comparison test. GM = Geometric mean fluorescence intensity.

mum CD203c expression level induced by casein was 592.4 ± 153.5 for the MA+ group and 248.2 ± 95.6 for the MA– group, and the CD203c SI was 3.1 ± 0.5 for the MA+ group and 1.7 ± 0.3 for the MA– group, with no significant difference between the groups.

ROC analyses were performed for CD203c SI. The AUC for egg allergy was 0.74 for egg white and 0.82 for ovomucoid in ROC analysis of HEA+ versus HEA–, and 0.72 and 0.84, respectively, in ROC analysis of REA+ versus REA– (fig. 2a), demonstrating that the parameter was higher after stimulation with ovomucoid in both ROC analyses. The cutoff value for CD203c SI in ROC analysis of HEA+ versus HEA– was 2.4 for egg white and 1.7 for ovomucoid. The sensitivity, specificity, PPV, NPV and efficiency were 73.7, 61.5, 84.8, 44.4 and 70.6%, respectively, for egg white and 80.0, 72.7, 90.3, 53.3 and 78.3%, respectively, for ovomucoid. The cutoff value for CD203c SI in ROC analysis of REA+ versus REA– was 1.7, the sensitivity, specificity, PPV, NPV and efficiency were 76.7, 62.5, 91.7, 33.3 and 74.5%, respectively, for egg white. The cutoff value was 1.6 for ovomucoid, and the sensitivity, specificity, PPV, NPV and efficiency were 82.5, 83.3, 97.1, 41.7 and 82.6%, respectively.

For milk allergy, the AUC for milk induced CD203c SI was higher than that for casein (0.84 vs. 0.67; fig. 2b). The cutoff value for CD203c SI was 1.9 for milk and 1.3 for casein. The sensitivity, specificity, PPV, NPV and efficiency were 88.9, 82.6, 85.7, 86.4 and 86.0% for milk and 66.7, 70.6, 73.7, 63.2, and 68.4% for casein, respectively.

As shown in figure 3a, for HEA, the threshold of CD203c expression for egg white and ovomucoid was ≥10 ng/ml. Eighteen of 19 patients (95%) were HEA+ when the threshold of CD203c expression for egg white was 10 ng/ml ($p < 0.01$). All of the 21 patients (100%) were HEA+ when the threshold of CD203c expression for ovomucoid was 10 ng/ml ($p < 0.01$). When diagnoses were

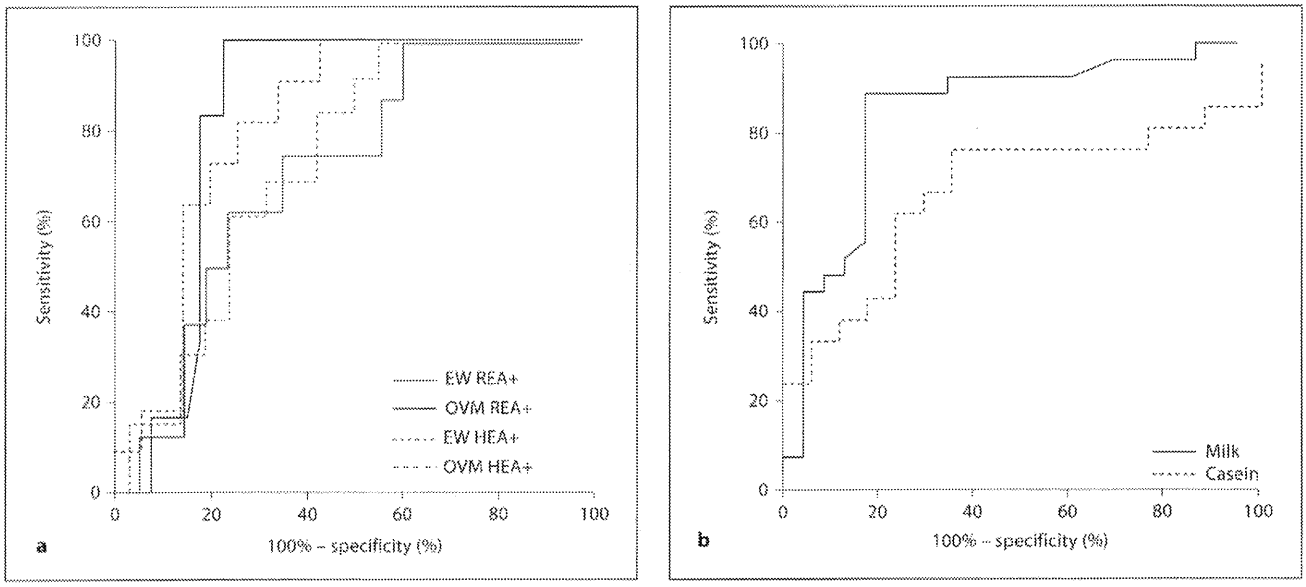


Fig. 2. ROC curves for CD203c SI induced by food antigen in patients with hen's egg allergy (**a**) and cow's milk allergy (**b**). OVM = Ovomucoid.

based on these threshold concentrations, sensitivity, specificity, PPV, NPV and efficiency were 47.4, 92.3, 94.7, 37.5 and 66.7%, respectively, for egg white and 60.0, 100.0, 100.0, 44.0 and 69.6%, respectively, for ovomucoid in HEA+ patients.

