

Histamine release-neutralization assay for sera of patients with atopic dermatitis and/or cholinergic urticaria is useful to screen type I hypersensitivity against sweat antigens

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Received: 18 October 2011 / Revised: 20 March 2012 / Accepted: 31 March 2012
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Abstract We previously reported that about 80 % of patients with atopic dermatitis and 60 % with cholinergic urticaria revealed type I allergy against sweat, by means of skin test against autologous sweat and/or histamine-release test for peripheral blood basophils with semi-purified sweat antigen. In this study, we developed an assay for sera to neutralize histamine-releasing activity of semi-purified sweat antigen. The semi-purified sweat antigen was pre-incubated with serially diluted sera for 30 min at 37 °C and was subjected to histamine-release activity. Histamine release-neutralization (HRN) activities were calculated by measuring the amount of histamine release from basophils in the presence or absence of semi-purified sweat antigen. Of 62 subjects, 39 showed positive histamine release (≥ 5 %) from their basophils in response to semi-purified sweat antigen, and sera of 34 out of 39 subjects (87.2 %) were also positive in HRN activity (≥ 10 %). The specificity of the HRN assay was 0.522. Moreover, HRN activities in sera were largely correlated with degrees of histamine release from peripheral blood basophils of the same donors in response to sweat antigen. To identify the substance that neutralizes histamine-release activity, we removed IgE and IgG from the sera of HRN (+) subjects by column chromatography. The HRN activities in 30 out

of 42 sera were largely reduced by the removal of IgG. On the other hand, sera of four subjects lost HRN activity by the removal of IgE, suggesting that the majority of HRN (+) subjects have serum IgG against the sweat antigen as well as IgE bound to peripheral basophils. Thus, the HRN assay maybe useful for the screening of type I allergy against sweat antigen.

Keywords Sweat allergy · Atopic dermatitis · Cholinergic urticaria · Histamine-release assay · Histamine release-neutralization assay

Abbreviations

HRA	Histamine-release assay
HRN	Histamine release-neutralization
IgE	Immunoglobulin E
IgG	Immunoglobulin G
CAP-FEIA	Capsulated-fluorezymeimmunoassay
ELISA	Enzyme-linked immunosorbent assay
ANOVA	Analysis of variance
FACS	Fluorescence activated cell sorter
ROC	Receiver-operator characteristic

Introduction

Atopic dermatitis is a chronically relapsing skin disease that frequently occurs in patients with a personal or family history of atopy [2]. It often develops on the face in babies, neck, cubital and popliteal fossae in infants. Adult patients may develop dermatitis in any region of the body, but frequently in the areas of the neck, trunk and fossae of elbows and knees [8]. Topical applications of corticosteroids and/or calcineurin inhibitors are the mainstay to suppress and relieve inflammation. However, identification

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and removal of aggregating factors are equally or even more important in the treatment of atopic dermatitis [6].

Various factors such as foods, emotional stress and environmental elements are known to worsen the symptoms of atopic dermatitis. Skin symptoms are also aggravated in hot and humid seasons [3]. However, the involvement of these factors cannot fully account for the above-mentioned characteristic distribution of skin lesions observed in many patients with atopic dermatitis. Therefore, we have focused on the involvement of sweat that increases in hot and humid seasons and tend to be retained in fossae in the body. We and other authors have shown that clinical symptoms of school children with atopic dermatitis were significantly improved by taking shower at schools in the summer season [10, 14, 15].

Moreover, the intradermal injection of autologous sweat elicited an immediate wheal-and-flare response in about 80 % of patients with atopic dermatitis [9].

We previously semi-purified and standardized the antigen from sweat on the surface of human body by a combination of various steps of chromatography. This revealed that autologous basophils of 74.6 % of patients with atopic dermatitis and 65.7 % of patients with cholinergic urticaria released histamine in response to the semi-purified sweat antigen prepared from the body surface of healthy volunteers, whereas only 8.7 % of healthy controls did so [17, 18]. We also have demonstrated that the release of histamine by sweat antigen is dependent on IgE of the patients with atopic dermatitis [9].

