

Semi-purification of the immunoglobulin E-sweat antigen acting on mast cells and basophils in atopic dermatitis

Tanaka A, Tanaka T, Suzuki H, Ishii K, Kameyoshi Y, Hide M. Semi-purification of the immunoglobulin E-sweat antigen acting on mast cells and basophils in atopic dermatitis.

Exp Dermatol 2006; 15: 283–290. © 2006 The Authors. Journal compilation © 2006 Blackwell Munksgaard

Background: Sweating aggravates the symptoms of atopic dermatitis (AD). We have recently reported positive skin reactions and histamine release from basophils in response to autologous sweat in patients with AD.

Objective: To characterize the biochemical and immunological properties of the substance in sweat that evokes histamine release and to study the usability of the basophil-histamine release test with the sweat antigen for AD.

Methods: Sweat collected from healthy volunteers was purified using chromatographies. Serum immunoglobulin (Ig)E of four patients with AD were purified using an affinity-chromatography column with anti-IgE antibodies. The amount of semi-purified sweat antigen (138 ng protein/ml) that induced a half-maximum reaction of basophils of a patient with AD was utilized for the basophil histamine release test. The involvement of specific IgE and high-affinity IgE receptor (FcεRI) in the reactions was examined using basophils of healthy volunteers, a human mast cell line (LAD2), and a rat basophilic leukemia cell line transfected with human α-subunit of FcεRI (RBL-48).

Results: The semi-purified sweat antigen induced histamine release from the basophils of 47 of 61 (74.6%) patients with AD and four of 46 (8.7%) healthy controls. Both basophils and mast cells sensitized with the patient-derived IgE showed degranulation upon stimulation with the sweat antigen. However, no reaction was observed when cells were sensitized with myeloma IgE or the antigen was treated with proteases.

Conclusion: The semi-purified standardized sweat antigen consists of a protein that induces degranulation of basophils and mast cells via antigen-specific IgE and FcεRI in patients with AD.

**A. Tanaka, T. Tanaka,
H. Suzuki, K. Ishii,
Y. Kameyoshi and M. Hide**

Department of Dermatology, Graduate School of
Biomedical Sciences, Hiroshima University,
Hiroshima, Japan

Key words: atopic dermatitis – IgE – purification
– sweat – type I allergy

Michihiro Hide
Department of Dermatology
Graduate School of Biomedical Sciences
Hiroshima University, 1-2-3, Kasumi
Minami-ku
Hiroshima 734-8551
Japan
Tel.: +81 82 257 5235
Fax: +81 82 257 5239
e-mail: mhide@hiroshima-u.ac.jp

Accepted for publication 30 November 2005

Introduction

Atopic dermatitis (AD) is a chronic pruritic skin disease based on an atopic background (1) and is often influenced by various triggering factors such as irritants, foods, aeroallergens, and emotional stress (2). Most patients with AD show high levels of total and specific serum immunoglobulin (Ig)E antibodies to a variety of allergens, especially those in food and inhalant allergens, such as chicken egg, dust mites, pollens, and molds (1). The IgE-dependent allergic reactions are therefore thought to play a major role in the pathogenesis of AD (3).

On the contrary, the characteristic distribution of skin lesions (cubital and popliteal fossa, neck, face, and wrist) and the aggravation after sweating, observed in many patients with AD, suggest the involvement of sweat in the pathogenesis of AD.

We recently reported that intradermal injection of autologous sweat elicited an immediate wheal-and-flare response in most patients with AD and that basophil-enriched peripheral leukocytes of patients with AD released histamine in response to sweat samples of both patients and healthy controls (4). We suggested the involvement of specific IgE in these reactions, showing that the

removal of IgE of the leukocytes with lactic acid treatment impaired the histamine release and the incubation with myeloma IgE before sensitization with serum of a patient with AD blocked the sweat-induced reaction (4). However, we could not deny the possibility that sweat-induced degranulation is not mediated by the IgE and the high-affinity IgE receptor (FcεRI) pathway because of the following reasons. First, serum rather than purified IgE was used for the sensitization of the leukocytes. Second, IgE also binds to the low-affinity IgE receptor (FcεRII) on lymphocytes (5) in the leukocytes' preparation for histamine release test employed in the previous study. Finally, IgE also binds to FcγRIII and causes degranulation of mouse mast cells (6).

Adachi and Aoki previously showed the binding of IgE in sera of patients with AD against concentrated sweat, but they observed no significant correlation between the binding activity and skin reaction to autologous sweat antigen (7). Moreover, Valenta and his colleagues have recently demonstrated the presence of auto-IgE in patients with severe AD (8, 9), but none of the identified antigens for such IgE has shown such a high reaction profile of the skin in patients as those induced by sweat samples.

Positive skin reaction to autologous sweat was selectively observed in the patients with AD rather than healthy controls (4). However, we cannot detect and compare precise degrees of this hypersensitivity by crude sweat materials, because the amount of histamine-releasing activity in sweat largely varied owing to the donors of sweat (4). To elucidate the role of sweat in the pathogenesis of AD, it is necessary to evaluate the prevalence and degree of sweat hypersensitivity using standardized sweat samples and to determine the precise mechanism of the reaction.

