TABLE I. Time course of chemokine-related genes with expressions upregulated more than 2-fold in the elicitation phase

Time (h)	No. of genes	Gene title	Gene symbol	Probe set ID	Change ratio
1	130				
3	263	Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1419209_at	2.2
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1441855_x_at	1
		Chemokine (C-X-C motif) ligand 2	*Cxcl2*	1449984_at	1.3
		Chemokine (C-C motif) ligand 7	*Ccl7*	1421228_at	1.3
		Chemokine (C-C motif) ligand 20	*Ccl20*	1422029_at	1.2
6	408	Chemokine (C-C motif) ligand 17	*Ccl17*	1419413_at	2.6
		Chemokine (C-C motif) ligand 20	*Ccl20*	1422029_at	2.6
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1419209_at	2.5
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1457644_s_at	1
		Chemokine (C-X-C motif) ligand 16	*Cxcl16*	1449195_s_at	1.2
		Chemokine (C-C motif) ligand 9	*Ccl9*	1417936_at	1
12	655	Chemokine (C-X-C motif) ligand 9	*Cxcl9*	1456907_at	4.5
		Chemokine (C-X-C motif) ligand 9	*Cxcl9*	1418652_at	2.9
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1419209_at	3.8
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1441855_x_at	2.2
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1457644_s_at	2.1
		Chemokine (C-X-C motif) ligand 16	*Cxcl16*	1449195_s_at	1.4
		Chemokine (C-C motif) ligand 9	*Ccl9*	1417936_at	1.2
		Chemokine (C-X-C motif) ligand 10	*Cxcl10*	1418930_at	2.8
		Chemokine (C-C motif) ligand 20	*Ccl20*	1422029_at	2.4
		Chemokine (C-X-C motif) ligand 2	*Cxcl2*	1449984_at	1.7
		Chemokine (C-C motif) ligand 2	*Ccl2*	1420380_at	1.2
24	902	Chemokine (C-X-C motif) ligand 9	*Cxcl9*	1418652_at	5.2
		Chemokine (C-X-C motif) ligand 9	*Cxcl9*	1456907_at	5.1
		Chemokine (C-X-C motif) ligand 10	*Cxcl10*	1418930_at	4
		Chemokine (C-C motif) ligand 17	*Ccl17*	1419413_at	2.5
		Chemokine (C-C motif) receptor 1	*Ccr1*	1419609_at	2.4
		Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1419209_at	2.2
		Chemokine (C-C motif) ligand 12	*Ccl12*	1419282_at	2.1
		Chemokine (C-C motif) ligand 2	*Ccl2*	1420380_at	2
		Chemokine (C-X-C motif) ligand 2	*Cxcl2*	1449984_at	1.8
		Chemokine (C-C motif) ligand 9	*Ccl9*	1448898_at	1.4
		Chemokine (C-C motif) ligand 9	*Ccl9*	1417936_at	1.2
		Chemokine (C-C motif) receptor 2	*Ccr2*	1421186_at	1.3
		Chemokine (C-C motif) ligand 7	*Ccl7*	1421228_at	1.3
		Chemokine (C-C motif) ligand 19	*Ccl19*	1449277_at	1.3
		Chemokine (C-X-C motif) ligand 16	*Cxcl16*	1449195_s_at	1.2
		Chemokine (C-C motif) ligand 8	*Ccl8*	1419684_at	1

genes at 24 hours. Because these results show the most prominent change in gene expression at 24 hours after elicitation, we chose this time and compared gene expression in the ears of vehicle-treated mice with that in ears of AE248-treated mice (Fig 3). Among the 902 genes, the signal intensity of 178 genes was significantly decreased in the AE248-treated group compared with that seen in the vehicle-treated group (cluster A), and signal intensity of 183 genes was significantly increased in the AE248-treated group (cluster B). The signal intensity of the other 541 genes was not significantly different between the groups. As for chemokine genes of significant signal intensity, cluster A includes genes for 3 chemokines: CXCL1, CXCL9, and CXCL16. Among them, expression of CXCL1 was most strongly suppressed by the AE248 treatment; the signal intensity decreased to 52% compared with the control intensity (Table II), and the average intensity of the other 2 chemokine genes, CXCL9 and CXCL16, decreased to 62% and 69%, respectively, compared with the control intensity. On the other hand, 4 chemokine genes, CCL19, CCL9, CCL8, and CCL12, were detected in cluster B. The average signal intensity of CCL19, CCL9, CCL8, and CCL12 in the AE248-treated group was 129%, 268%, 368%, and 404%, respectively, of that in the vehicle-treated group. Thus the treatment with AE248 did not decrease all of the chemokines upregulated 24 hours after the challenge. However, it nonetheless suppressed the inflammatory response in CHS, indicating that suppression of the expression of the chemokine genes in cluster A (ie, CXCL1, CXCL9, and CXCL16) has an important role in the CHS response. CXCL1 binds to CXCR2 and is one of the strong neutrophil-attracting chemokines. CXCL9 recruits T_H1 cells by binding cell-surface CXCR3 and contributes to the development of CHS.[5] CXCL16 is known to bind and activate the chemokine receptor CXCR6, which is expressed on T cells and natural killer T cells.[19] Given the critical role of CXCL1 and neutrophils in the development of CHS,[2,3] we further examined the roles of CXCL1 and neutrophils in CHS. We performed real-time RT-PCR analysis and confirmed that the expression of CXCL1 mRNA was

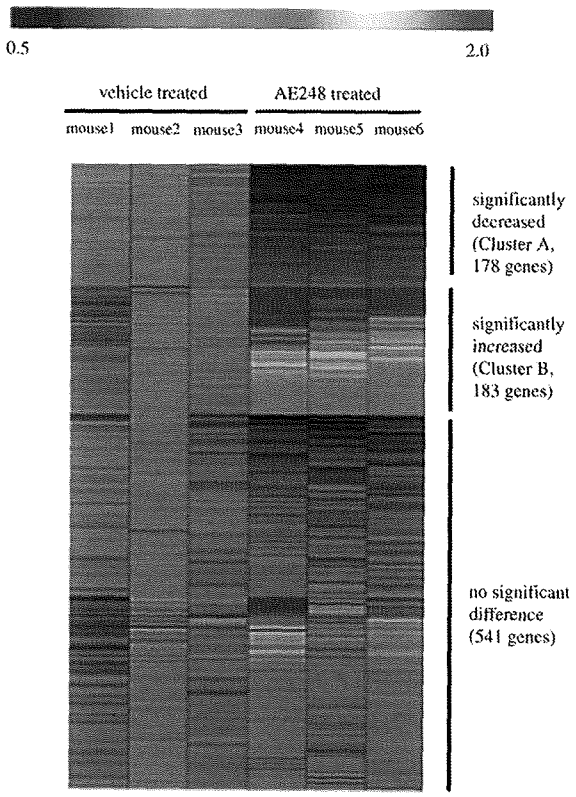

FIG 3. Microarray analysis for the effect of AE248 on gene expressions. Genes with a signal intensity that increased more than 2-fold in 24 hours at elicitation from baseline are selected (total of 902 genes). The signal intensity of each gene in the AE248-treated group (n = 3) was compared with the average signal intensity of each gene in the vehicle-treated group (n = 3), and the change ratio was indicated by means of color gradation.