These observations suggest that skin care to remove sweat and/or sweat antigen from the skin surface of patients with atopic dermatitis, especially those sensitized with sweat antigen, is important in the treatment of atopic dermatitis.

Therefore, the measurement of specific IgE against sweat antigen is an important issue not only in revealing the pathogenesis, but also for the treatment of atopic dermatitis. At present, skin tests and basophil histamine-release assay (HRA) are the only ways to prove the sensitivity of patients against sweat allergy [19, 20].

In this study, we developed a method for diagnosis of sweat allergy using sera, instead of basophils of the patients themselves, based on the characteristics of circulating antibodies of the patients to bind and neutralize the histamine-releasing activity of sweat antigen.

Materials and methods

Subjects

In total, 65 subjects (36 men and 29 women; 9–70 years of age, mean $\pm$ SD; 28.9 ± 13.8) were enrolled in this study. Atopic dermatitis was diagnosed by following the

guidelines for the management of atopic dermatitis by the Japanese Dermatological Association [16]. Cholinergic urticaria was confirmed by the development of numerous characteristic small wheals during or after exercise with sweating, according to the definition of cholinergic urticaria published by Magerl [13] and Zuberbier [21]. Thirty-seven subjects were patients with atopic dermatitis (21 men and 16 women, 9–48 years of age, mean $\pm$ SD 26.7 ± 9.8), 28 were patients with cholinergic urticaria (15 men and 13 women, 9–70 years of age, mean $\pm$ SD 31.7 ± 17.4) and 22 were apparently healthy volunteers without allergic symptoms (10 men and 12 women, 9–62 years of age, mean $\pm$ SD 30.8 ± 12.6). Blood samples of the patients were obtained together with those for routine clinical evaluations for their treatment. Blood samples of healthy volunteers were obtained for this study with informed consent. This study was approved by the ethics committee of Hiroshima University, Faculty of Medicine. All patients and healthy volunteers were informed of the aim and agreed to participate in the study.

Purification of sweat antigen

The semi-purified and standardized sweat antigen that induced histamine release from basophils of patients with atopic dermatitis was prepared as described previously [18] with slight modification. Briefly, sweat collected from healthy volunteers was mixed with 20 mM Tris so as to adjust the pH to 8.0 and loaded onto an anion exchange column (monoQ HR 10/10, Amersham Biosciences, Piscataway, NJ, USA), 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). 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). 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 HRA. Donors of sweat material were distinct from the subjects for HRA and histamine release-neutralization (HRN) assay.

Histamine-release assay (HRA)

HRA with basophils obtained from the peripheral blood were performed as described previously [9].

The amounts of histamine were measured by the HPLC system consisted of an LC-4A (Shimazu, Kyoto, Japan) with an RF-535 fluorescence monitor (Shimazu) operating at an emission wavelength of 450 nm and an excitation wavelength of 350 nm, as described by Koro et al. [11].

ELISA for measurement of IgE against sweat antigen

Wells of 96-well ELISA plate (Greiner bio-one, Germany) were incubated in the presence or absence of approximately 360 ng/ml semi-purified sweat antigen in phosphate buffered saline (PBS) at 4 °C overnight. Wells were then washed with PBS containing 0.05 % Tween-20 (Sigma, Tokyo, Japan). After blocking with PBS containing 5 % non-fat dry milk (Morinaga, Tokyo, Japan) for 1 h, wells were incubated for 2 h with 100 µl of sera diluted 100 times in PBS containing 0.3 % non-fat dry milk. After washing as above, wells were incubated with peroxidase-labeled goat anti-human IgE antibody (KPL, Gaithersburg, USA) for 1 h at room temperature. Following another series of washes, 100 µl of TMB peroxidase substrate (KPL, USA) was added to each well and incubated for 30 min at room temperature. The reaction was terminated by adding stop solution (KPL) and measured at 450 nm by an ELISA reader (Benchmark Plus, BIORAD, Hercules, USA). All assays were run in duplicate. To confirm if the plate for ELISA was sufficiently coated with sweat antigen, wells coated with or without semi-purified sweat antigen were incubated with basophils that release histamine in response to sweat antigen for 40 min at 37 °C. The cell suspension was collected by pipetting, centrifuged, followed by the measurement of histamine in supernatant and cell pellet as described for HRA.