In this study, we semi-purified the histamine-releasing activity in sweat and standardized it for more precise studies about the sensitivity, specificity, and the mechanism of the histamine release induced by the substance in sweat. Moreover, we also purified IgE from sera of patients with AD and demonstrated that the antigen in sweat is a protein that provokes degranulations of human basophils, human mast cell line (LAD2), and a rat mast cell line transfected with human FcεRI (RBL-48) via specific IgE and FcεRI.

Methods

Subjects

The following subjects were included in the study: 61 patients (33 men and 28 women; 2–43 years of age, mean $\pm$ SD

24.0 $\pm$ 7.5) with AD; 13 patients (10 men and 3 women; 29–73 years of age, mean $\pm$ SD 53.4 $\pm$ 17) with psoriasis vulgaris; and 46 healthy volunteers (29 men and 17 women; 1–52 years of age, mean $\pm$ SD 28.4 $\pm$ 8.9) without allergic symptoms. The protocol of the study was approved by the ethics committee of Hiroshima University, Faculty of Medicine. All patients and volunteers and/or their guardians were informed of the aim and protocol and agreed to participate in the study.

Cell lines

Rat basophil leukemia cell line RBL-2H3 cells were cultured as previously described (10). The RBL-48 cell line has been derived from RBL-2H3 transfected with the human α -subunit of FcεRI (10), kindly supplied as a gift by Dr Joko Kochan (Hoffmann-La Roche, Nutley, NJ, USA) and cultured as previously described (10). Human mast cell line LAD2 cells had been established from a patient with mast cell sarcoma/leukemia (11) and kindly supplied as a gift by Dr Cem Akin (National Institutes of Health, Bethesda, MD, USA). The cells were cultured as previously described (11).

Histamine-release assay

Histamine-release assays with peripheral blood leukocytes, containing about 1% basophils, were performed as described previously (4). IgE antibodies on the surface of basophils were removed by treatment with 10 mM lactic acid (pH 3.9; 4). For passive resensitization, the acid-treated basophils were incubated in calcium-free buffer solution (12, 13) in the presence or absence of 5 μ g/ml of the purified patient IgE (described below) at 37°C for 1.5 h. After sensitization, the cells were washed three times with buffer and exposed to the challenge stimuli.

Purification of IgE from sera of patients with AD

IgE were affinity purified from the sera of four patients with AD, whose peripheral leukocytes released histamine by the semi-purified sweat antigen (described below). A monoclonal antibody against human IgE antibody, a kind gift from Wakunaga Pharmaceutical Co. Ltd. (Osaka, Japan), was coupled to N-hydroxy-succinimide (NHS)-activated Sepharose 4FF (Amersham Biosciences Co. Piscataway, NJ, USA) according to the manufacturer's instructions. The gel was equilibrated in phosphate-buffered saline (PBS) and packed onto a 1 $\times$ 5 cm column (econo-column, Bio-Rad, Hercules, CA, USA) (human IgE affinity column). The serum was diluted with two volumes of PBS and applied to the affinity column. After washing the column thoroughly with PBS, the IgE antibody was eluted with 0.1 M glycine-HCl buffer (pH 2.7). To neutralize the pH immediately, we collected 1 ml of each fraction in a tube containing 0.25 ml of phosphate buffer (2 M, pH 7.2). IgE-containing fractions were pooled, filtered (0.22 μ m filter) (Millex-GV, Millipore Corp., Bedford, MA, USA), and stored at 4°C until use. The purity of the IgE preparation was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14). The bindability of purified IgE to the sweat antigen was assessed by dot blotting, using anti-human IgE labeled with alkaline phosphatase (Diagnostic Products Co., Los Angeles, CA, USA).

Purification of antigen in sweat

As previous study revealed no difference in the histamine release activity between the sweat of patients with AD and

that of healthy controls (4), sweat was collected from nine healthy volunteers using the methods described by Hide et al. (4), with a slight modification. Each subject took a nylon bag (Tade-En Co, Hiroshima, Japan), which covered an area from the foot to the shoulder of the subject, and sat in a small dry sauna bath box (Matsushita Electric Industrial Co. Osaka, Japan) at 70°C for about 30 min. The collected sweats (50–300 ml/volunteer) were immediately filtered (0.22- μ m filter) (250-ml Filter System; Corning, NY, USA) and stored at –70°C until use.

The antigen in sweat was partially purified as common histamine release activity for peripheral blood basophils of three patients with AD. Con A-Sepharose (Amersham Biosciences Co.) was added to sweat and mixed gently at 4°C overnight. After centrifugation at 700 $\times$ g, the supernatant (Con A-unbound material) was collected and mixed with 20 mM Tris so as to adjust the pH to 8.0. The pH-adjusted Con A-unbound material was loaded onto an anion-exchange column (monoQ HR 10/10, Amersham Biosciences Co.), which was previously equilibrated with 20 mM Tris-HCl, pH 8.0, and eluted by a linear gradient of NaCl up to 1 M using FPLC systemTM (AKTA explorer 10S and SMART system, Amersham Biosciences Co.). Eluted fractions with histamine-releasing activity were collected and diluted by 0.1% trifluoroacetic acid and loaded onto a reversed phase chromatography column (SOURCE 15RPC ST 4.6/100, Amersham Biosciences Co.). The column-bound material was separated, being eluted by a linear gradient of acetonitrile. Each fraction was freeze-dried, dissolved in PBS, and subjected to histamine-release assays. The fractions with histamine-releasing activity were pooled and used as the semi-purified sweat antigen.

