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ORIGINAL ARTICLE

Elevated Serum IgE against MGL_1304 in Patients with Atopic Dermatitis and Cholinergic Urticaria

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ABSTRACT

Background: MGL_1304 secreted by *Malassezia globosa* is contained in human sweat and induces histamine release from basophils in patients with atopic dermatitis (AD) at a high positive rate. The aims of this study were to establish the enzyme-linked immunosorbent assay (ELISA) measuring specific immunoglobulins against MGL_1304 and to investigate the levels of these immunoglobulins in sera of patients with various allergic diseases.

Methods: Purified MGL_1304 from human sweat (QRX) and recombinant MGL_1304 (rMGL_1304) were prepared for ELISA. To quantify the amount of MGL_1304-specific immunoglobulins, the standard serum was created by pooling sera of 20 patients with AD whose basophils released histamine in response to QRX. A monoclonal antibody which exhibited the highest neutralizing ability against QRX was established as Smith-2, and used as a capture antibody for the assay of QRX-specific IgE. A total of 156 subjects [normal controls (n = 23), AD (n = 63), cholinergic urticaria (CU) (n = 24), bronchial asthma (n = 32), and allergic rhinitis (n = 14)] were enrolled in this study.

Results: ELISA methods to quantify the specific IgE, IgG and IgG4 against MGL_1304 in sera were successfully established. Levels of QRX-specific IgE in sera of patients with AD and CU were significantly higher than those of normal controls. Moreover, the levels of QRX-specific IgE and rMGL_1304-specific IgE in patients with AD were significantly correlated with their disease severities.

Conclusions: These ELISA methods to quantify the specific immunoglobulins against MGL_1304 are easy and useful means to assess allergy to MGL_1304. MGL_1304 contained in sweat is an important antigen for patients with AD and CU.

KEY WORDS

atopic dermatitis, cholinergic urticaria, ELISA, *Malassezia globosa*, MGL_1304

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory pruritic, chronic or chronically relapsing skin disease occurring often in families with other atopic diseases.[1-3] The involvement of IgE has been suggested as an important factor in the pathogenesis of AD, as in other atopic diseases, such as asthma or allergic rhinitis. Moreover, high levels of total IgE in sera of patients with AD, and several reports presenting the effectiveness of omalizumab for AD[4,5] enforce the importance of IgE in AD. Recently, filaggrin shed light on the development of AD,[6] but mutations of filaggrin are also detected in patients with ichthyosis vulgaris without AD. Moreover, only 15% of mild-to-moderate AD and 50% of moderate-to-severe AD cases have revealed filaggrin mutations.[7] Thus, precise role of IgE in the pathogenesis of AD remains to be investigated.

Numerous triggering factors, such as irritants, aeroallergens, food, microbial organisms, and sweating, are also known to be involved in the development and/or the aggravation of AD.[3] We and other authors

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reported that skin test with autologous sweat was positive in the majority of patients with AD,[8-10] and clinical symptoms of children with AD significantly improved during the summer if they took showers at schools.[11,12] Moreover, we previously revealed that semi-purified sweat antigen induced histamine release from the basophils of 77% patients of AD[13] and 66% patients of cholinergic urticaria (CU),[14] and the histamine release by sweat antigen was mediated by specific IgE.[9,13] Finally, we extensively purified histamine release activity of the semi-purified sweat antigen by a combination of various chromatographies and identified a putative protein, MGL_1304 of *Malassezia globosa* (*M. globosa*), as a major allergen in human sweat.[15] MGL_1304 induced histamine release of basophils obtained from the majority of patients with AD.[15] In Japan, a commercial histamine release test (HRT) against sweat containing MGL_1304 has been available since 2010. However, HRT needs fresh blood cells of patients and its results are qualitative rather than quantitative. In the present study, to overcome the disadvantages of HRT, we established enzyme-linked immunosorbent assays (ELISAs) to measure specific IgE against native MGL_1304 collected from human sweat (QRX) and recombinant MGL_1304 (rMGL_1304) by using sera of patients with AD and other allergic diseases.