Although 18 of the 19 patients (95%) were REA+ when the threshold of CD203c expression for egg white was 10 ng/ml, no significant difference was seen between REA+ and REA– patients. All of the 30 patients (100%) were REA+ when the threshold of CD203c expression for ovomucoid was $\leq$1,000 ng/ml ($p < 0.01$). When diagnoses were based on these threshold concentrations, sensitivity, specificity, PPV, NPV and efficiency were 41.9, 87.5, 94.7, 21.9 and 49.0%, respectively, for egg white and 73.2, 100.0, 100.0, 31.3 and 76.1%, respectively, for ovomucoid in REA+ patients (fig. 3b).

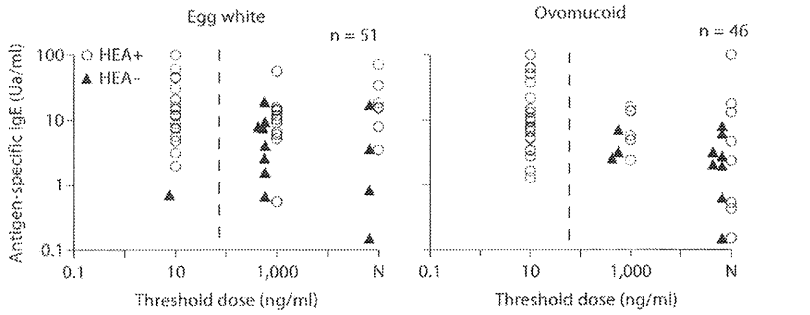
As shown in figure 3c, 24 of the 28 patients (86%) were MA+ when the threshold of CD203c expression for milk was $\leq$1,000 ng/ml ($p < 0.01$). Twelve of the 16 patients (75%) were MA+ when the threshold of CD203c expression for casein was $\leq$1,000 ng/ml (nonsignificant). When diagnoses were based on these threshold concentrations, sensitivity, specificity, PPV, NPV and efficiency for milk were 88.9, 82.6, 85.7, 86.4 and 52.6%, respectively, and 57.1, 76.5, 75.0, 59.1 and 52.6%, respectively, for casein in MA+ patients.

Discussion

Generally, indications for OFC are individually determined considering the patient's history and antigen-specific IgE levels. However, OFC may sometimes cause severe allergic reactions. In recent years, measurement of CD203c expression on basophils has become a promising diagnostic procedure for IgE-dependent allergy.

In the present study, we demonstrated that in patients with egg allergy, the maximum CD203c expression by ovomucoid was significantly higher in the HEA+ group, and in patients with milk allergy, the maximum CD203c expression by milk was also significantly higher in the MA+ group. In a study by Ocmant et al. [17], CD203c expression was up-regulated after ovalbumin stimulation in patients with egg allergy, and CD203c SI had a sensitivity of 62.5% and a specificity of 96.5% and could be useful in the diagnosis of FA. These findings suggested that the levels of CD203c expression induced by these antigens reflect the presence of egg and milk allergies.

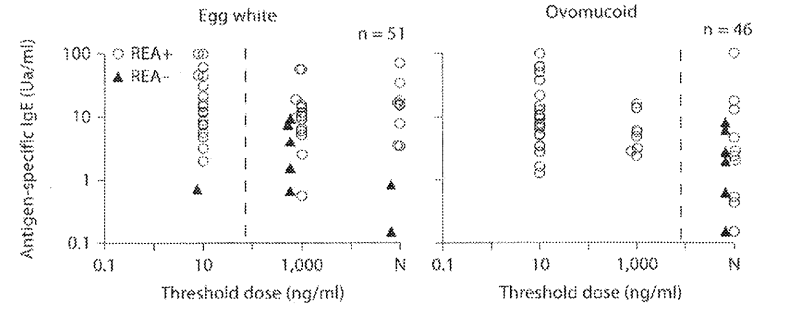
Regarding HEA, the AUC received from ROC analysis for ovomucoid CD203c SI was higher than for egg white (0.84 vs. 0.72, respectively). Similarly, regarding REA, the AUC received from ROC analysis for ovomucoid CD203c SI was also higher than for egg white (0.82 vs. 0.74, respectively). In addition, using the cutoff value obtained from



Threshold ng/ml	HEA+	HEA–	Total
≤10	18*	1	19
>10	20	12	32
Total	38	13	51

Threshold ng/ml	HEA+	HEA–	Total
≤10	21*	0	21
>10	14	11	25
Total	35	11	46

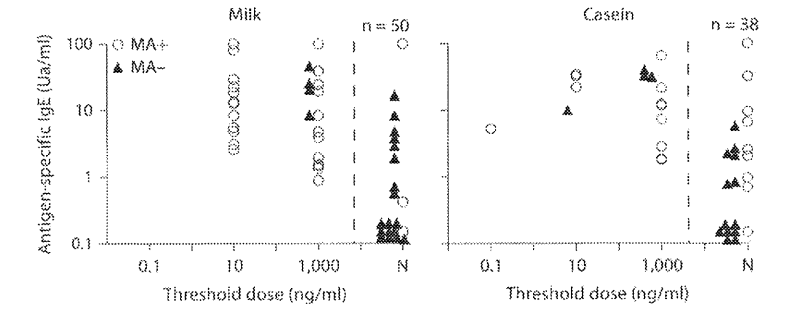
a



Threshold ng/ml	REA+	REA–	Total
≤10	18	1	19
>10	25	7	32
Total	43	8	51

Threshold ng/m	REA+	REA–	Total
≤1,000	30*	0	30
>1,000	10	6	16
Total	40	6	46

b



Threshold ng/ml	MA+	MA–	Total
≤1,000	24*	4	28
>1,000	3	19	22
Total	27	23	50

Threshold ng/ml	MA+	MA–	Total
≤1,000	12	4	16
>1,000	9	13	22
Total	21	17	38

c

Fig. 3. Diagnosis of FA and its relationship to antigen-specific IgE and threshold of CD203c expression. **a** Heated-egg allergy. **b** Raw-egg allergy. **c** Cow's milk allergy. * $p < 0.01$, χ^2 test. N = CD203c SI at the maximum concentration of antigen is <2.

the ROC analysis, the PPV of ovomucoid CD203c SI was higher than that for egg white in both HEA and REA. These results indicate that after stimulation with ovomucoid CD203c SI is a useful predictor of OFC-associated symptoms in egg allergy.