To estimate the molecular weight of the sweat antigen, we fractionated the semi-purified sweat antigen in 50 μ l PBS/fraction by a gel chromatography column (Superdex 75 PC 3.2/30, Amersham Biosciences Co.), using the SMART system at a flow rate of 50 μ l/min.

Enzyme treatment of the semi-purified sweat antigen

A total of 100 μ l of the semi-purified sweat antigen was incubated with several enzymes, proteinase K (2 mg/ml, Ambion, Austin, TX, USA), trypsin (0.1 mg/ml, Sigma-Aldrich Co. St Louis, MO, USA), DNase I (200 U/ml, Ambion), RNase A (51.5 U/ml, Ambion), N-glycanase (25 mU/ml, Takara, Kyoto, Japan), O-glycanase (Endo-N-acetylgalactosaminidase, 125 mU/ml; Calbiochem, San Diego, CA, USA), sialidase (neuraminidase, 1 U/ml, Roche Diagnostic, Tokyo, Japan), or O-sialoglycoprotein endopeptidase (96 μ g/ml, CEDARLANE, Ontario, Canada), at 37°C overnight. The enzyme-digested samples were incubated at 85°C for 15 min to destroy the enzymatic activities and stored at –20°C until use. Ten microliter of each sample was used for the histamine-release assay.

Visualization of specific IgE using Dot-blot analysis

The semi-purified sweat antigen diluted with PBS was absorbed to nylon membrane (Immobilon-P, Millipore, Billerica, MA, USA). After blocking with 10% skimmed milk at room temperature for 2 h, the membrane was incubated with 10% human serum diluted with 3% BSA/PBS at 4°C for one overnight. After washing with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), the membrane was incubated with horse radish peroxidase-labeled mouse monoclonal anti-human IgE antibody (Zymed laboratories, San Francisco, CA,

USA). IgE-binding materials were detected with chemiluminescence of ECL (Amersham Biosciences Co.).

β -Hexosaminidase-release assay

Degranulation of RBL cells or LAD2 cells was assessed by the release of β -hexosaminidase. The RBL-2H3 cells and the RBL-48 cells were sensitized with or without 50 ng/ml mouse anti-dinitrophenyl (DNP) IgE (Sigma-Aldrich Co.), 0.5 μ g/ml human myeloma IgE (Athens Research and Technology, Athens, GA, USA) or 0.5 μ g/ml of the purified patient IgE in the cell culture medium at 37°C for 4 days. The LAD2 cells were sensitized with 1 μ g/ml human myeloma IgE or 1 μ g/ml of the purified patient IgE in STEM PRO-34 SFM Complete Medium (Gibco, Grand Island, NY, USA) at 37°C for 6 days. The semi-purified sweat antigen, diluted with buffers for each of the cell-assays, was added to the culture of IgE-sensitized cells and incubated at 37°C for 30 min. A goat anti-human IgE antibody (Seikagaku Co. Tokyo, Japan; diluted 2500 and 1500 times for the RBL cells study and the LAD2 cells study, respectively) or 10 ng/ml DNP-human serum albumin (HSA) (Sigma-Aldrich Co.) were used as a positive control for the assays. Cells were pelleted through centrifugation, and the supernatants were analyzed for β -hexosaminidase release (15, 16).

Statistics

Data were summarized as mean $\pm$ SD. The statistical significance of differences was determined using ANOVA with post hoc examinations with the Scheffe's *F*-test for results shown in Fig. 3 or Fisher's PLSD test for results shown in Figs 4, 5, and 6. Any difference with a *P*-value of less than 0.05 was considered to be statistically significant. The tests were performed using Stat View version 5 for the Macintosh (Abacus concepts Inc, Berkeley, CA, USA).

Results

Semi-purification of the antigen in sweat

Purification of the antigen from pooled sweat was conducted 13 times and showed essentially identical results. In a representative purification shown here, a total volume of 400 ml sweat (74.2 mg protein content) was collected from three healthy volunteers. The histamine-release assay for the eluted fractions of 15 RPC column revealed two peaks of the activity (Fig. 1a). Fractions comprising the main peak of histamine-releasing activity for the basophils of all of three patients with AD (peak II, eluted with 30–40% acetonitrile) were pooled and used as semi-purified sweat antigen (1.18 mg protein content in 1 ml PBS) in the following studies. The effective concentration that induced a half of the maximum reaction of basophils of a patient with AD (EC₅₀) dropped to 138 ng/ml (the semi-purified sweat antigen) from 2.8 μ g/ml (crude sweat) in protein concentration. This concentration (EC₅₀) of the semi-purified sweat antigen was used as the standard concentration for the histamine-release assay.