METHODS

PATIENTS AND ETHICS

A total of 156 subjects were enrolled in this study. Sixty-three patients with AD (36 men and 27 women; 0-65 years of age, mean ± SD: 29.9 ± 11.5), 24 patients with cholinergic urticaria (CU) and no history of AD (14 men and 10 women; 7-65 years of age, mean ± SD: 24.0 ± 13.7) and 32 patients with bronchial asthma (BA) and without AD (15 men and 17 women; 20-82 years of age, mean ± SD: 54.6 ± 16.0) who visited Hiroshima University Hospital were included in this study. Fourteen patients with allergic rhinitis (AR) without other allergic diseases (4 men and 10 women; 22-75 years of age, mean ± SD: 33.2 ± 15.5) and 23 normal control subjects (NC) without allergic symptoms (15 men and 8 women; 22-44 years of age, mean ± SD: 26.2 ± 6.5) were recruited as volunteers and included in this study. The severity of AD was evaluated using severity index of Japanese guideline for AD.[3] This study was carried out in accordance with the guidelines stated in the Declaration of Helsinki and was approved by the Ethics Committee of Hiroshima University Institute of Biomedical & Health Sciences (Approved No 556, 630). Written informed consent was obtained from every subject.

PREPARATION OF PURIFIED MGL_1304 (QRX) AND RECOMBINANT MGL_1304

The semi-purified sweat antigen purified from human sweat of healthy volunteers described in the previous report,[13] QR (semi-purified sweat antigen), was used for histamine release test (HRT). QR was further fractionated by a gel chromatography column, and the fractions with histamine releasing activity were collected as purified MGL_1304 (QRX) as described previously.[15] QRX was used for immunoblot analysis and ELISA.

By using *Escherichia coli* (JM109) and pCold TF vectors coding MGL_1304, the poly histidine-tagged and trigger factor (TF)-fused rMGL_1304 protein (TF-rMGL_1304) was prepared as described previously.[15]

MONOCLONAL ANTIBODY AGAINST PURIFIED MGL_1304 (QRX)

A mouse monoclonal antibody, Smith-2,[15] which has neutralization ability against histamine release activity from basophils of patients with AD in response to QRX, was used for ELISA measuring QRX-specific IgE.

WESTERN BLOT ANALYSIS

Samples were loaded into a SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane as reported elsewhere.[16] The membranes were incubated with AD standard serum or anti-penta-His antibody (Qiagen, Hilden, Germany) at 4℃ overnight. The membrane bound primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

HISTAMINE RELEASE TEST

HRT with basophils obtained from peripheral blood were performed as described previously.[17] Cells were stimulated with 1 μg/ml of goat anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA) and 33 ng/ml of QR.

ESTABLISHMENT OF ELISA SYSTEMS FOR MEASUREMENT OF MGL_1304-SPECIFIC IMMUNOGLOBULINS

To quantify the amount of MGL_1304-specific immunoglobulins, the AD standard serum was prepared by pooling sera of 20 patients with AD whose basophils release histamine in response to QR.

Ninety-six well ELISA-plates (High-Binding, Greiner bio-one, Frickenhausen, Germany) were used for all ELISA. For the measurement of QRX-specific IgE, 96 well plates were directly coated with 1 μg/ml QRX for direct ELISA or 10 μg/ml Smith-2 antibody for sandwich ELISA in phosphate buffered saline (PBS) without any carrier proteins and incubated over night at 4℃. The remaining non-specific binding sites were blocked with 2% bovine serum albumin PBS (BSA-PBS) for 1 hour at room temperature. The plates coated with Smith-2 antibody were sequentially incubated with 1 μg/ml QRX in 0.1% BSA-PBS for 90 minutes at room temperature. The wells were incubated

with 100 μl of patient sera diluted 1 : 40 with 1% BSA-PBS at room temperature for 90 minutes and subsequently with peroxidase-labeled antibody to human IgE (ε-chain specific) (KPL, Gaithersburg, MD, USA) diluted 1 : 3000 with 1% BSA-PBS, at room temperature for 1 hour.

For the measurement of rMGL_1304-specific IgE, 96 well plates were directly coated with 3 μg/ml TF-rMGL_1304 or the same concentration of TF alone diluted in PBS without carrier protein, and incubated over night at 4℃. The remaining non-specific binding sites were blocked with 2% BSA-PBS for 1 hour at room temperature. The wells were incubated with 100 μl of patient sera diluted 1 : 20 with 1% BSA-PBS at room temperature for 2 hours and subsequently with peroxidase-labeled antibody to human IgE described above and diluted 1 : 3000 with 1% BSA-PBS, at room temperature for 1 hour.