Histamine release-neutralization assay

Sera were obtained from subjects who had been evaluated for sweat allergy by HRA using their own basophils and semi-purified sweat antigen.

Ten microliters of semi-purified sweat antigen at 358 ng/ml were mixed with or without 20 µl of 5-, 50- or 500-fold diluted sera and buffer to make up 50 µl reaction mixtures and incubated for 30 min at 37 °C. The reaction mixtures were subjective to HRA with 50 µl suspension of basophils that were obtained from other patients with atopic dermatitis. All basophils used for this assay released more than 30 % histamine in response to sweat antigen that had not been pretreated with sera of patients.

Histamine release-neutralization activity was defined as $\{(\text{histamine release induced by only semi-purified sweat antigen}) - (\text{histamine release induced by semi-purified sweat antigen pre-incubated with sera}) (\text{Net } \%) \} / (\text{histamine release induced by only semi-purified sweat antigen}) (\text{Net } \%) \times 100 (\%)$.

A cut-off value was set at 10 % of HRN activity based on the calculation of a receiver-operator characteristic curve (ROC curve) so as to obtain high sensitivity.

Immunoglobulin (Ig) E and IgG were removed from the sera by column chromatography using NHS-activated

Sephacrose 4 fast flow (Amersham Biosciences) combined with anti-IgE antibody (Bethyl Laboratories, Montgomery, TX, USA) to remove IgE, and that with Protein G (Amersham Biosciences) to remove IgG, respectively. Sera passing through a column of non-activated Sepharose 4 fast flow were used as control. The columns were prepared as described previously [20].

Depletion of IgE and IgG were confirmed by ELISA kits for IgE and IgG, respectively (Human IgE ELISA Quantitation Set and Human IgG ELISA Quantitation Set, Bethyl Laboratories, Montgomery, TX, USA).

Statistics

Data were summarized as mean $\pm$ SD. The statistical significance of differences was determined using one-way ANOVA with Bonferonni's post test for data as shown in Figs. 1, 2 and 4, Fisher's test in Fig. 3 and regression analysis in Fig. 5. Any difference with a *P* value of <0.05 was considered to be statistically significant. All tests were performed using GraphPad Prism version 5 for

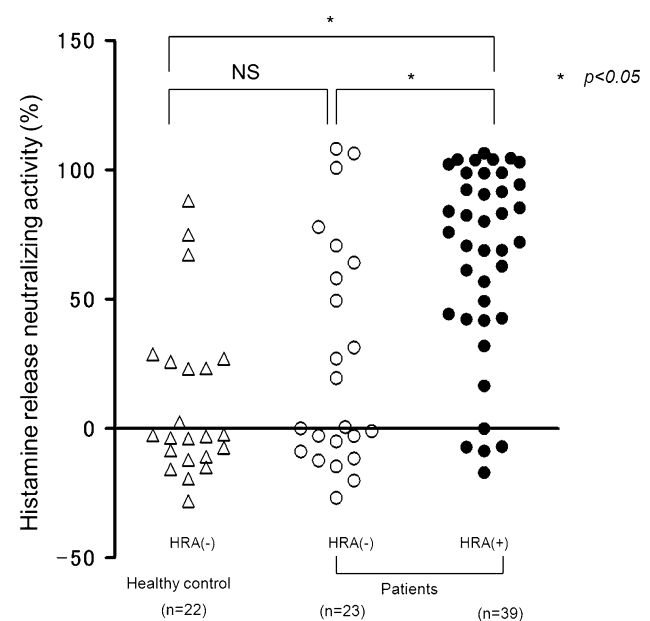


Fig. 1 Relationship between histamine release of basophils and histamine release-neutralizing activity in their sera. Of the 62 subjects, 39 showed histamine release from their basophils in response to semi-purified sweat antigen. Fifty times diluted sera of 34 out of these 39 subjects neutralized the histamine-release activity of sweat antigen. Sera of 12 out of 23 subjects, whose basophils did not release histamine, did not neutralize the histamine-release activity of sweat antigen. Negative values of HRN activities showed that the pre-incubation of semi-purified sweat antigen with sera enhanced, rather than inhibited the histamine release from basophils. The sensitivity and specificity of the whole HRN assay were calculated as 0.872 and 0.522, respectively. The statistical difference was determined by one-way ANOVA with Bonferonni's post test. **P* < 0.05 significantly different, NS not significantly