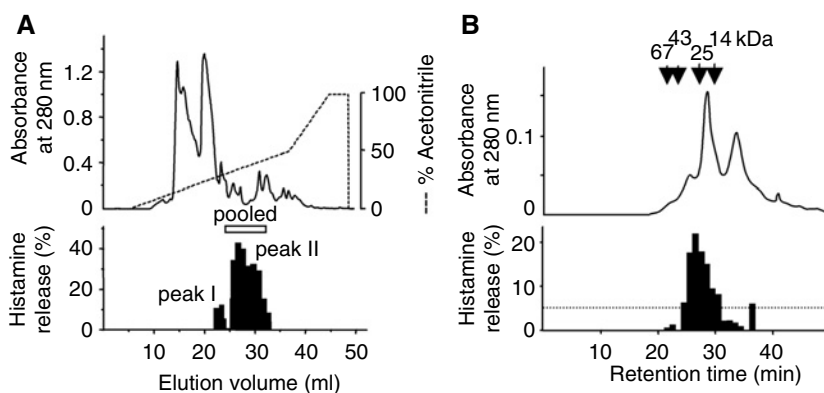


Figure 1. Chromatographic profiles of the histamine-releasing activity in sweat. (a) Elution profile on SOURCE 15RPC ST 4.6/100 column chromatography. The open bar represents fractions used as the semi-purified sweat antigen. (b) Superdex 75 PC 3.2/30 gel-filtration profile of the semi-purified sweat antigen. Histamine-releasing activity was tested using the leukocytes of a patient with atopic dermatitis (AD). The leukocytes of other two patients with AD showed the same histamine-release pattern.

For the estimation of molecular weight of sweat antigen, the semi-purified sweat antigen was fractionated using gel filtration (Fig. 1b). The histamine-releasing activity was detected in fractions corresponding to 15–30 kDa of globular proteins.

The histamine-releasing activity of the semi-purified sweat antigen was totally abolished by the treatment with proteinase K or trypsin, whereas the activity was reduced by none of the other enzymes (Fig. 2). Dot-blotted purified antigen, under non-denaturing condition using anti-human IgE labeled with horse radish peroxidase induced positive signals when incubated with sera of three patients but not with sera of two healthy controls (Fig. 3). However, we have not been able to show the binding of IgE to the

antigen with Western blotting under reducing or non-reducing condition (data not shown).

Histamine release from basophils by the semi-purified sweat antigen

Of 61 patients with AD, 47 (77.0%) showed more than 5% histamine release in response to the semi-purified sweat antigen, whereas only four of 46 (8.7%) healthy controls and none of 13 patients with psoriasis vulgaris did so (Fig. 4).

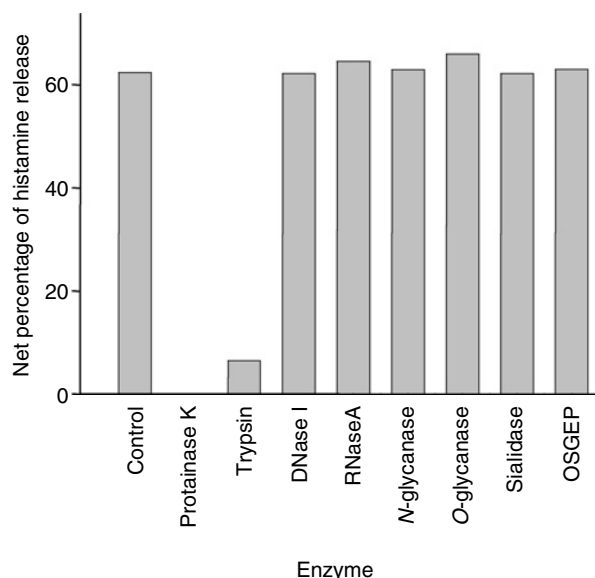


Figure 2. Histamine-releasing activity of the semi-purified sweat antigen after the treatment with several enzymes. The antigen was treated with each enzyme and heated to destroy the activity of the enzyme and examined for the histamine-releasing activity using basophils of a patient with atopic dermatitis. OSGEP: O-sialoglycoprotein endopeptidase.

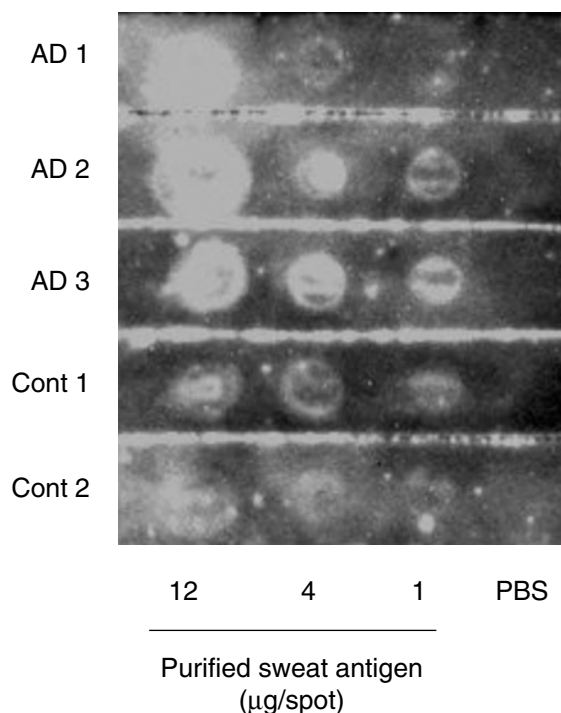


Figure 3. Visualization of the binding activity of immunoglobulin (Ig)E to the semi-purified sweat antigen using Dot-blot analysis. The semi-purified sweat antigen (0–12 µg) blotted on the membrane was incubated with 10% serum of three patients with atopic dermatitis (AD) (AD 1–3) and two healthy controls (cont 1 and cont 2). The binding activity of IgE to the antigen was visualized by anti-human IgE labeled with horse radish peroxidase and chemiluminescence (ECL).