For the measurement of rMGL_1304-specific IgG, 96 well plates were directly coated with 3 μg/ml TF-rMGL_1304 or TF alone in PBS without carrier protein, at 4℃ over night. The remaining non-specific binding sites were blocked with 5% skim-milk in PBS (skim-milk-PBS) for 1 hour at room temperature. The wells were incubated with 100 μl of patient sera diluted 1 : 200 in 1% skim-milk-PBS at room temperature for 2 hours and subsequently with peroxidase-labeled antibody against human IgG (Fc specific) (KPL) diluted 1 : 2000 in 1% skim-milk-PBS at room temperature for 1 hour.

For the measurement of rMGL_1304-specific IgG4, 96 well plates were directly coated with 3 μg/ml TF-rMGL_1304 or TF alone in PBS without carrier protein, and incubated over night at 4℃. The remaining non-specific binding sites were blocked with 5% skim-milk-PBS for 1 hour at room temperature. The wells were incubated with 100 μl of patient sera diluted 1 : 20 with 1% skim-milk-PBS at room temperature for 2 hours and subsequently with peroxidase-labeled antibody to human IgG4 (γ4 chain specific) (Southern Biotech) diluted 1 : 2500 with 1% skim-milk-PBS, at room temperature for 1 hour.

All incubations were followed by three washes with wash buffer (PBS with 0.05% Tween-20), and the antibody binding was visualized by incubation with TMB Microwell Peroxidase Substrate and TMB Stop Solution (KPL). The optical density at 450 nm (OD450) was then read with an automatic plate reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA). When we use TF-rMGL_1304 as an antigen, we calculated differences between OD450 obtained with TF-rMGL_1304 and that with TF alone. Samples were tested in duplicate, and the concentrations of specific antibodies (Units/ml) were calculated by using a standard curve obtained from a serial dilution of the AD standard serum stated above. The units of each specific antibody in one milliliter of the AD standard serum were defined as 1000 Units.

MEASUREMENT OF MALASSEZIA-SPECIFIC IgE AND TARC

Serum levels of *Malassezia*-specific IgE (U_A/ml) and TARC (pg/ml) were measured by using the ImmunoCAP 100 instrument (Phadia AB, Portage, MI, USA) and ELISA in SRL Inc. (Tokyo, Japan), respectively.

STATISTICAL ANALYSIS

Analyses were performed by the use of GRAPHPAD PRISM version 5.04 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM.

RESULTS

ESTABLISHMENT OF THE ELISA SYSTEM FOR IgE, IgG, AND IgG4 AGAINST MGL_1304

The semi-purified sweat antigen, QRX (17 kDa), TF (60 kDa) and TF-rMGL_1304 (77 kDa) were detected with immunoblots by using AD standard serum and/or anti-polyhistidine-tag antibody (Supplementary Fig. 1). However, with a direct ELISA using QRX as a coated antigen, we could hardly detect QRX-specific IgE (Fig. 1a), IgG and IgG4 (data not shown). The minimum detection limit of the sandwich ELISA for QRX-specific IgE by using Smith2-antibody as a coated (capture) antibody was greatly improved as compared with the direct ELISA (Fig. 1a). In contrast to IgE against QRX, IgG and IgG4 against QRX could not be detected by the sandwich ELISA using Smith2-antibody due to a cross activity of Smith2-antibody for the secondary antibodies against human IgG and IgG 4 (data not shown). However, specific bindings to rMGL_1304 by IgE, IgG and IgG4 were all successfully detected with the direct ELISA (Fig. 1b-d).

rMGL_1304-SPECIFIC IgE VS QRX-SPECIFIC IgE

The concentrations (Units/ml) of QRX-specific-IgE and rMGL_1304-specific-IgE in sera of patients with AD were highly correlated (Fig. 2a, R = 0.954, $P < 0.0001$, $N = 63$). The levels of QRX- or rMGL_1304-specific IgE in sera and histamine release induced by QR from basophils of the patients with AD were also correlated (Fig. 2b, R = 0.778, $P < 0.0001$, $N = 53$) (Fig. 2c, R = 0.783, $P < 0.0001$, $N = 53$). According to the ROC curves of QRX-specific IgE or rMGL_1304-specific IgE and HRT (Fig. 2d, e) among patients and NC, we defined the cut off values as 53 Units/ml for QRX specific-IgE (sensitivity 84.9%, specificity 85.7%, $P < 0.0001$, $N = 111$), and 33.6 Units/ml for rMGL_1304-IgE specific-IgE (sensitivity 81.8%, specificity 80.0%, $P < 0.0001$, $N = 111$).