significantly decreased in the AE248-treated group compared with that seen in the vehicle-treated group (data not shown). CXCL1 is produced mainly by keratinocytes in the elicitation phase of CHS. To confirm whether differences in gene expression result from differences in cell composition rather than from changes in gene expression in keratinocytes, we next examined the effect of AE248 on the mRNA expression of CXCL1 in keratinocytes from murine ear skin. We purified keratinocytes as shown in the Methods section of this article's Online Repository at www.jacionline.org. We then examined CXCL1 mRNA expression in purified keratinocytes and found that the AE248-treated group had significantly lower CXCL1 mRNA expression compared with that seen in the vehicle-treated group (Fig 4, *A*). We further examined the effect of AE248 on the production of CXCL1 by cultured keratinocytes *in vitro*. We found that keratinocytes activated with TNF-α produced a significant amount of CXCL1, and the administration of AE248 significantly suppressed the CXCL1 production (Fig 4, *B*). We next compared neutrophil (polymorphonuclear leukocytes [PMNs]) infiltration in the ears of the vehicle-treated and AE248-treated mice, as shown in the Methods section. We detected a number of PMNs in the dermis of the ear 24 hours after elicitation, whereas only a few cells were detected in the dermis of control animals. We also detected PMNs in the ears of the AE248-treated mice. However, the number of PMNs was significantly reduced in the AE248-treated mice compared with that seen in the vehicle-treated group (Fig 4, *C*). Because the average signal intensity of CXCL2 in microarray analysis, which is another important chemokine for neutrophil recruitment, was 44% of the vehicle-treated group in the AE248-treated group, we examined the CXCL2 mRNA expression in purified keratinocytes. We found that the AE248-treated group had significantly lower CXCL2 mRNA expression compared with that seen in the vehicle-treated group (see Fig E1 in this article's Online Repository at www.jacionline.org). These results combined together suggest a possibility that EP_3 exerts its anti-inflammatory effect by acting directly on keratinocytes and inhibiting neutrophil infiltration through downregulation of neutrophil-recruiting chemokines.

Involvement of endogenous PGE_2-EP_3 signaling in the development of CHS

We next examined the involvement of endogenous PG signaling in the development of CHS by applying indomethacin topically to the elicitation site. Because the effects of indomethacin were hard to detect in the usual CHS protocol (data not shown), we adopted the repetitive challenge model of CHS (repeated-challenge CHS) and examined the effect of indomethacin on the model as in the Methods section. Mice treated with indomethacin showed significantly increased ear swelling compared with the vehicle-treated mice (Fig 5, *A*), suggesting that endogenous PG produced locally in the challenged skin plays a suppressive role in inflammation of repeated-challenge CHS. We next examined the involvement of PGE_2-EP_3 signaling in this process by subjecting *Ptger3*$^{-/-}$ mice to repeated-challenge CHS. Similarly to the indomethacin-treated mice, *Ptger3*$^{-/-}$ mice exhibited significantly increased ear swelling compared with that of WT mice, and this enhancement continued 72 hours after elicitation (Fig 5, *B*). HE staining showed increased inflammatory cell infiltration in *Ptger3*$^{-/-}$ mice compared with that seen in WT mice (Fig 5, *C*). These results suggest that PGE_2-EP_3 signaling functions endogenously to negatively modulate the development of repeated-challenge CHS.

DISCUSSION

In the present study we have made the following findings. First, systemic administration of AE248, an EP_3 agonist, during the elicitation phase, can suppress inflammation in mice with CHS. Data of X-gal staining and immunohistochemical analysis demonstrated predominant EP_3 expression in keratinocytes, and topical application of AE248 to the ear skin resulted in suppression of CHS. Microarray analyses revealed that administration of AE248 modulates CHS-induced gene expression in the lesional skin either way; a decrease and an increase were demonstrated in clusters A and B, respectively. Among the chemokine genes regulated by AE248, CXCL1 was the most strongly suppressed. Consistently, AE248 suppressed CXCL1 production by TNF-α–activated keratinocytes *in vitro*. Finally, local treatment with indomethacin or the loss of EP_3 exacerbated inflammatory response to the repeated-challenge CHS. These results suggest that endogenous PGE_2 acts on EP_3 in keratinocytes *in situ* in the skin to modulate the extent of inflammation of CHS and that stimulation of PGE_2-EP_3 signaling with exogenously added agonist can control allergic inflammation in the skin.

The importance of keratinocytes in inflammatory skin diseases, such as contact dermatitis,[2,4] atopic dermatitis,[20] and psoriasis,[21,22]

TABLE II. Top 20 genes with signal intensities that were significantly decreased in AE248-treated mice compared with those in vehicle-treated mice

Gene title	Gene symbol	Probe set ID	Signal intensity		Percentage of AE248/vehicle
			Vehicle	AE248	
Solute carrier family 26, member 4	*Slc26a4*	1419725_at	2431	664.6	27.3
Matrix metallopeptidase 10	*Mmp10*	1420450_at	3371	1152	34.2
PG-endoperoxide Synthase 2	*Ptgs2*	1417262_at	3377	1345	39.8
SH2 domain containing 5	*Sh2d5*	1436100_at	2729	1118	41
Small proline-rich protein 2I	*Sprr2i*	1422963_at	13751	6136	44.6
Serine (or cysteine) peptidase inhibitor, clade E, member 1	*Serpine1*	1419149_at	801.6	359	44.8
Interferon-activated gene 202B	*Ifi202b*	1457666_s_at	15766	7122	45.1
Solute carrier family 29 (nucleoside transporters), member 2	*Slc29a2*	1447748_x_at	359.4	166.6	46.4
Fos-like antigen 1	*Fosl1*	1417487_at	3827	1785	46.6
IL-6	*Il6*	1450297_at	2010	955.7	47.6
Heparin-binding EGF-like growth factor	*Hbegf*	1418349_at	3090	1490	48.2
Cardiotrophin-like cytokine factor 1	*Clcf1*	1437270_a_at	1047	530.9	50.7
Chemokine (C-X-C motif) ligand 1	*Cxcl1*	1419209_at	4085	2131	52.2
Nucleolar complex–associated 2 homolog *(S cerevisiae)*	*Noc2l*	1424323_at	1638	867.1	52.9
RIKEN cDNA 2310002A05 gene /// hypothetical protein LOC630971	*2310002A05Rik /// LOC630971*	1456248_at	25273	13462	53.3
Similar to late cornified envelope protein	*LOC545548*	1456001_at	19223	10243	53.3
Sulfiredoxin 1 homolog *(S cerevisiae)*	*Srxn1*	1426875_s_at	4664	2525	54.1
Defensin β 3	*Defb3*	1421806_at	17218	9328	54.2
Serine (or cysteine) peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 9	*Serpina9*	1429285_at	1362	750.5	55.1
RIKEN cDNA 2310007F04 gene	*2310007F04Rik*	1429641_x_at	9952	5505	55.3