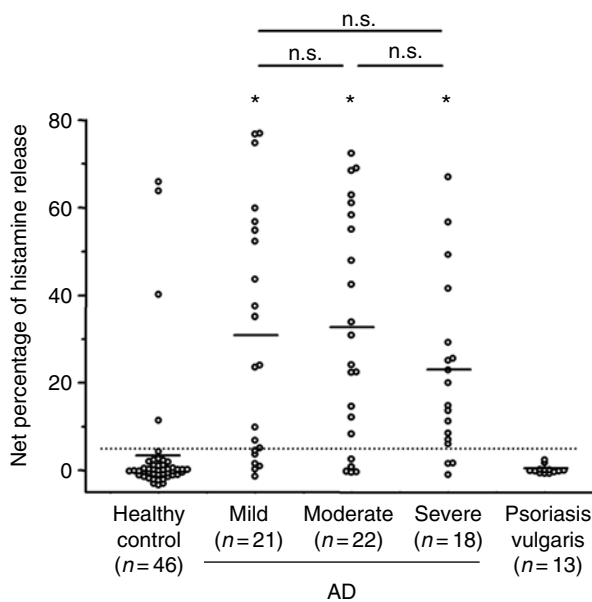


Figure 4. Histamine release from leukocytes in response to the semi-purified sweat antigen. Leukocytes of healthy controls, patients with atopic dermatitis (AD), and patients with psoriasis vulgaris were stimulated with the standard concentration (EC_{50} ; 138 ng/ml) of the semi-purified sweat antigen. Horizontal line, mean values; dotted line, cut-off level (5%) of histamine release from basophils. * $P < 0.001$ in comparison with healthy controls. n.s., not significant.

The leukocytes of all donors tested in this study released substantial amounts of histamine in response to goat anti-human IgE antibody. To examine the correlation between disease severity and the extent of histamine release, we divided patients into three groups based on the severity, according to the grading of AD by Rajka and Langeland (17). The means $\pm$ SD of net percentage histamine release of 21 patients with mild dermatitis, 22 patients with moderate dermatitis, and 18 patients with severe dermatitis were $30.9 \pm 28.2\%$, $32.3 \pm 26.0\%$, and $22.3 \pm 19.9\%$, respectively. The extents of histamine release were not statistically correlated with disease severity of AD.

Effect of IgE removal and passive sensitization of basophils with the patient IgE

To study whether the histamine release in response to the semi-purified sweat antigen was mediated by IgE, we utilized healthy volunteers' basophils that released histamine in response to anti-IgE antibodies but not to the semi-purified sweat antigen. When the cells were sensitized with IgE purified from sera of four patients with AD (AD 1–4), they released histamine in response to both the semi-purified sweat antigen

(1.5 μ g/ml) and anti-IgE antibodies (Fig. 5). These results demonstrated that the release of histamine from basophils is mediated by IgE of the patients with AD.

Degranulation of RBL cells and LAD2 cells by semi-purified sweat antigen via specific IgE

Both LAD2 cells sensitized with myeloma IgE and those sensitized with the patient IgE (AD 1) released β -hexosaminidase in response to anti-human IgE antibody (Fig. 6). However, only those sensitized with the patient IgE released β -hexosaminidase in response to the semi-purified sweat antigen in a concentration-dependent manner (0.46–4.6 μ g protein/ml), indicating that the release of β -hexosaminidase from LAD2 cells is an antigen-specific IgE-mediated reaction (Fig. 6).

Similarly, both RBL-48 cells sensitized with myeloma IgE and those sensitized with the patient IgE (AD 1) released β -hexosaminidase in response to anti-human IgE antibody (Fig. 7a). However, only those incubated with the patient IgE released β -hexosaminidase in response to the semi-purified sweat antigen (1.5 μ g/ml) (Fig. 7a). On the contrary, RBL-2H3 cells, the parental cell line that does not bind human IgE, did not release β -hexosaminidase, regardless of the same sensitization procedures (Fig. 7b). This result was

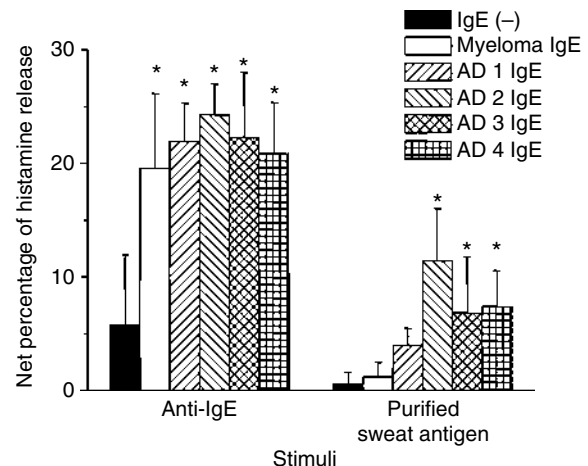


Figure 5. Effect of sensitization with immunoglobulin (Ig)E of patients with atopic dermatitis (AD) on histamine release from basophils. Basophils of a healthy volunteer were treated with lactic acid to remove endogenous IgE and passively sensitized with or without IgE (–), human myeloma IgE (myeloma IgE), or purified patients IgE (AD 1–4 IgE). The cells were stimulated with anti-IgE antibody or the semi-purified sweat antigen (1.5 μ g/ml), respectively. Basophils of other three healthy volunteers showed the same reaction pattern. Data are mean $\pm$ SD of four independent experiments done in duplicate. * $P < 0.05$ vs. IgE (–).