LEVELS OF QRX- AND rMGL_1304-SPECIFIC IgE IN PATIENTS WITH EACH DISEASE, AND CORRELATIONS WITH SEVERITY OF AD

The levels of QRX-specific IgE in sera of patients with AD and those of patients with CU were significantly higher than those of NC ($P < 0.001$, $P < 0.05$) and pa-

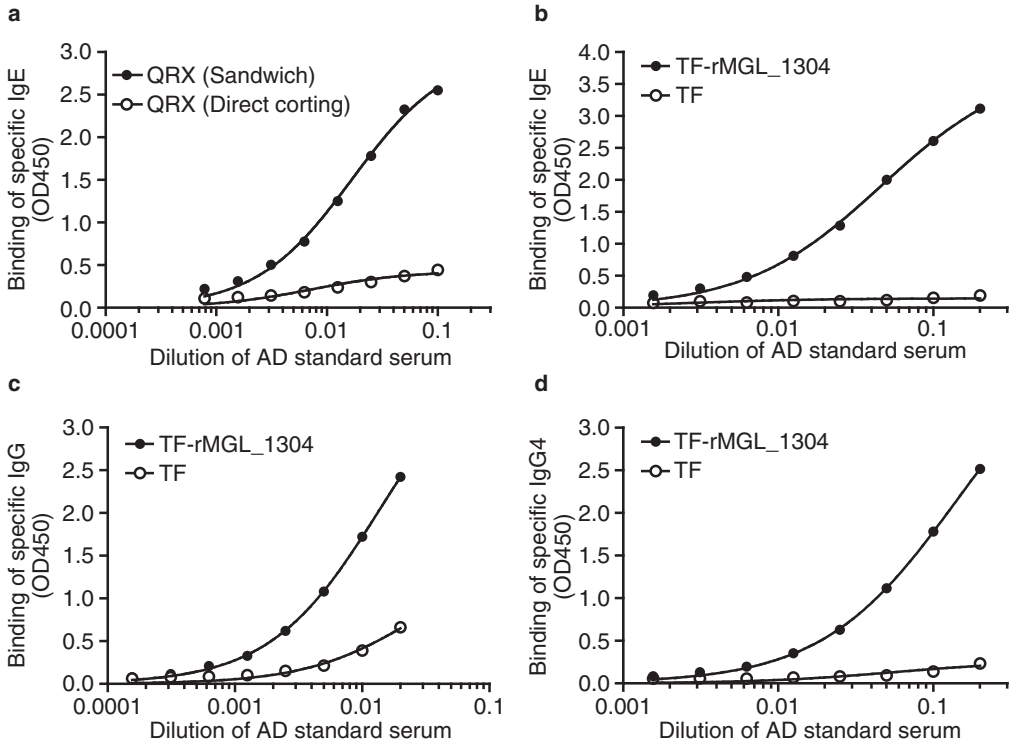

Fig. 1 Standard curves of MGL_1304-specific immunoglobulins plotted by using AD standard serum in ELISA. QRX-specific IgE in the serial diluted AD standard serum was measured by direct and sandwich ELISA (**a**). rMGL_1304-specific IgE (**b**), rMGL_1304-specific IgG (**c**), and rMGL_1304-specific IgG4 (**d**) in the serial diluted AD standard serum were measured by direct ELISA. Each panel showed a representative data expressed as means of OD450 values done in duplicate.

tients with BA ($P < 0.001$, $P < 0.01$) (Fig. 3a). Likewise, the level of rMGL_1304-specific IgE in sera of patients with AD was significantly higher than those of NC ($P < 0.05$) and patients with BA ($P < 0.001$) (Fig. 3b). The level of rMGL_1304 specific-IgE in sera of patients with CU was significantly higher than that with BA ($P < 0.001$), but not that of NC. According to the cut off values defined by ROC curves, the rate of QRX-specific IgE-positive patients with AD was significantly higher than those of NC ($P < 0.01$). However, there were no significant differences between NC and CU in the rate of patients with positive QRX-specific IgE and rMGL_1304-specific IgE in sera, respectively (Table 1). Moreover, the rate of rMGL_1304-specific IgE-positive patients with BA was significantly lower than that in NC ($P < 0.01$). Disease severities and the levels of QRX-specific IgE and rMGL_1304-specific IgE in patients with AD were significantly correlated (Fig. 3c, R = 0.5468, $P < 0.0001$) (Fig. 3d, R = 0.448, $P < 0.0001$).