When the threshold of egg white-induced CD203c expression was used in the diagnosis of HEA, sensitivity, specificity and PPV were 47.4, 92.3 and 94.7%, respectively, for egg white and 60.0, 100.0 and 100.0%, respectively, for ovomucoid. When the threshold of CD203c expression was used in the diagnosis of REA, sensitivity, specificity and PPV were 41.9, 87.5 and 94.7%, respectively, for egg white and 75.0, 100.0 and 100.0%, respectively, for ovomucoid, indicating the higher PPV for CD203c expression than CD203c SI.

Antigen-specific IgE levels were reported to be instrumental in predicting OFC outcomes in children [4]. However, the outcomes of OFCs depend on the individual patient even if antigen-specific IgE levels are the same. The threshold for CD203c also depends on the individual patient even if antigen-specific IgE levels are the same. When the threshold for CD203c after stimulation by egg white or ovomucoid is 10 ng/ml, OFCs are likely to elicit allergic reactions regardless of antigen-specific IgE levels. To our knowledge, our study is the first report dealing with diagnostic analyses of the threshold for antigen-induced CD203c. On the other hand, patients with birch pollen allergy complicated with oral allergy syndrome had low concentrations and thresholds to birch pollen-induced histamine release compared to those with birch pollen allergy not complicated with oral allergy syndrome [20], suggesting that CD203c expression may be enhanced at lower concentrations in patients with allergic symptoms compared to patients without them.

Sensitivity and specificity of CD203 SI or the threshold of CD203c expression slightly differed between HEA and REA. Sensitivity and specificity after stimulation by egg white were almost similar between HEA and REA, whereas those after stimulation by ovomucoid were higher for HEA than REA. Eggs are sensitive to thermal denaturation [21], and antigenicity may depend on the heating conditions of the food tested. Ovomucoid, an egg white allergen, is rather resistant to heat [22]. Ovomucoid-specific IgE levels were high in patients with HEA [23]. This implies that patients with HEA responded to heat-resistant ovomucoid, and ovomucoid may thus be a better stimulatory allergen in the diagnosis of such patients.

The benefit of milk-induced CD203c expression on basophils in the diagnosis of milk allergy has not been documented previously. In the present study, the AUC received from ROC analysis for milk-induced CD203c SI was higher than for casein (0.84 vs. 0.67, respectively). In addition, using the cutoff value obtained from ROC analysis, sensitivity, specificity and PPV of milk-induced CD203c SI were 88.9, 82.6 and 85.7%, respectively, and those after stimulation by casein were 66.7, 70.6 and 73.7%, respectively. The PPV of milk-induced CD203c SI was higher than that for casein.

When the threshold of milk-induced CD203c expression was used in the diagnosis of milk allergy, sensitivity, specificity and PPV were 88.9, 82.6 and 85.7%, respectively. These results were similar to those obtained when the CD203c SI was used. There was no significant difference between MA+ and MA– patients using the threshold of casein-induced CD203c expression. These data indicate that using a threshold of milk-induced CD203c expression $\leq$1,000 ng/ml, OFCs are likely to elicit allergic reactions regardless of antigen-specific IgE levels.

In conclusion, OFCs are likely to elicit allergic reactions in egg or milk allergy patients when the threshold of ovomucoid-induced CD203c is 10 mg/ml or when the threshold of milk-induced CD203c is $\leq$1,000 ng/ml. Consequently, analysis of CD203c expression on basophils may be beneficial in the diagnosis of FA.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.

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Utility of the Peripheral Blood Basophil Histamine Release Test in the Diagnosis of Hen's Egg, Cow's Milk, and Wheat Allergy in Children

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Key Words
Food allergy · Histamine release · Hen's egg · Cow's milk · Wheat

Abstract
Background: The diagnosis of food allergy (FA) is made by oral food challenge tests (OFCs) that occasionally produce serious symptoms in patients; therefore, whether to perform OFCs should be carefully considered. The utility of the histamine release test (HRT) in the diagnosis of childhood FA has not been fully examined. ***Methods:*** Sixty-four subjects with suspected hen's egg allergy, cow's milk allergy (CMA), and wheat allergy (WA) were enrolled. The diagnosis of FA was made based on the outcomes of OFCs or a convincing history of symptoms after food ingestion within 6 months before or after sample collection. HRT was performed using an HRT Shionogi kit. The threshold of histamine release (HRT threshold), which was defined as the minimum concentration of food antigen to induce a 10% net histamine release, was analyzed in association with FA diagnosis. ***Results:*** Receiver operating characteristic analysis showed that the HRT threshold was useful in the diagnosis of heated egg allergy (HEA), raw egg allergy (REA), CMA, and WA. We were able to determine the cutoff value for the HRT threshold in relation to outcomes of OFCs. The cutoff value was 6 ng/ml of egg white antigen in HEA and REA ($p < 0.01$), 40 ng/ml of milk antigen in CMA ($p < 0.01$), and 500 ng/ml of wheat antigen in WA ($p < 0.05$). The efficiency was 70.3% for HEA, 78.0% for REA, 77.6% for CMA, and 70.7% for WA. ***Conclusions:*** We conclude that the HRT threshold measurement for egg white, milk, and wheat antigen is related to outcomes of OFCs and is useful in determining when OFCs should be performed.