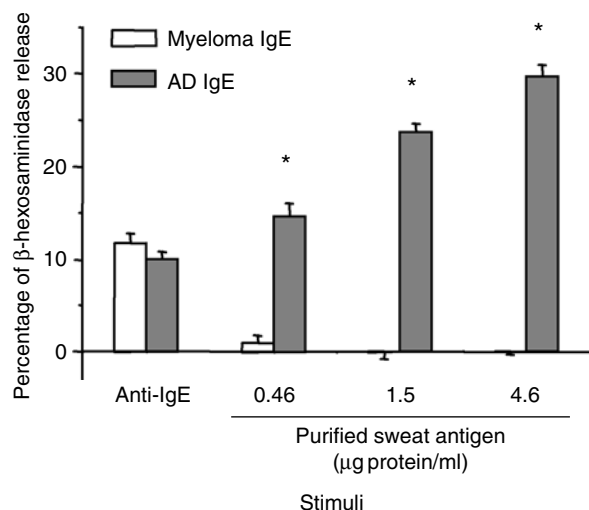


Figure 6. β -Hexosaminidase release from LAD2 cells. LAD2 cells were incubated with human myeloma immunoglobulin (IgE) (myeloma IgE) or with purified patient IgE (AD 1 IgE). The cells were stimulated with anti-IgE antibody or the purified sweat antigen, and the supernatant was analyzed for β -hexosaminidase release. Data are mean $\pm$ SD of four independent experiments done in duplicate. * $P < 0.05$ vs. myeloma IgE.

not due to a general dysfunction of these cells, because RBL-2H3 cells sensitized with mouse anti-DNP IgE released the equivalent amount of β -hexosaminidase in response to DNP-HSA, as did the RBL-48 cells (Fig. 7). These results demonstrated that human $\text{Fc}\epsilon\text{RI}\alpha$ as well as antigen-specific IgE is essential for the release of β -hexosaminidase stimulated by the semi-purified sweat antigen.

Discussion

In this study, we demonstrated a high specificity and a sensitivity of basophil-histamine release test to the semi-purified sweat antigen for patients with AD and a crucial involvement of specific-IgE and $\text{Fc}\epsilon\text{RI}$ in this reaction.

Both the nature of constituents and the amounts of antigen(s) in sweat may vary among individuals (4). To standardize the antigen in sweat, we partially purified the antigen with histamine-releasing activity using step-by-step column chromatography. The result of histamine release tests using this preparation suggested that the semi-purified sweat antigen contains major, if not all, the antigens in sweat, because both the incidence of positive reactions in the patient group and that in control group were similar to that obtained in skin tests with crude sweat samples in previous studies (4, 7). Moreover, essentially identical results in the purification

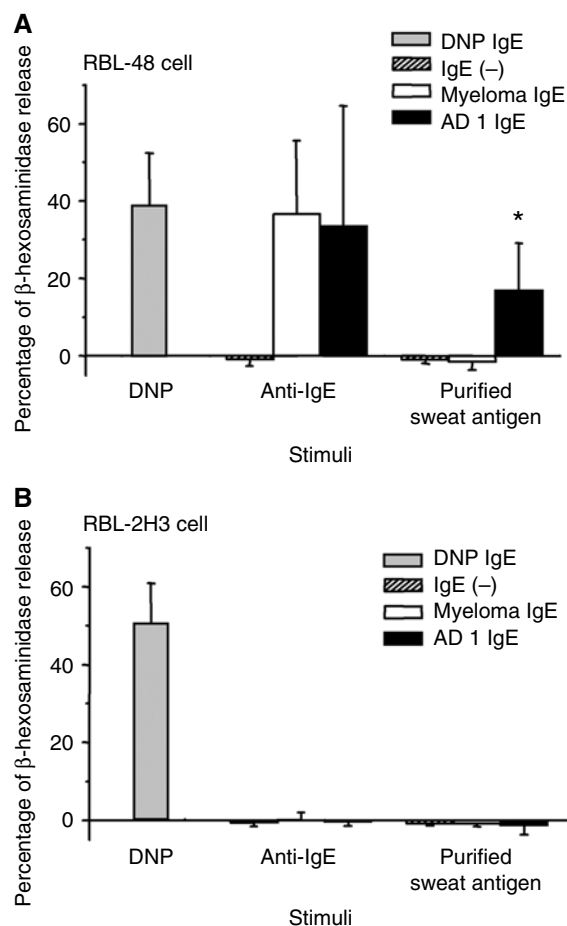


Figure 7. β -Hexosaminidase release from RBL cells. RBL-48 cells (a) and RBL-2H3 cells (b) were incubated with or without IgE (-), mouse anti-DNP IgE (DNP IgE), human myeloma IgE (myeloma IgE), or purified patient IgE (AD 1 IgE). The cells were stimulated with DNP-HSA, anti-IgE antibody, or the semi-purified sweat antigen (1.5 $\mu\text{g}/\text{ml}$). Data are mean $\pm$ SD of four independent experiments done in duplicate. * $P < 0.05$ vs. IgE (-).

from sweat of different donors suggest that the antigens in the semi-purified antigen are commonly contained in sweat.