LEVELS OF rMGL_1304-SPECIFIC IgG AND IgG4 IN PATIENTS WITH EACH DISEASE, AND CORRELATIONS WITH SEVERITIES OF AD

The levels of rMGL_1304-specific IgG in patients with AD was significantly higher than that of BA ($P < 0.01$), but the difference in the levels of rMGL_1304-specific IgG4 in patients with AD and those of the other diseases was not apparent (Fig. 4a, b). The disease severities and the levels of rMGL_1304-specific IgG and IgG4 in patients with AD were weakly but significantly correlated (Fig. 4c, R = 0.3292, $P = 0.0024$) (Fig. 4d, R = 0.3823, $P = 0.0004$).

MGL_1304-SPECIFIC IgE IS CORRELATED WITH rMGL_1304-SPECIFIC IgG AND IgG4 IN PATIENTS WITH AD BUT NOT IN PATIENTS WITH CU

The levels of rMGL_1304-specific IgE and rMGL_1304-specific IgG or IgG4 in patients with AD were significantly correlated (Fig. 5a, R = 0.469, $P = 0.0002$, $N = 60$) (Fig. 5b, R = 0.511, $P < 0.0001$, $N = 60$). The levels of total serum IgE and that of rMGL_1304-specific IgE or QRX-specific IgE in patients with AD were even more significantly correlated (Fig. 5c, R = 0.703, $P < 0.0001$, $N = 49$) (Fig. 5d, R = 0.697, $P < 0.0001$, $N = 49$). Moreover, the serum levels of QRX-specific IgE and rMGL_1304-specific IgE in patients with AD were weakly but significantly correlated with serum TARC (Supplementary Fig. 2a, R = 0.266, $P = 0.0367$, $N = 62$) (Supplementary Fig. 2b, R = 0.300, $P = 0.018$, $N = 62$). However, no correlations were ob-