The average signal intensity of each gene was compared between vehicle-treated mice and AE248-treated mice.
EGF, Epidermal growth factor.

has now received much attention. In CHS keratinocytes produce various chemokines and regulate inflammatory cell infiltration. For example, it was previously shown that blockade of CCL27, which is produced from keratinocytes and attracts memory T cells to the skin, reduced T-cell infiltration and led to the suppression of CHS,[4] and inhibition of CCL8 produced from keratinocytes suppressed CHS.[22] In this study we found that EP_3 is expressed in keratinocytes and that administration of EP_3 agonist suppresses CXCL1 mRNA expression and its production in keratinocytes. CXCL1 is a strong attractant of neutrophils and is produced mainly by keratinocytes in the elicitation phase of CHS.[2,3] Recently, it has been reported that infiltration of neutrophils is required for the development of inflammation in CHS.[2] Depletion of neutrophils in hapten-challenged mice decreased the number of IFN-γ–producing T cells at elicitation sites, which resulted in the inhibition of CHS response, and injection of neutrophils into ears restored the CHS response.[2,3] Administration of anti-CXCL1 serum in the elicitation phase significantly inhibited the neutrophil infiltration in the challenged ear and suppressed CHS.[3] Furthermore, it has been reported that corticosteroids exert their anti-inflammatory effects in contact dermatitis mainly by targeting neutrophils and macrophages.[23] These findings are consistent with our results described above and suggest that one of the anti-inflammatory effects of AE248 is through EP_3-mediated downregulation of CXCL1

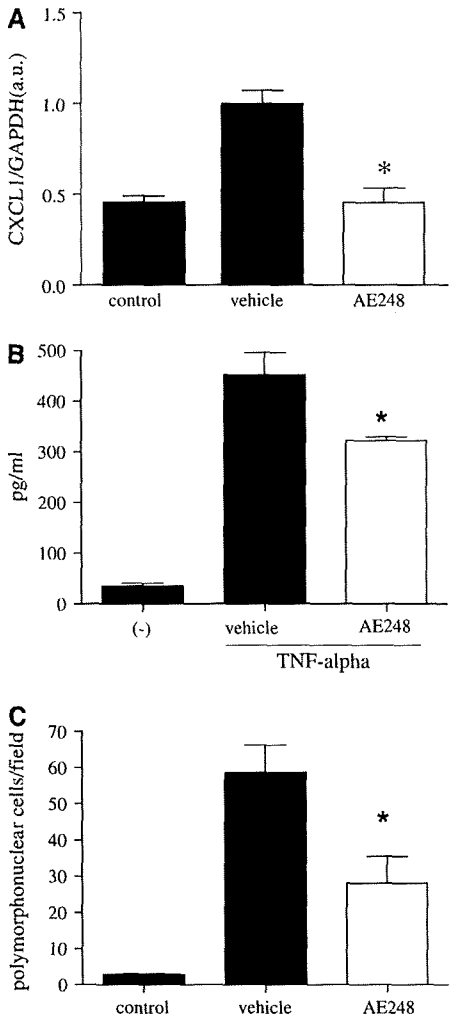

FIG 4. Effect of AE248 on CXCL1 production and neutrophil accumulation at the elicitation site. **A,** Real-time RT-PCR analysis on mRNA expression of CXCL1 in the keratinocytes in hapten-challenged ears of vehicle- or AE248-treated mice (n = 3 per group). **B,** ELISA analysis of CXCL1 production from cultured keratinocytes (n = 3 per group). Results are expressed as means ± SEMs and are representative of 3 independent experiments. **C,** Number of polymorphonuclear cells in the skin (n = 4 per group). *$P < .05$ versus the vehicle-treated group.

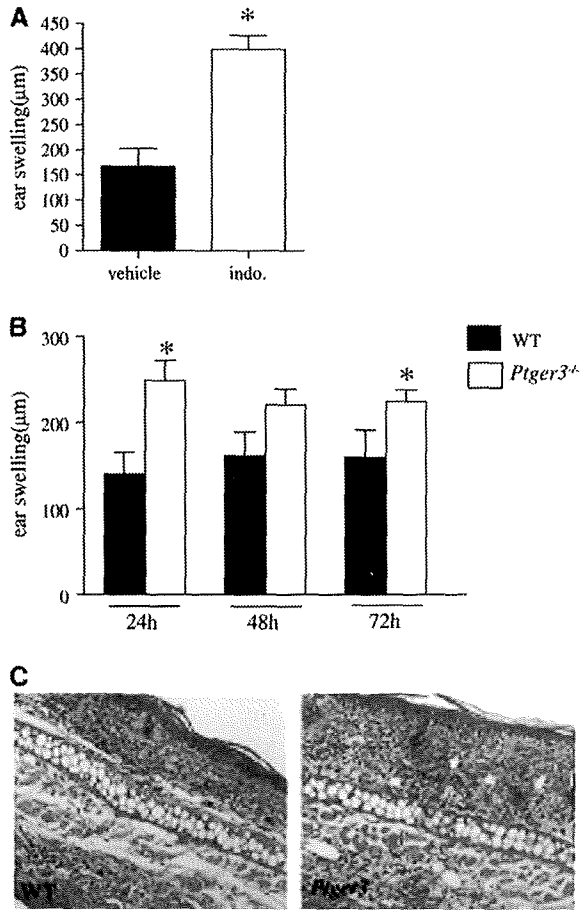

FIG 5. Enhanced inflammation of indomethacin *(indo)*-treated mice or *Ptger3*$^{-/-}$ mice in repeated-challenge CHS. **A,** Effect of topical administration of indomethacin on murine CHS (n = 4 per group). **B,** Increased ear swelling of *Ptger3*$^{-/-}$ mice in the CHS group (n = 4 per group). *$P < .05$ versus WT mice. *Data are representative of 3 independent experiments.* **C,** Representative HE staining of ear skin in WT and *Ptger3*$^{-/-}$ mice with repeated-challenge CHS at 24 hours after the fourth challenge.