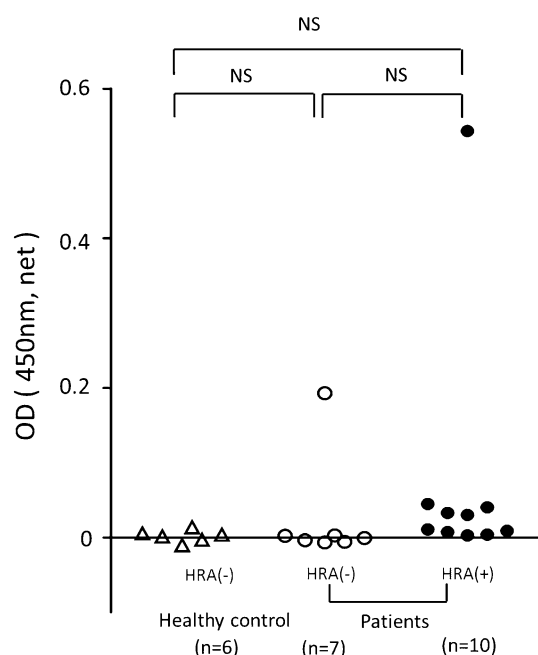


Fig. 2 ELISA for serum IgE against semi-purified sweat antigen sera of 23 subjects (ten HRA (+) patients, seven HRA (–) patients and six healthy controls) were tested for IgE against semi-purified sweat antigen by ELISA. No significant difference between healthy control and patients was observed regardless of the result of HRA. The statistical difference was determined by one-way ANOVA with Bonferonni's post test. *NS* the difference was not significant

Windows (GraphPad Software, Inc, San Diego, California, USA).

Results

Relationship between results of HRA with subjects' basophils and results of HRN assay with their sera

Among 65 subjects, two patients with atopic dermatitis and one patient with cholinergic urticaria released 2 % or less histamine in response to anti-IgE antibody (non-responders) and, therefore, were excluded. Of the 62 subjects, 39 showed more than 5 % histamine release from their basophils in response to semi-purified sweat antigen [HRA (+) subjects].

When pre-incubated with the semi-purified sweat antigen, sera of 34 out of 39 HRA (+) subjects showed more than 10 % HRN activities. On the other hand, sera of 14 out of 22 healthy volunteers and 12 out of 23 subjects, whose basophils did not release histamine more than 5 % in response to the semi-purified sweat antigen [HRA (–) subjects], did not neutralize the histamine-release activity of sweat antigen neither (Fig. 1).

Defining the cut-off value for positive HRN assay and HRA as 10 and 5 %, respectively, the sensitivity and

specificity of the whole HRN assay were calculated as 0.872 and 0.522, respectively.

ELISA for serum IgE against semi-purified sweat antigen

To detect the binding of serum IgE to sweat antigen, an ELISA plate was coated with or without semi-purified sweat antigen and incubated with sera of 23 subjects [ten HRA (+) patients, seven HRA (–) patients and six healthy controls] and a suspension of basophils, which released histamine in response to the semi-purified sweat antigen by HRA. Basophils in wells coated with sweat antigen released 44.4 % net histamine, indicating that wells were coated with sufficient amount of sweat antigen. Nevertheless, sera of only two patients, one with HRA (+) and another with HRA (–), showed an apparent binding of IgE to the surface of ELISA plate. No significant difference between healthy control and patients was observed regardless of the result of HRA (Fig. 2).

Inter-assay variations of HRN assay with basophils of different donors

To evaluate the reliability of the HRN assay, we performed assays using basophils of three volunteers with atopic dermatitis (Fig. 3). These donors were all different from subjects whose sera were tested. Histamine release (Net %) in response to semi-purified sweat antigen from these basophils was 74.2 % (donor 1), 33.6 % (donor 2) and 56.5 % (donor 3), respectively.