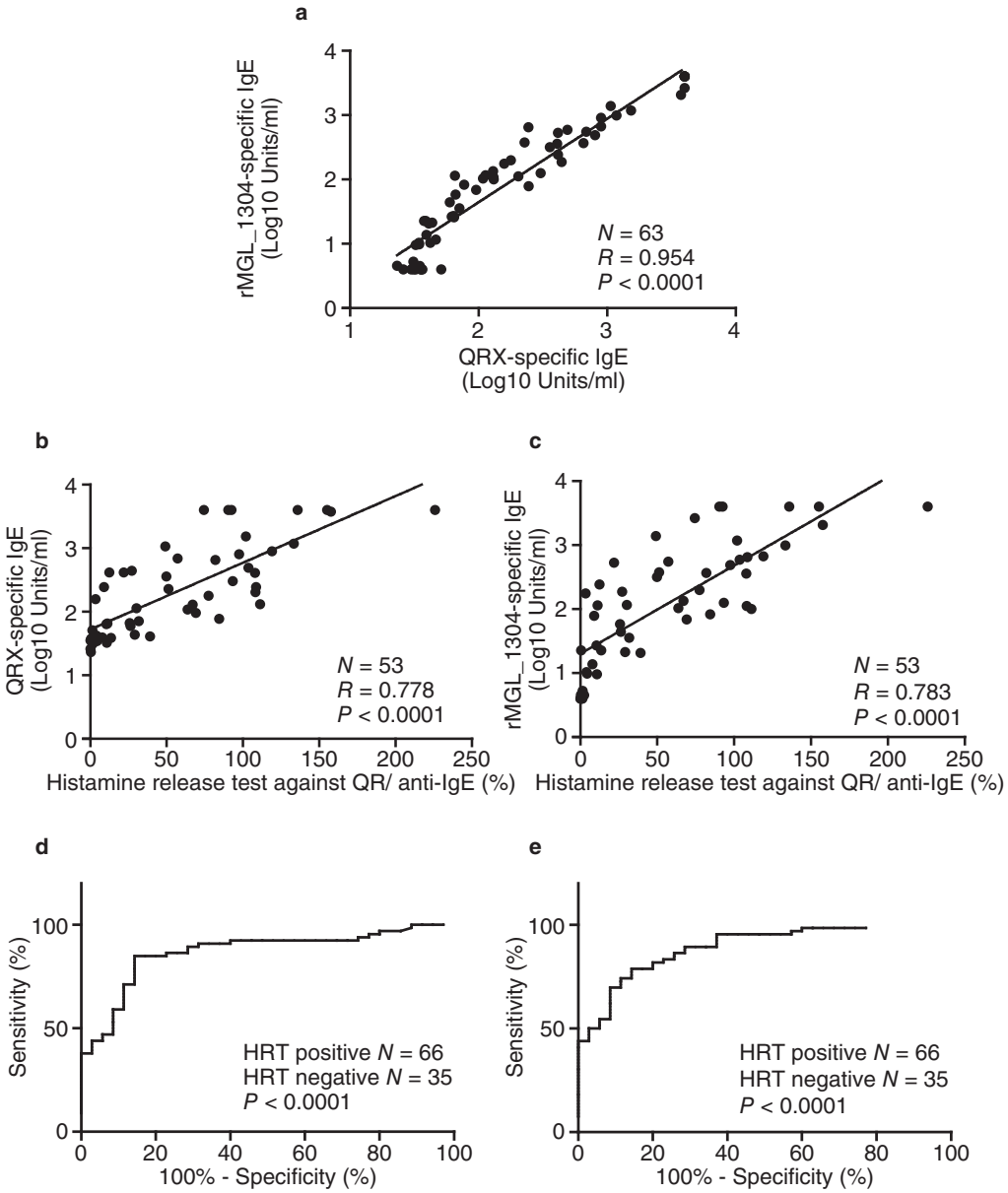

Fig. 2 Correlation between QRX-specific IgE, rMGL_1304-specific IgE, and basophil histamine release against QR. Levels of QRX- and rMGL_1304-specific IgE in sera of patients with AD (**a**). Histamine releases of basophils induced by QR are correlated with serum levels of QRX-specific IgE (**b**) and rMGL_1304-specific IgE (**c**) in patients with AD. Histamine release was expressed as (net histamine release induced by QR) / (net histamine release induced by anti-IgE) × 100 (%). ROC curves of QRX-specific IgE (**d**) and rMGL_1304-specific IgE (**e**) were created.

tained between rMGL_1304-specific IgE and rMGL_1304-specific IgG or IgG4, or between rMGL_1304 specific IgE or QRX-specific IgE and total serum IgE in patients with CU (Fig. 6). No correlation was observed between QRX-specific IgE or rMGL_1304 specific IgE and the number of peripheral eosinophils in patients with CU (Supplementary Fig. 3).

MALASSEZIA-SPECIFIC IgE AND MGL_1304-SPECIFIC IgE WERE CORRELATED, BUT NOT COMPLETELY MATCHED

The levels of QRX-specific IgE or rMGL_1304-specific IgE and the levels of *Malassezia*-specific IgE were significantly correlated (Fig. 7a, R = 0.729, $P < 0.0001$, $N = 59$) (Fig. 7b, R = 0.730, $P < 0.0001$, $N = 59$), but not completely matched in patients with AD. The levels of *Malassezia*-specific IgE in patients with AD were