production in keratinocytes and inhibition of neutrophil infiltration in the skin. On the other hand, our study showed that the EP_3 stimulation might have an effect on other chemokines opposite to that expected from the previous studies. For example, the expression of CCL8 was increased more than 3-fold in the AE248-treated group. In addition, we did not detect significant expression of CCL27 in our model. Given the finding that inhibition of CCL8 leads to suppression of inflammation, as described above, it is intriguing that EP_3 stimulation can reduce the inflammatory reaction in spite of such enhanced CCL8 gene expression.Unraveling this apparent discrepancy might help to reveal intricate relations among chemokines in inducing skin inflammation and define more correctly how EP_3 modulates their interaction. Taken together, chemokines from keratinocytes contribute much to the inflammation of CHS, and regulation of their production can be a useful strategy for the treatment of allergic dermatitis.

It should mentioned here that EP_3 is also expressed in mast cells in skin[24] and that mast cells can regulate the elicitation of CHS.[25,26] Indeed, expression of CXCL2 mRNA, which mast cells produce and was reported to promote neutrophil infiltration in elicitation sites, decreased to 44% of the control expression with EP_3 stimulation, although the difference was not statistically significant. However, we found that CXCL2 mRNA expression was significantly decreased in AE248-treated ears on purified keratinocytes. In addition, data of bone marrow chimera experiments indicate that most of the effect of AE248 was through stromal cells, suggesting that the anti-inflammatory effect of EP_3 derived from its action on keratinocytes.

EP_3 has 3 splice variants in the mouse, and 8 splice variants in human subjects,[27] among which at least 3 EP_3 variants are expressed in human keratinocytes.[10] They can couple Gs, Gi, and Gq and use cyclic AMP or Ca^{2+} as second messengers. EP_3 signaling can use ceramide as a second messenger in keratinocytes.[10] Our study does not clarify which signaling mechanism of EP_3 is responsible for its anti-inflammatory effect. The fact that a small dose of AE248 can induce anti-inflammatory effects is consistent with the Gi pathway because the cyclic AMP decrease mediated by Gi occurs at lower agonist

concentrations than other signaling. Indeed, the CB1 cannabinoid receptor that couples to Gi was recently reported to act on keratinocytes and suppress CHS inflammation.[28] However, this article reports that stimulation of CB1 inhibits CCL8 production from keratinocytes, which, as described, is opposite to our findings. These results indicate that multiple signaling pathways might function downstream of EP_3. It was shown recently that the Ca^{2+} signaling from EP_3 can inhibit nuclear factor κB activation in keratinocytes,[29] which is consistent with regulation of expression of CXCL1 mRNA by nuclear factor κB activity.[30] The precise molecular mechanisms underlying the anti-inflammatory effect of EP_3 remain to be elucidated.

Our results might also explain the anti-inflammatory effect of PGE_2 in skin inflammation and the adverse effect of NSAIDs in inflammatory skin diseases, such as psoriasis. Psoriasis is one of the inflammatory skin diseases, and constitutive activation of keratinocytes has been suggested as one of its causes.[21,22] Histologically, neutrophil infiltration in the epidermis is one of its characteristic features. Although PGE_2 is generally considered an inflammatory mediator by increasing vasodilation and edema formation, the anti-inflammatory effect of PGE_2 has been suggested in patients with psoriasis[31] or in animal models of neutrophil infiltration in skin.[32] On the other hand, administration of NSAIDs sometimes causes the exacerbation of psoriasis.[33,34] Thus far, the increase in levels of leukotrienes (LTs), such as LTB_4, a strong chemoattractant for neutrophils or T cells, has been suggested as one of its causative mechanisms.[33,34] Because arachidonic acid is used by both COX and lipoxygenase and NSAIDs block only COX activity, the use of NSAIDs might divert arachidonate metabolism to the lipoxygenase pathway, which leads to the increase of LTB_4. Such an argument was also made in aspirin-induced asthma. However, our study appears against such diversion mechanism. Alternatively, the PGE_2 pathway somehow modulates LT production. Our results suggest that one of the therapeutic effects of PGE_2 and an adverse effect of NSAIDs in skin inflammation is through modulation of PGE_2-EP_3 signaling.

In conclusion, stimulation of EP_3 signaling suppresses skin inflammation in CHS. Regulation of EP_3 signaling and keratinocyte function might be a novel approach for the treatment of skin inflammation, including allergy.

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Clinical implications: EP_3 in keratinocytes can be a target for the treatment of allergic skin inflammation.

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METHODS

mRNA extraction of purified keratinocytes from murine ear skin

The hairs on murine ear skin were removed with depilatory cream. After removing hairs, the ears were split into dorsal and ventral halves, and the cartilage was removed. Then the skin was floated on 0.25% trypsin/EDTA for 30 minutes at 37°C and separated into epidermis and dermis. Single epidermal cell suspension (EC suspension) was done by means of vigorous trituration of the epidermal sheet. Because EC suspensions are mixtures of keratinocytes, Langerhans cells, and γδ T cells, we purified keratinocytes by removing Langerhans cells and γδ T cells from the EC suspension with the autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) using magnetic microbeads coated with anti-mouse CD45 antibody. The purity of keratinocytes is greater than 99%. Then we extracted RNA from keratinocytes with the RNeasy mini kit (Qiagen).

Real time RT-PCR primer

The primer sequences of CXCL2 were 5′-GCC TAT CGC CAA TGA GC-3′ (forward) and 5′-TGG ACA ATT TTC TGA ACC AAG-3′ (reverse).