Sera collected from ten patients with atopic dermatitis and seven with cholinergic urticaria were studied by basophils of three donors, and sera from six apparently healthy volunteers were studied by basophils of donors 2 and 3. Sera of 9 out of 13 HRA (+) subjects showed more than 10 % HRN activities (sensitivity 0.692) in all three assays with basophils of different donors. Serum of one HRA (–) subject showed more than 10 % (49 %) HRN activity using basophils of donor 2, but <10 % HRN activity (–1.0 and 7.0 %) using basophils of the other donors (donors 1 and 3). Sera of the other nine HRA (–) subjects showed the same results in assays using basophils of two (donors 2 and 3) or three donors (donors 1–3).

Effect of serum dilution in HRN assay

To optimize the condition for the HRN assay, we examined the effect of serum dilutions on HRN activities. Serum HRN activities of nine HRA (+) subjects were detected in a dose-dependent manner. Although sera of all subjects showed more than 80 % HRN activity at $\times 1/5$ dilution, the

Fig. 3 Inter-assay variations of HRN assay with basophils of different donors. 50-fold diluted sera of 9 out of 13 HRA (+) subjects showed more than 10 % HRN activities (sensitivity 0.692) in all three assays with different three donors of basophils. Serum of one HRA (-) subject^a was assessed as HRN (+) using basophils of donor 2, and as HRN (-) using basophils of donors 1 and 3. Sera of the other nine HRA (-) subjects showed the same results of HRN using basophils of two or three donors. *NS* not significant from HRA (-) by Fisher's test

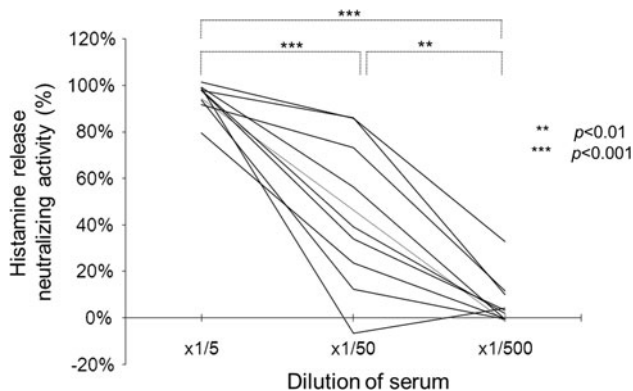
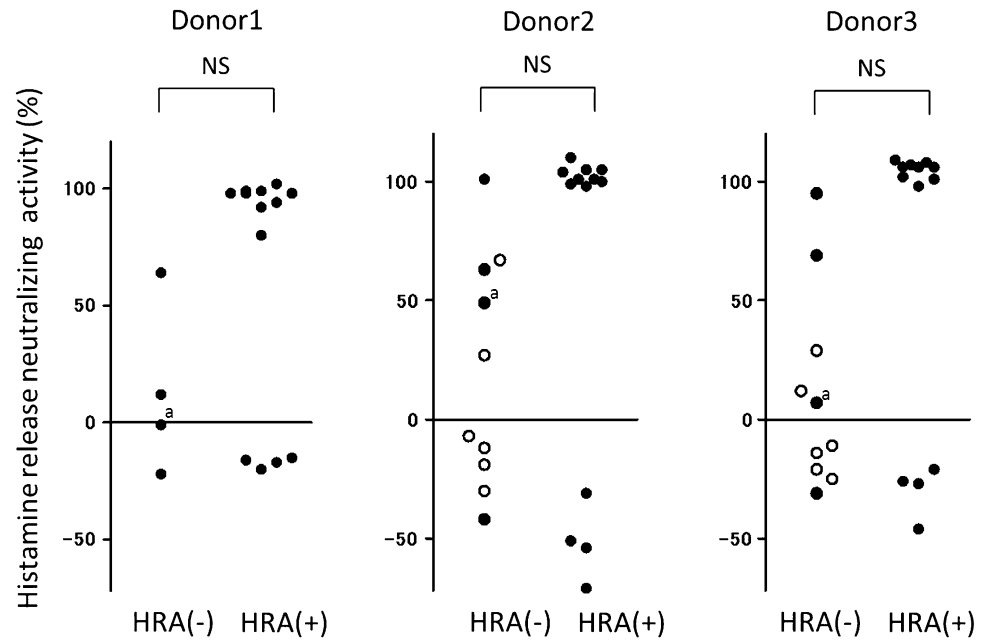