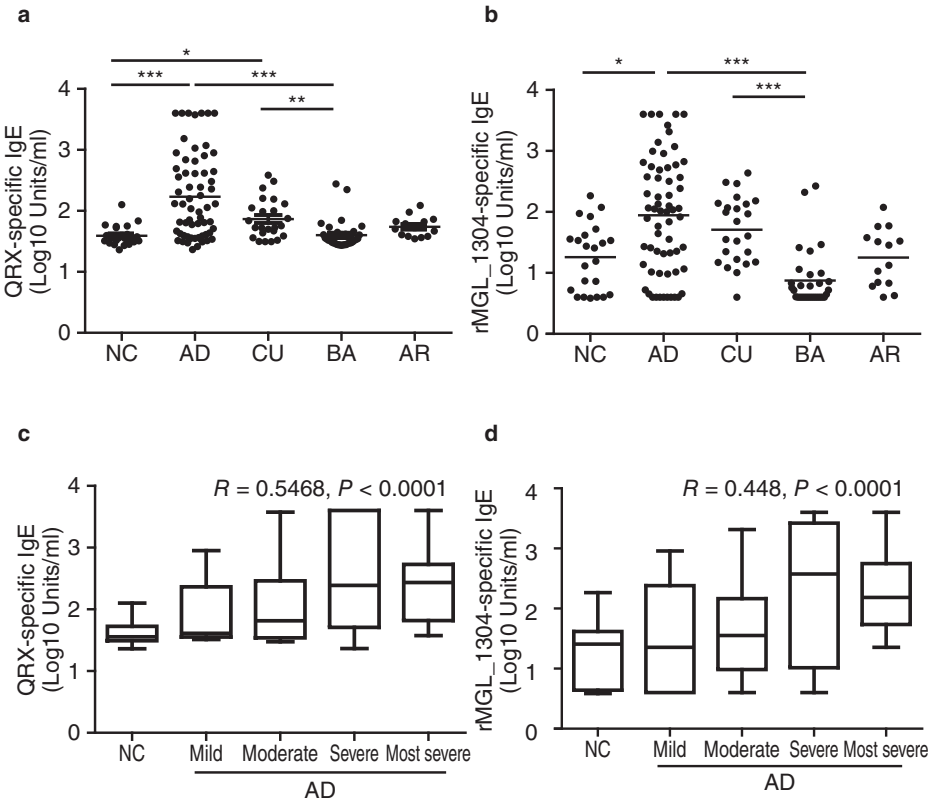

Fig. 3 Comparisons of serum levels of QRX- and rMGL_1304-specific IgE among diseases and correlations with severities of AD. Serum levels of QRX-specific IgE (**a**) and rMGL_1304-specific IgE (**b**) in patients with each disease were plotted and analyzed by Kruskal-Wallis test. The serum levels of QRX-specific IgE and rMGL_1304-specific IgE in patients showing various severities of AD were plotted and analyzed by Spearman rank correlation (**c, d**). NC, normal controls; AD, atopic dermatitis; CU, cholinergic urticaria; BA, bronchial asthma; AR, allergic rhinitis. *P < 0.05, **P < 0.01, ***P < 0.001.

Table 1 Number of patients with positive or negative QRX-specific IgE and rMGL_1304-specific IgE in each disease

QRX-specific IgE	NC	AD	CU	BA	AR
Positive	6	42	13	6	7
Negative	17	21	11	26	7
Positive rate	26.1	66.7	56.5	18.8	50.0
P-value	-	0.0012**	0.0753	0.5296	0.1713

rMGL_1304-specific IgE	NC	AD	CU	BA	AR
Positive	9	39	14	2	4
Negative	14	24	10	30	10
Positive rate	39.1	61.9	58.3	6.3	28.6
P-value	-	0.0857	0.2476	0.0048**	0.7245

Positive rates were compared with NC by Chi-square test. NC, normal controls; AD, atopic dermatitis; CU, cholinergic urticaria; BA, bronchial asthma; AR, allergic rhinitis. **P < 0.001.

also highly correlated with total serum IgE (Supplementary Fig. 4a, R = 0.749, P < 0.0001, N = 0.46), and weakly correlated with eosinophils (%) (Supplementary Fig. 4b, R = 0.283, P = 0.0491, N = 49).

DISCUSSION

In this study, we established ELISA systems to measure specific IgE, IgG and IgG4 against native and