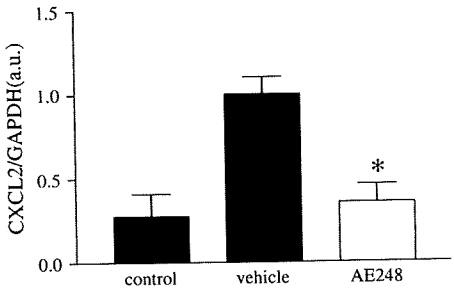

FIG E1. Real-time RT-PCR analysis on mRNA expression of CXCL2 in the purified keratinocytes in hapten-challenged ears of vehicle- or AE248-treated mice (n = 3 per group). Results are expressed as means ± SEMs. *$P < .05$ versus the vehicle-treated group. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

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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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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 up-regulated 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)

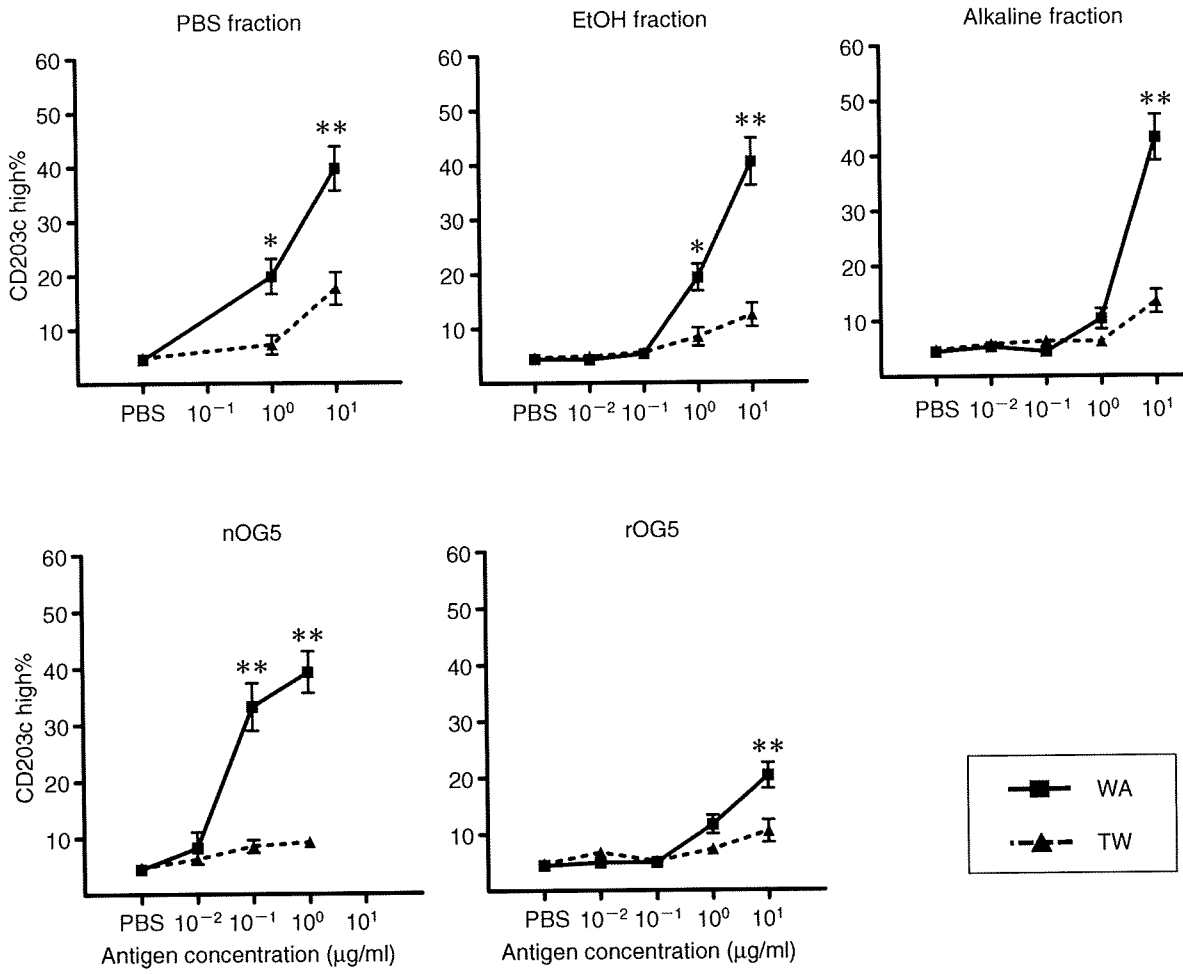

Fig. 1 Expression of CD203c on basophils induced by various wheat protein extracts and gliadins. WA, subjects with definite wheat allergy ($n = 5$); TW, subjects tolerant to wheat ($n = 5$). Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$, Dunn's multiple comparison test.

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/

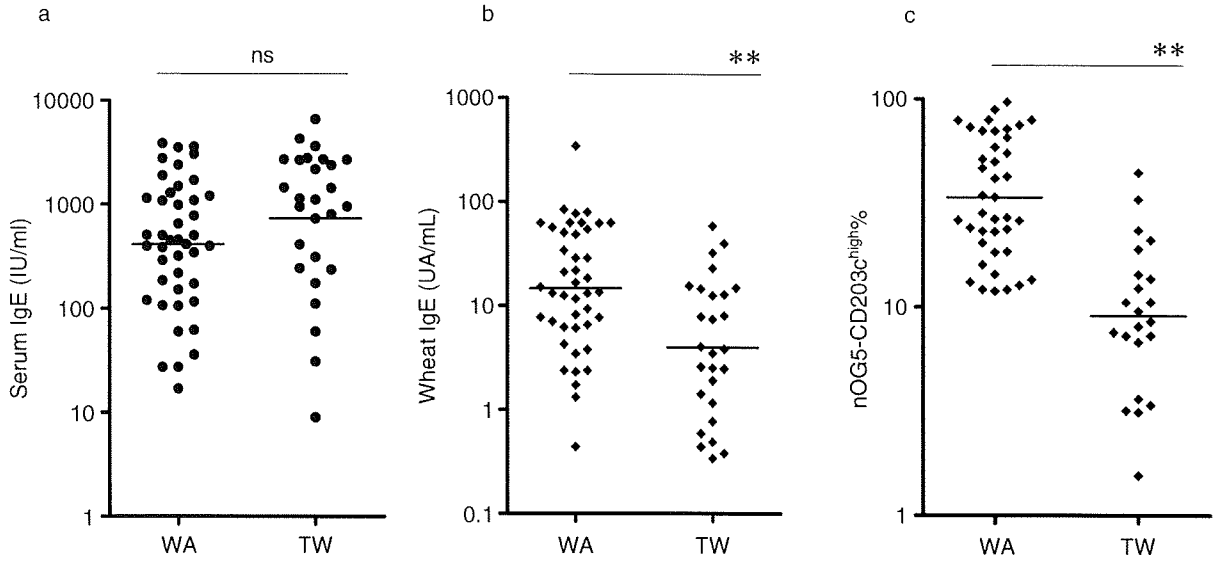

Fig. 2 Total IgE (**a**) , wheat-specific IgE (**b**) , and nOG5-induced CD203c expression on basophils (**c**) in subjects with wheat allergy (WA) and tolerance to wheat (TW) . Concentration of nOG5 used was 1 μg/ml. $**P < 0.01$, Mann-Whitney U test.