The semi-purified sweat antigen induces degranulation of basophils and mast cells via the antigen-specific IgE in the sera of the patients with AD and $\text{Fc}\epsilon\text{RI}$ on the cell surface. The basophils of healthy donors, which did not react to the semi-purified sweat antigen, released histamine after the sensitization with all of the purified patient IgE. Moreover, LAD2 (human mast cell line) cells sensitized with the IgE showed β -hexosaminidase release in response to the semi-purified sweat antigen, whereas those sensitized with myeloma IgE did not. The necessity of IgE binding to $\text{Fc}\epsilon\text{RI}$ was demonstrated using RBL-48 cells, which express the α -subunit of human $\text{Fc}\epsilon\text{RI}$. We demonstrated that RBL-48

cells sensitized with the purified IgE released β -hexosaminidase in response to the semi-purified sweat antigen, but neither RBL-48 cells sensitized with myeloma IgE nor their parental RBL-2H3 reacted to the same antigen.

The hypersensitivity against the semi-purified sweat antigen was selectively observed in patients with AD, and, unexpectedly, there was no correlation between the extent of the reactions and disease severities. The basophil-histamine release test showed high sensitivity (77.0%) and specificity (91.3%) for patients with AD and healthy controls. Many patients with AD are associated with high serum IgE levels and positive reactions in the skin prick test to various environmental and/or food antigens (18, 19). House dust mite is one of the commonest antigens to which many patients with AD are sensitive (20). However, many healthy individuals also show positive reactions to this antigen (42%) (19). On the contrary, the incident of sensitization of patients with AD against the other allergens, such as foods, pollens, and molds, is not higher than 70% (19). Therefore, the sweat antigen is quite different from such common environmental allergens in both sensitivity and specificity for patients with AD. Although the precise mechanism of developing eczema by sweat allergy remains unclear, a number of studies have demonstrated that the activation of mast cells by antigens results in not only the release of histamine but also those of other mediators and cytokines, which may induce and/or exacerbate the inflammation (21), observed in AD. In addition, the tests for type I hypersensitivity to this sweat antigen, such as the histamine-release test and the radioallergosorbent test, should be helpful in making an accurate diagnosis of AD, which is usually made clinically, based on the features of the skin and history of the patient and his/her family, taking into account the level of serum IgE and/or reactions in skin prick test (22, 23). Recently, Fukunaga et al. (24) reported that the majority of patients with cholinergic urticaria are highly sensitive to autologous sweat antigen. Some of their patients without AD developed wheal-and-flare reactions in response to the autologous sweat injection, as well as patients with AD. Type I hypersensitivity against sweat antigen may be a common feature shared by AD and cholinergic urticaria, which are often complicated to each other.

The antigen in sweat has not been identified yet. The loss of histamine-releasing activity by the treatment with proteases, but not the other enzymes, suggests that the proteinaceous

structure is essential for the antigenicity. However, neither a single band in SDS-PAGE nor a sharp peak of the absorbance in chromatography, corresponding to the histamine-releasing activity, has been obtained, despite vigorous purification efforts using the combinations of the chromatography methods. The binding activity of IgE to the antigen was detected in dot-blot analysis (Fig. 3) but not in Western blotting (data not shown). These results suggest that the biochemical characteristics of the antigen are largely different from those of common proteins, aside from the possibility that our preparation contains an insufficient amount and/or purity of the antigen for detection. It is known that fungi and bacteria colonize the skin surface of many patients with AD (25). However, the incidence of the patients' basophil-histamine releases in response to the semi-purified sweat antigen seems too high to be explained by a simple contamination of *Malassezia furfur* antigens, or staphylococcal antigens (SEA and SEB), because the incidence of sensitization in patients against these antigens were reported as 40–65 (26) and 34% (27), respectively. Moreover, the histamine-release test did not reveal any difference between sweat samples collected from patients and those from healthy volunteers, in terms of the amounts of antigens (4). Therefore, it is likely that the antigen in the semi-purified sweat antigen is an endogenously produced substance rather than a contaminating exogenous substance, such as bacteria on the skin. Valenta et al. (9) and Natter et al. (8) have identified several intracellular proteins from a human epithelial cell line as autoantigens for IgE in sera of patients with severe skin manifestation of AD. However, those proteins produced by recombinant technology induced no or merely mild skin reactions in patients whose sera have been demonstrated to contain IgE against them using Western blotting (8). Moreover, patients with psoriasis vulgaris, another inflammatory skin disease with pathological epithelial cell proliferation, showed negative results in the histamine-release test to the semi-purified sweat antigen, and the extents of histamine release to the antigen were not correlated with the severity of eczema. Therefore, we speculate that the antigen in the semi-purified sweat antigen derives from sweat glands rather than epithelial cells.

In conclusion, a large population of patients with AD have type I allergy to the antigen in sweat. The *in vitro* basophil-histamine release test with the semi-purified sweat antigen reported in this study should be a potential tool for the

diagnosis of AD because of the high sensitivity and specificity for AD. Efforts are underway in our laboratory to carry out a definitive identification of the sweat antigen and to develop solid phase immunoassays, such as enzyme-linked immunosorbent assay, for IgE to the antigen. The measures against sweat allergy, such as the use of clothes with a function to effectively remove sweat antigen and the continuous injection of sweat antigen for desensitization, may contribute to the treatment of AD.