Introduction

A definitive diagnosis of food allergy (FA) is generally made using oral food challenge tests (OFCs). Since OFCs occasionally induce severe reactions, whether to perform OFCs should be carefully considered. Antigen-specific IgE levels, a useful predictor of reaction symptoms in OFCs, have been widely used in clinical practice [1–4]. However, antigen-specific IgE is highly sensitive but less specific, and the development of more reliable tests for FA diagnosis is being pursued.

When a causative antigen is bound to specific IgE on the surface of basophil cell membranes, cross-linking of the high-affinity IgE receptor (FcεRI) occurs, inducing

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the release of histamine and the production of leukotrienes and cytokines [5, 6]. The expression of FcεRI on the surface of basophils correlates with serum IgE levels [7]. Moreover, it has been reported that basophils primed with IL-3 enhance histamine release in response to IgE-dependent activation [8]. In this way, basophils are influenced by biological conditions. Therefore, we hypothesized that examination of the histamine release test (HRT) is distinct from simply measuring antigen-specific IgE.

Recently, basophil activation tests have been reported to be useful in the diagnosis of IgE-dependent allergies and have been evaluated in association with a variety of different allergies [9–16]. More recently, Ocmant et al. [15] reported that corresponding performances of ovalbumin and peanut-induced basophil activation tests may be helpful in the diagnosis of egg and peanut allergies. Wanich et al. [17] reported that patients were able to tolerate heated cow's milk have suppressed basophil expression of milk-induced CD63.

On the other hand, the development of HRT in the 1960s [18] did not lead to its widespread use outside of research laboratories due to the large volume of blood required and the short testing window after blood sampling [19]. In recent years, a novel HRT method has been developed that is more convenient and requires a smaller volume of blood. This HRT is commercially available for diagnostic examinations in Japan [20].

Various reports have described the utility of HRT in the diagnosis of FA [21–28]. Norgaard et al. [26] examined the utility of HRT in diagnosing hen's egg allergy and cow's milk allergy (CMA) in adults [28]. These reports suggested that improving the quality of allergen preparations may allow HRT to become a valuable method in FA diagnosis. Lau et al. [25] examined the utility of HRT in the diagnosis of hen's egg allergy in children and indicated that HRT was not effective in predicting outcomes of OFCs. However, in past reports, the utility of HRT in childhood FA diagnosis was not fully examined as many of the subjects were adults and the number of children included in the studies was low [21–28]. The objective of this study was to examine the utility of HRT in the diagnosis of FA in children.

Subjects and Methods

Subjects

Sixty-four subjects (50 males and 14 females; mean age 5.8 ± 3.7 years) with suspected FA, and who had received follow-up care at the Division of Pediatrics of Sagamihara National Hospital between July 2005 and October 2005, were recruited for this study. The diagnosis of FA was made using the results of OFCs or a convincing history of symptoms after food ingestion within 6 months before or after sample collection. Ethics approval was obtained through the Institutional Review Boards at Sagamihara National Hospital. Written informed consent was given by the child or by the child's parents prior to enrolment.

Grouping of Subjects Based on Acquisition of Food Tolerance

Of the subjects, those who were tested for HRT and antigen-specific IgE were grouped as follows: for egg allergy, subjects with heated egg allergy (HEA) who reacted after ingestion of heated eggs [HEA(+)] and those without HEA who did not develop symptoms after ingestion of heated eggs [HEA(–)], and subjects with raw-egg allergy who reacted after ingestion of raw eggs [REA(+)] and those without raw egg allergy (REA) who did not develop symptoms after ingestion of raw eggs [REA(–)]; for CMA, subjects with CMA [CMA(+)] who developed symptoms after drinking milk and those without CMA [CMA(–)] who did not develop symptoms after drinking milk; for wheat allergy (WA), subjects with WA [WA(+)] who developed symptoms after eating wheat and those without WA [WA(–)] who did not develop symptoms after eating wheat.

Oral Food Challenge Tests

OFCs were performed using an open challenge or a single-blind challenge [29]. The open challenge test was performed using heated egg (about 30 g) for HEA, yogurt (35 g) or cow's milk (200 ml) for CMA, and udon (made from wheat flour; 15–50 g) for WA. The single-blind challenge test employed freeze-dried powder (raw egg, cow's milk, and wheat) provided by QP Co., Ltd. Institute, Tokyo, Japan [30]. The initial dose was 1/16 of the amount of the total challenge dose, increasing gradually at 15-min intervals for a period of 1 h, and subjects were carefully monitored for the subsequent 24 h.