Fig. 4 Effect of serum dilution in HRN assay. Histamine release-neutralizing activities were detected in sera of nine histamine release positive subjects in a dose-dependent manner. Although sera of all subjects showed more than 80 % HRN activity at $\times 1/5$ dilution, the activity in one subject at $\times 1/50$ dilution, and in 8 subjects at $\times 1/500$ dilution became $<10\%$. The statistical difference was determined by one-way ANOVA with Bonferonni's post test. $**P < 0.01$, $***P < 0.001$ significantly different

activity in one subject at $\times 1/50$ dilution, and 8 subjects at $\times 1/500$ dilution became $<10\%$ (Fig. 4).

HRN activity in sera of patients with atopic dermatitis and those with cholinergic urticaria

Histamine release from basophils in response to semi-purified sweat antigen and HRN activity in sera of the donor of the basophils exhibited a significant correlation ($r = 0.3461$, $P < 0.0001$) (Fig. 5). Among 35 patients with atopic dermatitis, 26 out of 29 HRA (+) subjects and 26 out of 30 HRN (+) subjects were positive in HRN assay

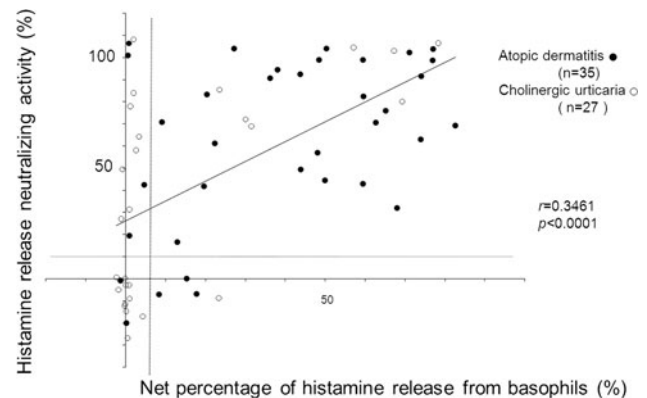


Fig. 5 Histamine release-neutralizing activity in sera of 35 patients with atopic dermatitis and those of 27 patients with cholinergic urticaria were positively correlated with histamine release of their basophils in response to semi-purified sweat antigen. The statistical analysis was determined by regression analysis; r and P values for all subjects were 0.346 and <0.001 . Those for subjects with atopic dermatitis were 0.227 and 0.0038, and those for subjects with cholinergic urticaria were 0.392 and 0.0003, respectively

(sensitivity 0.90, positive predictive value 0.87). Similarly, eight out of ten HRA (+) subjects, and eight out of six HRN (+) subjects with cholinergic urticaria were positive in HRN assay (sensitivity 0.80, positive predictive value 0.50). Nine out of 17 HRA (-) subjects were negative in HRN assay (specificity 0.53) (Table 1).

HRN activity in sera depleted of IgE or IgG

Sera of 42 HRN (+) subjects were treated with a column with anti-IgE and that with Protein G, to remove IgE and IgG, respectively. Sera of 30 subjects lost HRN activities

Table 1 Diagnostic evaluation of histamine release-neutralization test for atopic dermatitis and cholinergic urticaria

	Histamine release from basophils		Case
	Positive	Negative	
Atopic dermatitis ^a			
Inhibition of histamine release			
Positive	26	4	30
Negative	3	2	5
	29	6	35
Cholinergic urticaria ^b			
Inhibition of histamine release			
Positive	8	8	16
Negative	2	9	11
	10	17	27

^a Sensitivity 26/29 = 0.90, specificity 2/6 = 0.30, positive predictive value 26/30 = 0.87

^b Sensitivity 8/10 = 0.80, specificity 9/17 = 0.53, positive predictive value 8/16 = 0.50

by the removal of IgG. On the other hand, sera of four subjects lost HRN activities by the removal of IgE (Fig. 6a). Among four subjects whose sera lost HRN activity by IgE removal, sera of one subject completely lost HRN activity by IgG removal (subject No. 61), whereas HRN activity of another subject's sera was not affected by the same treatment (subject No. 53) (Fig. 6b). The HRN activity in sera of the other two subjects was partially lost by the treatment.