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

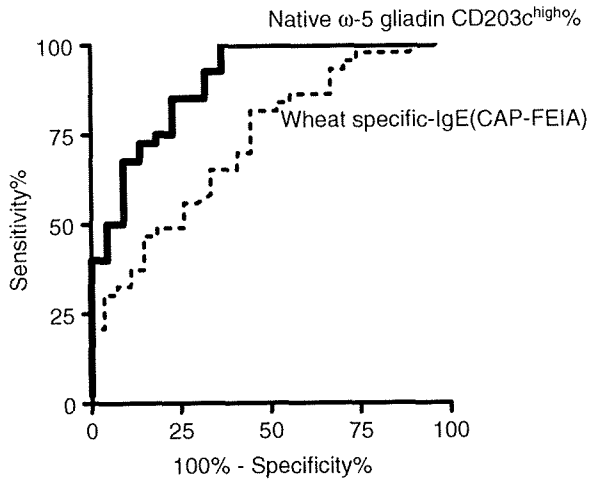

Fig. 3 ROC curves for native ω-5 gliadin-induced CD203c expression test and wheat specific IgE test. Horizontal bars indicate geometric means.

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.

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REVIEW ARTICLE

Food-Dependent Exercise-Induced Anaphylaxis—Importance of Omega-5 Gliadin and HMW-Glutenin as Causative Antigens for Wheat-Dependent Exercise-Induced Anaphylaxis—

Eishin Morita[1], Hiroaki Matsuo[2], Yuko Chinuki[1], Hitoshi Takahashi[1], Jörgen Dahlström[3] and Akira Tanaka[4]

ABSTRACT

Food-dependent exercise-induced anaphylaxis (FDEIA) is a special form of food allergy where a food-intake alone does not induce any symptoms. However, allergic symptoms are elicited when triggering factors such as exercise or aspirin-intake are added after ingestion of the causative food. The most frequent causative foodstuff in Japan is wheat. The triggering factors, both exercise and aspirin-intake, facilitate allergen absorption from the gastrointestinal tract, resulting in allergic symptoms in the patients with wheat-dependent exercise-induced anaphylaxis (WDEIA). Analysis using purified wheat proteins revealed that approximately 80% of the patients with WDEIA have IgE reacting to omega-5 gliadin and the remaining of the patients to high molecular weight glutenin (HMW-glutenin). Simultaneous measurement of specific IgE to omega-5 gliadin and HMW-glutenin was found to be highly useful in diagnosing WDEIA compared with the routine diagnostic system for wheat.

KEY WORDS

allergen, anaphylaxis, exercise, gliadin, wheat

INTRODUCTION

Food allergy is an IgE-mediated immediate-type reaction including a series of symptoms elicited after ingestion of food. The allergic response changes with age: immediate-type reaction and atopic dermatitis are common in infants, but food-dependent exercise-induced anaphylaxis (FDEIA) is frequent in adolescence and adults. FDEIA is a peculiar form of food allergy where a food-intake alone does not induce any symptoms. However allergic symptoms are elicited when triggering factors such as exercise are added after ingestion of the causative food. The symptoms appear usually on skin, mucous membrane, respiratory tract, and gastrointestinal tracts, and as anaphylaxis at a high incidence. Elicitation of the allergic symptoms is known to be dependent on amount of the food ingested.[1] Combination of food intake was necessary in some cases of FDEIA in provoking their symptoms.[2,3] In many case reports strenuous exercise, such as playing basketball and tennis or running, triggered anaphylaxis after ingesting specific food(s).[4-6] However, milder exercise often induced the symptoms. One patient was reported who required exercise before eating food to induce the symptoms.[4] Triggering factors in FDEIA includes the patient's general condition, drugs, alcohol, and atmospheric and seasonal conditions in addition to ex-

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Table 1 Triggering factors in FDEIA

Triggering factors	References
Foods (amounts, sort and combination)	1-3
Exercise (strength, type of exercise, duration and timing after meal)	4-6
General conditions (fatigue, sleep, common cold, stress, menstruation)	6-8
Drugs (aspirin, NSAIDs)	6, 9, 10
Atmospheric condition/seasonal	7
Alcohol	3

ercise as listed in Table 1. Fatigue, cold, and lack of sleep appeared to affect the development of the anaphylactic symptoms.[6] Some cases of FDEIA induced only in winter were described.[7] There have also been some cases in which a menstrual cycle affected the onset of FDEIA.[6,8] Aspirin intake has been well-documented to induce symptoms or to provoke more severe symptoms compared with symptoms without aspirin intake.[6,9,10] Aspirin also induces symptoms in combination with food ingestion even without exercise challenge.[10]

CAUSATIVE FOODS AND ALLERGENS RESPONSIBLE FOR FDEIA

Various kind of foods appear to be responsible for FDEIA, including shellfish,[6,9,11-13] wheat products,[2,6,7,9,10,13,14] vegetables,[3,13,15,16] fruits,[6,13,17] nuts,[13] egg,[13,18] mushrooms,[19] corn,[13,20] garlic,[13] pork/beef,[13] rice,[13] and cows milk.[8] A recent review indicates that the foodstuffs involved in FDEIA are characteristic in Japan.[21] In European countries, vegetables are the most common food allergens. Of these, tomatoes were found to be the most frequent. However, in Japan, wheat is the most frequent causative food and accounts for approximately 60% of the total cases.

Wheat gluten has been speculated as a causative protein for wheat-dependent exercise-induced anaphylaxis (WDEIA).[5] As a result of increasing numbers patients with WDEIA, wheat allergens have been intensively investigated.[22-24] Wheat protein is fractionated into salt-insoluble protein and salt-soluble non-gluten proteins. The latter consisted of water-soluble albumins and water-insoluble globulins. The former are called gluten and can be fractionated into two further categories of proteins in according to solubility in 70% ethanol. The ethanol-soluble proteins are named gliadins and the ethanol-insoluble proteins are glutenins. Omega-5 gliadin has been recently identified as a major allergen in WDEIA.[22,23] Analysis using a panel of purified gliadins and glutenins revealed that approximately 80% of the patients with WDEIA have IgE antibodies reacting to omega-5 gliadin and the remainder patients to high molecular weight glutenin (HMW-glutenin).[24] When IgE-binding epitopes were investigated using sera of WDEIA patients, four epitopes consisting seven amino acids, QQIPQQQ, QQFPQQQ, QQSPEQQ and QQSPQQQ, were found to be dominant epitopes in omega-5 gliadin, and three epitopes QQPGQ, QQPGQGQQ and QQSGQGQ were identified in HMW glutenin.[24,25] Mutational analysis of the QQIPQQQ and QQFPQQQ peptides indicated that five common amino acids at position 1 (Q), 4 (P), 5 (Q), 6 (Q) and 7 (Q) were critical for IgE-binding in omega-5 gliadin.[24]