Histamine Release Test

The HRT was performed using an HRT Shionogi kit (Shionogi & Co., Ltd., Osaka, Japan) [20]. First, 2 ml of whole blood was drawn into plastic tubes containing EDTA2Na. Blood (20 μl) and anti-leukocyte antibody- (BA312) coated magnetic beads (100 μl) were added to each well of a 96-well microplate and incubated for 10 min at room temperature on a plate mixer. After incubation, a magnetic device was inserted in the 96-well microplate and the basophil-bead complexes were captured for 4 min on the magnetic device at room temperature. The basophil-bead complexes were transferred to another microplate, which was coated with streptavidin for enzyme-linked immunosorbent assay (ELISA) and contained 100 μl of anti-IgE; digitonin (200 μg/ml) for total histamine; HEPES-buffered saline with human serum albumin containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% glucose (HACMG) for spontaneous histamine release, and/or food antigen in the respective wells. The microplate was incubated at 37°C for 60 min, with the basophil-bead complexes remaining on the magnetic device. After incubation, the magnetic device was removed and histamine content was measured by ELISA. The following antigens were used: anti-IgE antibody [31], egg white, milk, and wheat (Greer Laboratories, Inc., Lenoir, N.C., USA). The antigens were diluted in HACMG to final concentrations of egg white at 0.5, 6, 70, 830, and 10,000 ng/ml; milk at 0.4, 4, 40,

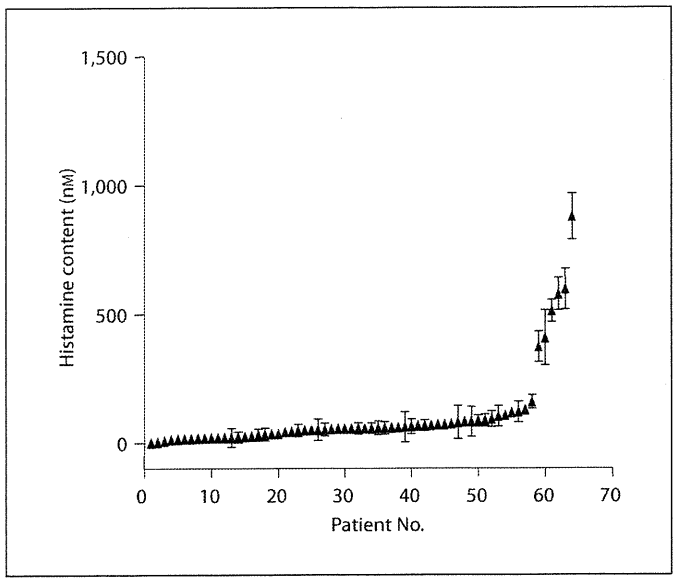

Fig. 1. Distribution of spontaneous histamine release (n = 64). Plots and bars represent means ± SD.

Table 1. Characteristics of subjects

Food antigen	Diagnosis of food allergy	Subjects, n	Average age, years	Antigen-specific IgE (Ua/ml)
Hen's egg (heated) (n = 64)	HEA(+)	38	4.9 ± 3.4	40.0 ± 36.7
	HEA(–)	26	7.1 ± 3.8**	13.9 ± 18.8*
Hen's egg (raw) (n = 50)	REA(+)	38	4.9 ± 3.4	40.0 ± 36.7
	REA(–)	12	6.6 ± 4.6	7.9 ± 14.3
Cow's milk (n = 49)	CMA(+)	27	4.7 ± 3.4	23.8 ± 32.9
	CMA(–)	22	8.2 ± 3.9*	4.6 ± 10.0*
Wheat (n = 41)	WA(+)	12	3.6 ± 3.0	16.9 ± 26.0
	WA(–)	29	7.1 ± 4.1*	7.3 ± 14.0

The diagnosis of food allergy was made based on the results of food challenge tests and a convincing history of food allergies within the past 6 months [HEA(+), REA(+), CMA(+), and WA(+)]. HEA(–), REA(–), CMA(–), and WA(–) denote no symptoms after ingesting a causative food. Mann-Whitney U test, * $p < 0.05$; ** $p < 0.01$.

400, and 4,000 ng/ml; anti-IgE antibody, and wheat at 0.5, 5, 50, 500, and 5,000 ng/ml. Antigen-induced histamine release was measured in duplicate and total histamine content and spontaneous histamine release were measured using 10 wells. HRT was performed in duplicate on the sampling day. Spontaneous histamine release was expressed as means ± standard deviation (SD). Antigen- (anti-IgE antibody, egg white, milk, and wheat) induced histamine release was calculated as: (antigen-induced histamine release – spontaneous histamine release)/total histamine content × 100 [24]. The maximum of percent histamine release (Max. of %HR) is defined as the peak value of antigen-induced histamine release [22] and the threshold of HRT (HRT threshold) was determined as the minimum concentration required to induce a 10% net histamine release.

Antigen-Specific IgE Antibody

Serum was obtained from all subjects on the HRT sampling day. Antigen-specific IgE antibodies to egg white, milk, and wheat were measured using ImmunoCAP (Phadia AB, Uppsala, Sweden). The assay's detection limit was 0.35 kUa/l. We divided the subjects into 2 group according to a cutoff value which was 95% of a positive predictive value of antigen specific IgE levels reported by Komata et al. [1] and Sampson [2].

Statistical Analysis

Results are expressed as means ± SD or means ± standard error (SEM). Comparisons between groups were performed using the Mann-Whitney U test and the χ^2 test. $p < 0.05$ was considered statistically significant. ROC curve analyses were performed to analyze antigen-specific IgE, Max. of %HR, and the HRT threshold. The cutoff value for the HRT threshold was based on the lowest statistically significant food antigen concentration, and sensitivity, specificity, and efficiency were calculated using the cutoff values. These data were calculated using Graph Pad Prism (version 5; GraphPad Software, Inc., Calif., USA).