Discussion

In this study, we developed an assay for sera to neutralize histamine-releasing activity of semi-purified sweat antigen. Using this assay, we revealed that sera of patients with atopic dermatitis and cholinergic urticaria have HRN activities in correlation with histamine-release activity of their basophils.

We previously reported that about 80 % of patients with atopic dermatitis and 60 % of cholinergic urticaria revealed type I allergy against sweat, by means of skin test against autologous sweat and/or histamine-release test for peripheral blood basophils (cut-off value 10 %) with semi-purified sweat antigen [19, 20].

In this study, 31 out of 35 (88.6 %) subjects with atopic dermatitis and 10 out of 27 (37.0 %) subjects with cholinergic urticaria were positive for histamine release in response to semi-purified sweat antigen from basophils. Although the proportion of positive subjects in atopic dermatitis was similar to the previous report [20], the proportion of positive subjects in cholinergic urticaria recruited in this study was lower than that in the previous

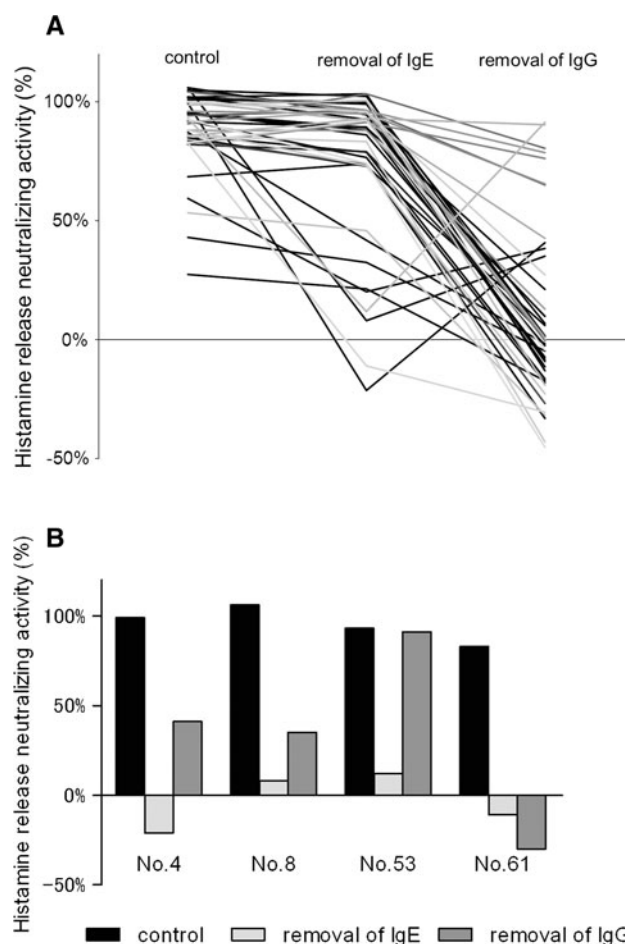


Fig. 6 Histamine release-neutralization activity in sera depleted of IgE or IgG. Among four subjects whose sera lost HRN activity by IgE removal, sera of one subject completely lost HRN activity by IgG removal

study. The reason for such a discrepancy is unclear. However, the incidence of patients with cholinergic urticaria complicated with atopic dermatitis was 51.4 % in the previous report [19], but 25.9 % (7 out of 27 subjects) in this report. Therefore, the low incidence of complication with atopic dermatitis in this study may account for the low incidence of sweat allergy in patients with cholinergic urticaria observed in this study [19].