USEFUL MEASUREMENT OF OMEGA-5 GLIADIN-SPECIFIC IGE IN THE PATIENTS WITH WDEIA

An enzymatic immunoassay system is a standardized procedure to detect allergen-specific IgE such as CAP-FEIA (ImmunoCAP, Phadia, Sweden) and now widely used for the diagnosis of food allergy. Detection of food-specific IgE concentrations has been reported to be useful in predicting clinical reactivity in egg, milk, peanut, and fish allergy.[26,27] Measurement of gluten-specific IgE as well as wheat-specific IgE is possible in the diagnosis of WDEIA using the ImmunoCAP, however more than 50% of patients with definite WDEIA are negative using these tests, indicating that the patients with WDEIA have very low levels of allergen-specific IgE in their sera (Table 2). In addition, a considerable number of the patients with atopic dermatitis (AD) have positive CAP scores for gluten as well as wheat, although the patients have not experienced episode of immediate-type allergic reactions after ingestion of wheat products (Table 2). Thus, the measurement of food-specific IgE using the ImmunoCAP is not a satisfactory tool for diagnosis of FDEIA.

Recently, recombinant food allergens, which are consistent in quality, have been produced and tried to apply for diagnosis in many food allergies.[28,29] Measurement of specific IgE to omega-5 gliadin and HMW-glutenin was found to be highly useful in diagnosing WDEIA when compared with the routine diagnostic ImmunoCAP for wheat and gluten.[30-32] As shown in Table 1, 82.0% (32/39) of the patients with WDEIA were found to have IgE reacting to recombinant omega-5 gliadin and 92.3% (36/39) of the patients were positive in combination of the recombinant omega-5 gliadin-specific IgE test and the recombinant HMW-glutenin-specific IgE test, whereas

Table 2 Positive rate of ω-5 gliadin-specific IgE and HMW glutenin-specific IgE measurement in the patients with WDEIA and atopic dermatitis (AD)

CAP-FEIA	WDEIA† (*n* = 39)	AD‡ (*n* = 16)	Healthy (*n* = 12)
	%	%	%
Wheat	41.0	87.5	0
Gluten	43.5	18.7	0
Wheat and/or gluten	51.3	87.5	0
ω-5 gliadin	82.0	0.0	0
HMW-glutenin	12.8	12.5	0
ω-5 gliadin and/or HMW-glutenin	92.3	12.5	0

†Positive rate was determined as specific IgE (kUa/L) ≥ 0.7.

‡AD patients had positive IgE antibodies to wheat ≥ 0.34 (kUa/L) but no episodes of of immediate-type allergic reactions.

Table 3 Age associated positive rates of recombinant ω-5 gliadin-specific IgE in the patients with WDEIA

Group	Age	*n* (f/m)	Positive rates of CAP-FEIA (%)		
			Wheat	Gluten	ω-5 gliadin
1	under 20	13 (2/11)	38.4	46.1	46.1
2	20-30	14 (7/7)	35.7	50.0	92.8
3	over 30	28 (11/17)	40.7	47.6	92.8
Total		55 (20/35)	40.0	47.3	81.8

gluten- and wheat-specific IgE tests positively recognized only 43.5% (17/39) and 41.0% (16/39) of these patients, respectively. In addition, specific IgE values to the recombinant allergen were much higher than that to gluten in most patients with WDEIA, indicating a higher capability of the recombinant allergen on the ImmunoCAP to detect allergen-specific IgE.[31] This is due to higher content of IgE-binding epitopes in the recombinant allergen molecules, since gluten contains only 5% of omega-5 gliadin and 9% of HMW-glutenin. When the patients with AD who had specific IgE antibodies to wheat but no obvious allergic reactions to wheat products were tested using the ImmunoCAP with recombinant proteins, positive rates were markedly decreased from 87.5% for gluten to 0% for omega-5 gliadin and 12.5% for HMW-glutenin, indicating higher specificity of the tests (Table 2).

The positive rate of the omega-5 gliadin-specific IgE was rather specific to the adult-patients with WDEIA as shown in Table 3. In the group of the aged patients with WDEIA, the test identified 92.8% of the patients positively, whereas the test only 46.1% of the children with WDEIA. On the other hand, most of the children negative to the omega-5 gliadin-specific IgE had specific IgE to the HMW-glutenin. These observations were further confirmed in the European patients with WDEIA using the omega-5 gliadin ImmunoCAP.[33]

Measurement of specific IgE to omega-5 gliadin was also useful in identifying the AD children who have allergic symptoms against wheat products.[34] The mean concentration of serum IgE specific to omega-5-gliadin in the children with wheat allergy was significantly higher than those in children with no wheat allergy. In addition, children reacting with severe symptoms upon wheat-challenge tests had significantly increased levels of serum omega-5 gliadin-specific IgE compared to children with moderate, mild or no symptoms.[34] In contrast, recent studies in German and American children show that omega-5 gliadin-specific IgE antibodies did not correlate with the outcomes of oral food challenges in wheat-sensitized children with suspected wheat allergy.[35] These results suggest that wheat allergens need to be further investigated in the children with wheat allergy.

MECHANISMS ELICITING ALLERGIC SYMPTOMS IN FDEIA

WDEIA is considered to be IgE-mediated hypersensitivity to wheat allergens, because IgE antibodies against the wheat allergens are detected in their sera using recombinant wheat proteins in the most patients with WDEIA.[31] The mechanisms by which exercise induces the reaction have been controversial. A case of WDEIA, in which pretreatment with sodium bicarbonate inhibits the reappearance of anaphylactic symptoms as well as elevation in plasma histamine