Results

Characteristics of the subjects are summarized in table 1. Thirty-six of the subjects (6.3%) presented with bronchial asthma and 51 (79.7%) with atopic dermatitis. There were 64 subjects with HEA [HEA(+) vs. HEA(–); 38 vs. 26], 50 with REA [REA(+) vs. REA(–); 38 vs. 12], 49 with CMA [CMA(+) vs. CMA(–); 27 vs. 22], and 41 with WA [WA(+) vs. WA(–); 12 vs. 29]. The number of subjects who had received OFCs was as follows: 37 for HEA, 25 for REA, 15 for CMA, and 9 for WA. The mean age of the subjects was significantly higher in HEA(–), REA(–), CMA(–), and WA(–) groups than in the respective (+) groups. The mean antigen-specific IgE level was significantly higher in HEA(+), REA(+), and CMA(+) groups than in the respective (–) groups; no significant differences were observed in WA.

Six of the 64 subjects showed elevated spontaneous histamine releases greater than 40%. The other 58 subjects showed relatively low spontaneous histamine releases, with a mean percent spontaneous histamine release of 9.6 ± 7.4% (fig. 1). The mean percent anti-IgE induced histamine release was 73.2 ± 18.8%. There was no subject

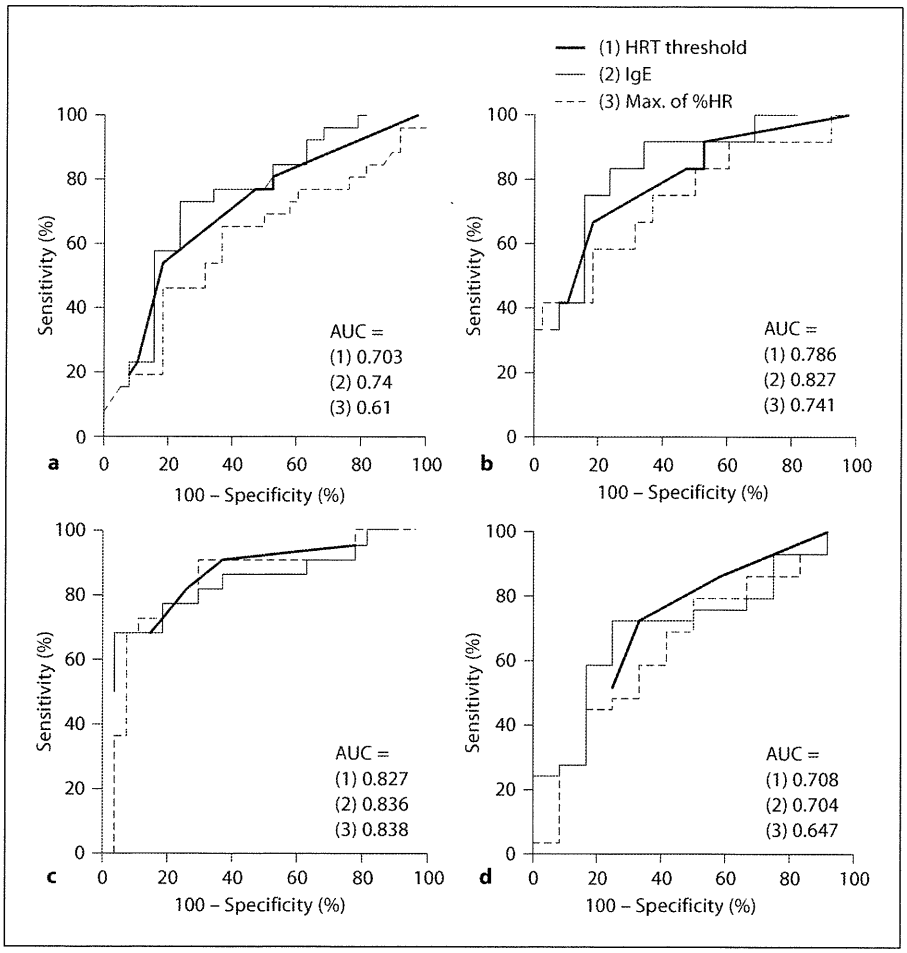

Fig. 2. ROC curves for HRT and antigen-specific IgE to egg white, cow's milk, and wheat. **a** HEA. **b** REA. **c** CMA. **d** WA.

whose anti-IgE induced histamine release was less than 10%.

The results of ROC analysis for the HRT threshold, Max. of %HR, and antigen-specific IgE levels are shown in figure 2. By ROC analysis, the area under the curve (AUC) of milk was found to be highest with 0.827 for the HRT threshold and 0.838 for Max. of %HR, respectively. The AUCs of the HRT threshold in HEA, REA, and WA were higher than that of Max. of %HR, though that of the HRT threshold in CMA was similar to that of Max. of %HR. The AUCs of antigen-specific IgE levels (0.74, 0.827, and 0.836) were higher than that of HRT threshold in the subjects with HEA, REA, and CMA, respectively.

The correlation between the HRT threshold and antigen-specific IgE levels is shown in figure 3a–d. The cutoff value for the HRT threshold was 6 ng/ml of egg white antigen in HEA ($p < 0.01$); the sensitivity, specificity, and efficiency were 81.6, 53.8, and 70.3%, respectively. Similarly, the cutoff value for the HRT threshold was 6 ng/ml of egg white antigen in REA, 40 ng/ml of milk antigen in CMA ($p < 0.01$), and 500 ng/ml of wheat antigen in WA ($p < 0.05$). The sensitivity was 81.6, 74.0, and 66.7%; specificity was 66.7, 81.8, and 72.4%, and efficiency was 78.0, 77.6, and 70.7%, respectively.