Acknowledgements

We thank our clinical staff for assistance in collecting the peripheral blood, Dr Faiz Kermani for his review of the manuscript, and Dr Cem Akin (National Institute of Health, MD) for supplying LAD2 cells, Dr Joko Kochan (Hoffmann-La Roche, NJ, USA) for supplying RBL 48 cells, and Wakunaga Pharmaceutical Co. for supplying a monoclonal antibody against human IgE antibody. This work was supported by Cooperative Link of Unique Science and Technology for Economy Revitalization from Hiroshima Prefectural Institute of Industrial Science and Technology, Japan.

References

- Beltrani V S. The clinical spectrum of atopic dermatitis. *J Allergy Clin Immunol* 1999; 104: S87–S98.
- Morren M A, Przybilla B, Bamelis M, Heykants B, Reynaers A, Degreef H. Atopic dermatitis: triggering factors. *J Am Acad Dermatol* 1994; 31: 467–473.
- Leung D Y. Pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* 1999; 104: S99–S108.
- Hide M, Tanaka T, Yamamura Y, Koro O, Yamamoto S. IgE-mediated hypersensitivity against human sweat antigen in patients with atopic dermatitis. *Acta Derm Venereol* 2002; 82: 335–340.
- Melewicz F M, Plummer J M, Spiegelberg H L. Comparison of the Fc receptors for IgE on human lymphocytes and monocytes. *J Immunol* 1982; 129: 563–569.
- Takizawa F, Adamczewski M, Kinet J P. Identification of the low affinity receptor for immunoglobulin E on mouse mast cells and macrophages as FcγRII and FcγRIII. *J Exp Med* 1992; 176: 469–475.
- Adachi K, Aoki T. IgE antibody to sweat in atopic dermatitis. *Acta Derm Venereol Suppl* 1989; 144: 83–87.
- Natter S, Seiberler S, Hufnagl P et al. Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients. *FASEB J* 1998; 12: 1559–1569.
- Valenta R, Natter S, Seiberler S et al. Molecular characterization of an autoallergen, Hom s I, identified by serum IgE from atopic dermatitis patients. *J Invest Dermatol* 1998; 111: 1178–1183.
- Gilfillan A M, Kado-Fong H, Wiggan G A, Hakimi J, Kent U, Kochan J P. Conservation of signal transduction mechanisms via the human FcεRIα after transfection into a rat mast cell line, RBL 2H3. *J Immunol* 1992; 149: 2445–2451.
- Kirshenbaum A S, Akin C, Wu Y et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res* 2003; 27: 677–682.
- Hide M, Francis D M, Grattan C E H, Hakimi J, Kochan J P, Greaves M W. Autoantibodies against the high affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 1993; 328: 1599–1604.
- Grattan C E H, Francis D M, Hide M, Greaves M W. Detection of circulating histamine releasing autoantibodies with functional properties of anti-IgE in chronic urticaria. *Clin Exp Allergy* 1991; 21: 695–704.
- Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227 (5259): 680–685.
- Stutchfield J, Cockcroft S. Correlation between secretion and phospholipase D activation in differentiated HL60 cells. *Biochem J* 1993; 293: 649–655.
- Suzuki Y, Yoshimaru T, Matsui T et al. FcεRI signaling of mast cells activates intracellular production of hydrogen peroxide: role in the regulation of calcium signals. *J Immunol* 2003; 171: 6119–6127.
- Rajka G, Langeland T. Grading of the severity of atopic dermatitis. *Acta Derm Venereol Suppl* 1989; 144: 13–14.
- Schmid-Grendelmeier P, Simon D, Simon H U, Akdis C A, Wuthrich B. Epidemiology, clinical features, and immunology of the 'intrinsic' (non-IgE-mediated) type of atopic dermatitis (constitutional dermatitis). *Allergy* 2001; 56: 841–849.
- Tanaka M, Aiba S, Matsumura N et al. IgE-mediated hypersensitivity and contact sensitivity to multiple environmental allergens in atopic dermatitis. *Arch Dermatol* 1994; 130: 1393–1401.
- Beltrani V S. The role of house dust mites and other aeroallergens in atopic dermatitis. *Clin Dermatol* 2003; 21: 177–182.
- Stassen M, Hultner L, Schmitt E. Classical and alternative pathways of mast cell activation. *Crit Rev Immunol* 2002; 22: 115–140.
- Williams H C, Burney P G, Hay R J et al. The U.K. Working Party's diagnostic criteria for atopic dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br J Dermatol* 1994; 131: 383–396.
- Rudikoff D, Lebwohl M. Atopic dermatitis. *Lancet* 1998; 351: 1715–1721.
- Fukunaga A, Bito T, Tsuru K et al. Responsiveness to autologous sweat and serum in cholinergic urticaria classifies its clinical subtypes. *J Allergy Clin Immunol* 2005; 116: 397–402.
- Ring J, Abeck D, Neuber K. Atopic eczema: role of microorganisms on the skin surface. *Allergy* 1992; 47: 265–269.
- Lindborg M, Magnusson C G, Zargari A et al. Selective cloning of allergens from the skin colonizing yeast *Malassezia furfur* by phage surface display technology. *J Invest Dermatol* 1999; 113: 156–161.
- Bunikowski R, Mielke M, Skarabis H et al. Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J Allergy Clin Immunol* 1999; 103: 119–124.