When cut-off values for HRN activity were defined as 10 %, sera of 34 out of 39 HRA (+) subjects (87.2 %) were also positive in HRN activity. On the other hand, sera of 11 out of 23 HRA (-) subjects (47.8 %) were positive in HRN activity. The sensitivity and specificity were calculated as 0.872 and 0.522, respectively. The specificity of the HRN assay appeared to be low. However, the specificity of CAP-PEIA to measure IgE to common food antigens, such as cow's milk, hen's egg, wheat and soy for oral food challenge were also in a similar range 38–53 % [4]. In clinical practice, serum tests, such as CAP-PEIA and ELISA, are widely used for the evaluation of type I hypersensitivity

because of their convenience and reproducibility. However, they may not be as sensitive as skin tests or in vivo challenge tests [16]. In vivo tests, such as skin test and antigen challenge test, are more reliable, but may be painful, and could evoke an anaphylactic response when a patient is extremely sensitive to a particular antigen [5]. Moreover, intradermal injections of antigen may sensitize subjects who are not sensitized to the antigen. Both mast cells and basophils, which are sensitized with the same repertoire of IgE in individual subjects, release various substances, including histamine, arachidonic acid metabolites and cytokines in response to the antigen. They also express several molecules on their surface, such as CD203c and CD69. Therefore, the in vitro histamine-release test and cell surface analysis by fluorescence activated cell sorter (FACS) for basophils of peripheral blood is sensitive, safe and gives reliable information regarding antigens that cause type I hypersensitivity. Griese et al. [7] reported that the histamine-release test showed higher sensitivity and specificity than skin test or RAST analysis based on the comparison with bronchoprovocation of extrinsic asthmatic children. Moreover, the basophil activation test based on CD203c upregulation has been validated as a reliable tool for the diagnosis of IgE-mediated allergies [18]. Thus, detection of basophils' reaction against antigens may be a useful indicator of type I hypersensitivity. In fact, HRA using basophils of patients is fairly sensitive and reliable to evaluate the hypersensitivity against sweat antigen as well.

However, tests using patients' basophils essentially need to be performed on the day or next day of blood sampling. Moreover, it is not easy to analyze multiple samples simultaneously. Furthermore, basophils of certain populations of subjects may not release histamine even by anti-IgE, a positive control (non-responder) [12].

On the other hand, we could not detect a sufficient amount of specific binding of IgE to sweat antigen in the sera of patients with atopic dermatitis and cholinergic urticaria by ELISA using semi-purified sweat antigens (Fig. 2). Thus, we developed a method to evaluate sweat allergy using the sera of patients and basophils from other patients who have been proved to be sensitive to sweat antigen.

To identify the substance that neutralizes histamine-release activity, we removed IgE and IgG from the sera of 42 HRN (+) subjects by column chromatography. Sera of 36 subjects lost HRN activities by the removal of IgG. On the other hand, sera of four subjects lost HRN activities by the removal of IgE. These results indicate that a certain population of HRN (+) subjects has IgE against sweat antigen, but a majority of HRN (+) subjects have IgG against the sweat antigen in their sera. The reason why the removal of either IgE or IgG results in the loss of HRN activity in sera of subject No. 61 maybe due to the

combination of IgG against IgE that binds sweat antigen or IgE against IgG that binds the antigen.

Although the role of such IgG that neutralize histamine-release activity of sweat antigen is unclear, the activities of HRN were co-related with HRA of their basophils in response to sweat antigens. It is known that the level of antigen-specific IgG (especially IgG4) increases as a result of immunotherapies against mite and pollen in allergic rhinitis [1]. Therefore, serum HRN activity may represent homeostatic activities of HRA (+) subjects.

Taken together, HRN (+) activity appears to indicate the type I hypersensitivity against sweat antigen.

Further studies with large populations of subjects with atopic and non-atopic diseases are necessary to establish this assay as a screening test for sweat allergy.

Acknowledgments We thank Ms Kazue Uchida for the expert technical assistances, and Dr Faiz Kermani for his manuscript review. This work was supported in part by Health Science Research Grants from Ministry of Health, Welfare and Labor, Japan.

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