As shown in table 2a–d, in the subjects with HEA, although levels lower than 25.5 Ua/ml for egg white-specific IgE were detected in 20/38 HEA(+) subjects, 14 (70%) of these subjects had an HRT threshold of less than 6 ng/ml of egg white antigen. On the other hand, in the subjects with HEA(–), levels lower than the cutoff value for antigen-specific IgE were detected in 21 subjects; 8 (38%) of these subjects had an HRT threshold of less than 6 ng/ml of egg white antigen. Similarly, in REA(+) and CMA(+) subjects the ratio of subjects who were below the cutoff value for the HRT threshold was significantly high.

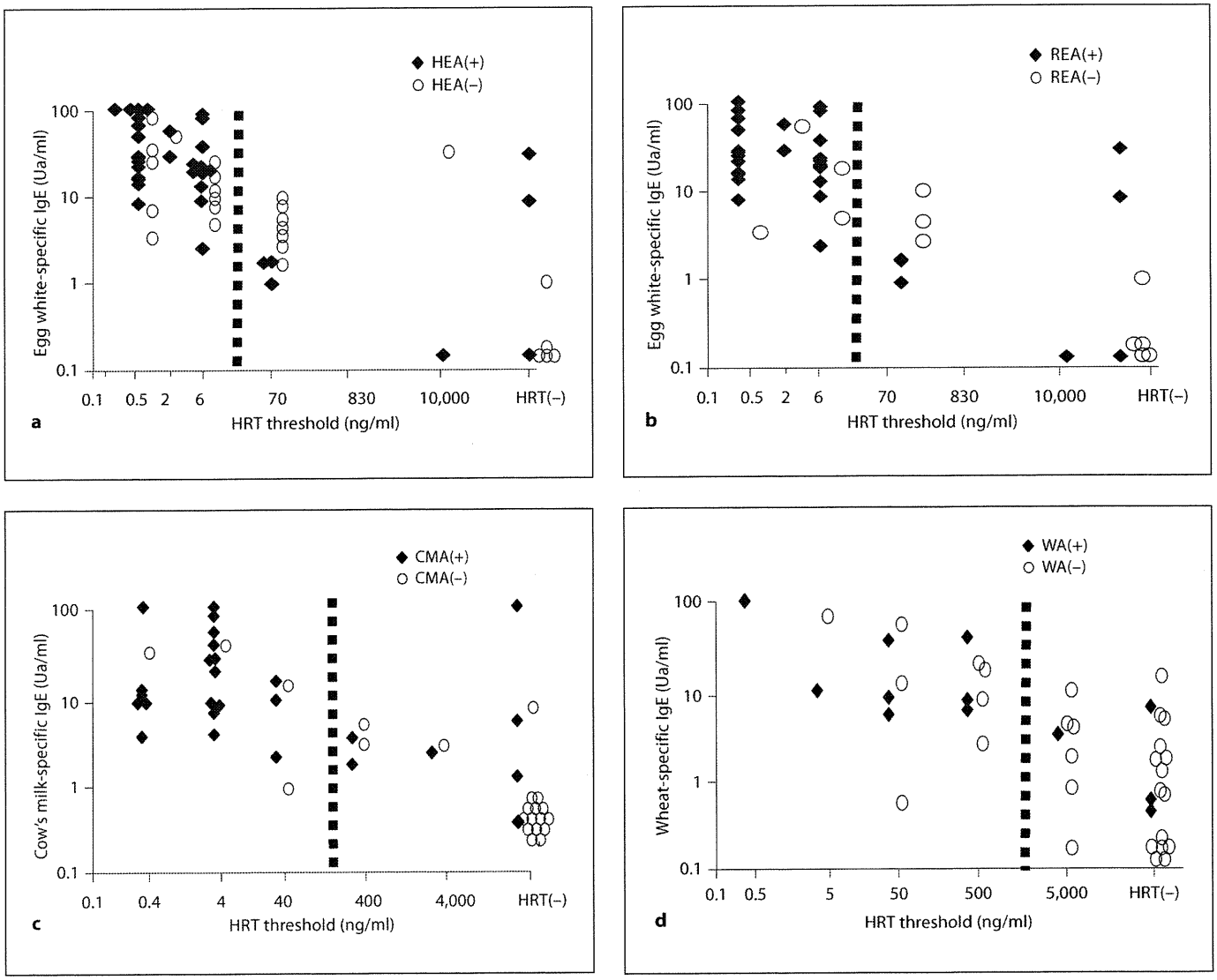

Fig. 3. Correlation between antigen-specific IgE and the HRT threshold. The diagnosis of FA was made using the results of an FCT and a convincing history of food allergies within the past 6 months [HEA(+), REA(+), CMA(+), and WA(+)]. HEA(–), REA(–), CMA(–), and WA(–) denote no symptoms after ingesting a causative food. The threshold of HRT was determined as the minimum concentration of antigen to induce a 10% net histamine release. HRT(–) means that the net percent histamine release by the maximum concentration of antigen is less than 10%. **a** HEA (n = 64). **b** REA (n = 50). **c** CMA (n = 49). **d** WA (n = 41).