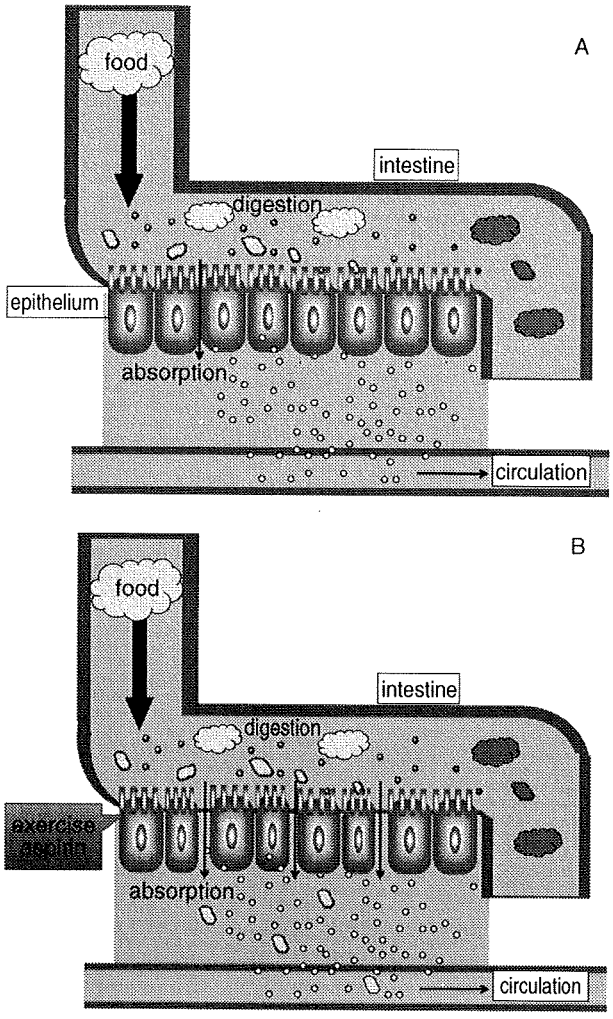

Fig. 1 Schematic figures of food allergen absorption in gastrointestinal tract in the patients with FDEIA. **A**: Ingested foods are digested in the intestine, and no immunoreactive allergens entered into circulation. **B**: Exercise and/or aspirin enhance absorption of undigested immunoreactive allergens into circulation.

levels following wheat and exercise provocation, was reported, suggesting a decrease in blood pH associated with exercise may play some role in mast cell activation.[36] On the other hand, when serum gliadin levels were evaluated using a gliadin-specific sandwich enzyme-linked immunosorbent assay, immunoreactive gliadins were found to appear in the sera of patients during the wheat challenge test combined with exercise, and these gliadins were concurrent with allergic symptoms.[37] The result indicates that exercise enhances absorption of allergens from the gastrointestinal tract as undigested forms (Fig. 1). The exercise-induced enhancement of allergen absorption was even seen in healthy subjects where the levels of serum gliadins were monitored before and after eating wheat,[37] indicating that the exercise-enhancement in antigen absorption occurs not only in allergic patients but also in healthy subjects.

A fact that has been well-established is that the symptoms are triggered by aspirin intake in the patients with FDEIA.[38,39] This was observed even in the patients with FDEIA who had no previous history of aspirin hypersensitivity or precipitation of symptoms by non-steroidal anti-inflammatory drugs.[38] Two possible mechanisms by which aspirin/non-steroidal anti-inflammatory drugs induce the symptoms have been considered: (1) aspirin enhances antigen uptake across the intestinal epithelium into circulating blood, and (2) aspirin itself activates mast cells in combination with IgE cross-linking of antigen. An enhancement of serum gliadin levels by aspirin was also demonstrated in combined challenge testing with wheat and aspirin, supporting the former hypothesis that aspirin as well as exercise, facilitates allergen absorption from the gastrointestinal tract (Fig. 1).[37] However, the latter possibility is supported by the fact that skin prick test was enhanced by pre-treatment with oral aspirin in five of eight patients with WDEIA.[39] The aspirin-induced enhancement of the allergic symptoms is possibly due to an inhibition of cyclooxygenase, as the symptoms can be diminished by co-ingestion of prostaglandin E1 (data in preparation).

Recently, a case of aspirin-associated WDEIA, which was elicited during low dose aspirin therapy for prevention of cerebral or myocardial infarction, was reported.[40] Taken into consideration with the data reported by Matsuo *et al.* that an increase in the serum gliadin level was observed in 5 of 7 subjects by administration of low dose aspirin (100 mg),[41] low-dose aspirin therapy may be also a risk factor for WDEIA.

An enhancement of aspirin is involved in induction of not only WDEIA but also various foodstuff-associated FDEIA because some cases of other foodstuff-associated FDEIA have been reported.[42]

MONITORING OF SERUM GLIADIN LEVELS IN CHALLENGE TESTS

Food-challenge test is now widely utilized to identify causative foods and to evaluate the outgrowing of food allergy. However, the test becomes rather complicated in case of FDEIA as ingestion of the causative food alone usually does not induce symptoms. A combination of the food intake and a second triggering factor, such as intense physical exercise, is necessary. Thus, for the definitive diagnosis of FDEIA, the challenge tests consist of three steps: food-challenge alone, exercise-challenge alone, a combination of food- and exercise-challenge.[37] With these procedures, standard food allergy as well as exercise-induced anaphylaxis can be eliminated. Recently, as described in the introduction and the mechanism section, aspirin is well-known triggering factor in inducing symptoms in combination with food-intake, even

in the patients with FDEIA who had no previous history of aspirin hypersensitivity. A combination challenge of food and aspirin-pre-treatment and/or triple combination challenge of aspirin-pretreatment, food and exercise can be performed as subsequent steps.[37]

If symptoms are induced by the combination challenge tests, the diagnosis of FDEIA is definite and the causative food is determined. However, in cases where the challenge test is negative, FDEIA cannot be excluded, as the symptoms are not always evoked by the challenge test. A false-negative challenge could be possible when either insufficient amount of food is ingested or an inappropriate triggering factor is challenged. According to the results that serum gliadin levels correlate well with allergic symptoms in the patients with WDEIA,[37] a monitoring of serum gliadin levels could be a useful marker in evaluating the challenge test. In the case of a negative challenge test, WDEIA could be excluded if a marked increase of serum gliadin levels was detected. Conversely, WDEIA could not be ruled out if the serum gliadin levels were under the detection limit. In the latter condition, additional challenge tests should be done to simulate the situation of episodes more strictly. In this case monitoring of the serum gliadin levels is also useful in assessing the strength of the challenge tests.

CONCLUSION

Wheat allergens were analyzed for WDEIA and it was found that omega-5 gliadin and HMW-glutenin are major allergens. Simultaneous detection of specific IgE to both recombinant omega-5 gliadin and recombinant HMW-glutenin is a reliable tool in identifying the patients with WDEIA. On the other hand, immunoreactive gliadins appeared in the sera of patients during the challenge tests with both wheat-exercise and wheat-aspirin challenges in parallel with allergic symptoms. These findings suggest that exercise and aspirin facilitate allergen absorption from the gastrointestinal tract in FDEIA.

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