Discussion

We examined the utility of HRT in the diagnosis of hen's egg allergy, CMA, and WA in children. The results of our study suggest that the HRT threshold for egg white, milk, and wheat antigen is related to the outcome of OFC after ingestion of a causative food and is useful in determining when OFC should be performed.

Six of 64 subjects (9.4%) showed high spontaneous histamine releases of 40% or more. Sampson et al. [32] reported that FA patients with atopic dermatitis who had eliminated the offending food allergen from their diet had a significantly lower rate of histamine release. It was suggested that mononuclear cells were being continuously stimulated by the offending food antigen, producing a histamine-releasing factor that results in basophil histamine release. As a result, basophils from these patients

were found to have high rates of spontaneous histamine release. In this study, 5 of 6 subjects with high spontaneous histamine release had concomitant atopic dermatitis. However, their atopic dermatitis was well controlled at the time of HRT measurement. All 6 subjects eliminated the offending food. Our cases did not agree with these reports and we plan to pursue this discrepancy further in future studies.

To evaluate the diagnostic utility of HRT in the diagnosis of HEA, REA, CMA, and WA, ROC analysis for each test was performed. In subjects with HEA, REA, and WA, the AUC of the HRT threshold was higher than that of Max. of %HR. Kleine-Tebbe et al. [24] also reported that birch pollen-allergic patients with oral allergy syndrome had lower thresholds of birch pollen-induced histamine release than did patients without oral allergy syndrome. From these results, it was thought that the HRT threshold was more useful than the Max. of %HR for the outcome prediction of OFCs.

Lau et al. [25] examined the utility of HRT in the diagnosis of HEA in patients 3.5 months to 12 years of age. They reported that HRT was not an effective predictor of the outcome of OFCs in childhood egg allergy because the sensitivity of HRT was 64.7%, the specificity was 40.0%, and the efficiency was 55.6%. However, the results of our study showed that the HRT threshold for egg white in subjects with HEA was 81.6% for sensitivity, 53.8% for specificity, and 70.3% for efficiency and that of REA was 81.6% for sensitivity, 66.7% for specificity, and 78.0% for efficiency. The efficiency of our data was higher than that in the report of Lau et al. [25]. It has been reported that differences in HRT sensitivity may be due to several factors, such as the quality of allergen extracts [28]. Lau et al. [25] employed a different basophil-stimulating antigen, i.e. ovalbumin, while we used raw egg white antigen. Furthermore, they decided that a percentage of histamine release of 30% or more was the HRT cutoff value. The results of our study suggest that the HRT threshold is more effective than the Max. of %HR for predicting the outcome of OFCs. These might be the reasons that the results of Lau et al. [25] differed from ours.

It has been reported that the antigen-specific IgE levels could predict the results of OFC after ingesting a causative food; cutoff values were set to a positive predictive value of 95% or greater [1, 2, 33]. In the patients with HEA and REA, the AUCs of antigen-specific IgE levels were higher than that of the HRT threshold. However, about 50% of subjects appeared to have symptoms caused by ingesting a causative antigen when antigen-specific IgE levels were below the cutoff value.

Table 2. Diagnosis of FA and its relationship to egg-, milk-, and wheat-specific IgE and the HRT threshold

a Heated egg

HEA(+)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤6	17 (94%)	14 (70%)	31 (82%)
	>6	1 (6%)	6 (30%)	7 (28%)
Total		18	20	38
HEA(–)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤6	4 (80%)	8 (38%)	12 (46%)
	>6	1 (10%)	13 (62%)	14 (54%)
Total		5	21	26

b Raw egg

REA(+)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤6	17 (94%)	14 (70%)	31 (82%)
	>6	1 (6%)	6 (30%)	7 (28%)
Total		18	20	38
REA(–)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤6	1 (100%)	3 (27%)	4 (33%)
	>6	0	8 (73%)	8 (67%)
Total		1	11	12

c Cow's milk

CMA(+)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤40	4 (80%)	16 (73%)	20 (74%)
	>40	1 (10%)	6 (27%)	7 (26%)
Total		5	22	27
CMA(–)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤40	0	4 (18%)	4 (18%)
	>40	0	18 (82%)	18 (82%)
Total		0	22	22

d Wheat

WA(+)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤500	3 (100%)	5 (44%)	8 (67%)
	>500	0	4 (56%)	4 (33%)
Total		3	9	12
WA(–)		>IgE	≤IgE	Total
HRT threshold, ng/ml	≤500	2 (100%)	6 (22%)	8 (28%)
	>500	0	21 (78%)	21 (72%)
Total		2	27	29

The cutoff value was decided based on previous reports. >IgE = Greater than the cutoff value of antigen-specific IgE levels; <IgE = lower than the cutoff value of antigen-specific IgE levels.

On the other hand, as shown in figure 3a–d, the HRT threshold differed in each case and was widely distributed, from low to high concentrations, in the subjects in which antigen-specific IgE levels were similar. When the HRT threshold was below the cutoff value, the ratio of subjects [HEA (+), REA (+), and CMA (+)] was significantly greater even if antigen-specific IgE levels were below the cutoff value.

In conclusion, the HRT thresholds for egg white, milk, and wheat antigen are related to the outcome of OFCs after ingesting a causative food and are useful in determining when OFCs should be performed, even if antigen-specific IgE levels are lower than the cutoff value. Therefore, measuring egg white-, milk-, and wheat-induced HRT could decrease the number of necessary OFCs and be useful in determining when OFCs should be performed.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the contents of this article.

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