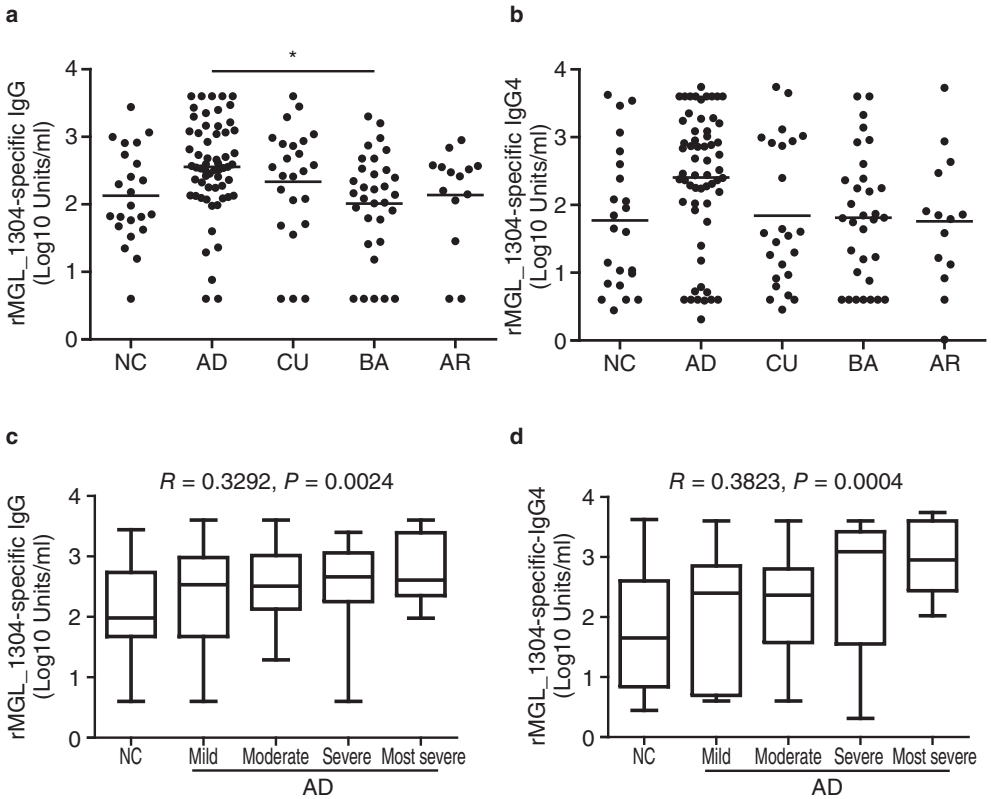

Fig. 4 Serum levels of rMGL_1304-specific IgG and IgG4 in diseases and their correlations with severities of AD. Serum levels of rMGL_1304-specific IgG (**a**) and IgG4 (**b**) in each disease were plotted and analyzed by Kruskal-Wallis test. The serum levels of rMGL_1304-specific IgG (**c**) and IgG4 (**d**) in patients with various severities of AD were plotted and analyzed by Spearman rank correlation. *$P < 0.05$.

rMGL_1304 in sera of patients with AD and other diseases. MGL_1304 is a secretory protein of *M. globosa* and a major antigen contained in human sweat and highly induces histamine release from basophils of many patients with AD.

By using a monoclonal antibody against purified MGL_1304 (QRX), Smith2, as a capture antibody, we could detect the specific IgE against QRX in sera of patients with AD with a high sensitivity. We have already reported that IgE from patients with AD recognized the steric structure, but not a short amino-acid sequence of MGL_1304.[15] The difficulty of the direct ELISA using QRX might be due to its antigenic property. In contrast to QRX, TF-rMGL_1304 could be applied to the direct ELISA to measure rMGL_1304-specific IgE, IgG and IgG4 without the capture antibody. This advantage of TF-rMGL_1304 over QRX could be due to the tag protein and/or TF that might 'raise' MGL_1304 on the ELISA plate so as to make its epitope more easily accessible by human antibodies. Moreover, data of QRX-specific IgE and rMGL_1304-specific IgE in sera of patients with AD are highly correlated. However, QRX specific-IgE showed higher sensitivity and specificity against HRT than rMGL_1304. Such differences may be due to a post-translational modification of native MGL_1304 in QRX, but which is lacked by rMGL_1304 synthesized by *Escherichia coli*. Since, QRX is purified from sweat of human volunteers, it is extremely scarce and difficult to obtain in sufficient amounts for assays in routine clinical practices. We are currently establishing a method to purify large amounts of native MGL_1304 from culture supernatant of *M. globosa*.

In patients with AD, the serum levels of specific IgE against MGL_1304 were significantly higher than those of NC. Moreover, the levels of MGL_1304-specific IgE in patients with AD were well correlated with disease severities of AD and serum levels of total IgE. These results indicate that the measurement of MGL_1304-specific IgE is useful not only to evaluate the allergy to MGL_1304 in sweat but also to evaluate the disease severity of AD. However, serum levels of MGL_1304-specific IgE showed only a weak correlation with serum TARC. It might be due to more rapid changes of TARC than MGL_1304-specific IgE in association with disease severities.[3,18] Therefore, the levels of QRX- and rMGL_1304-specific IgE are likely more suitable for the evaluation of long-term disease

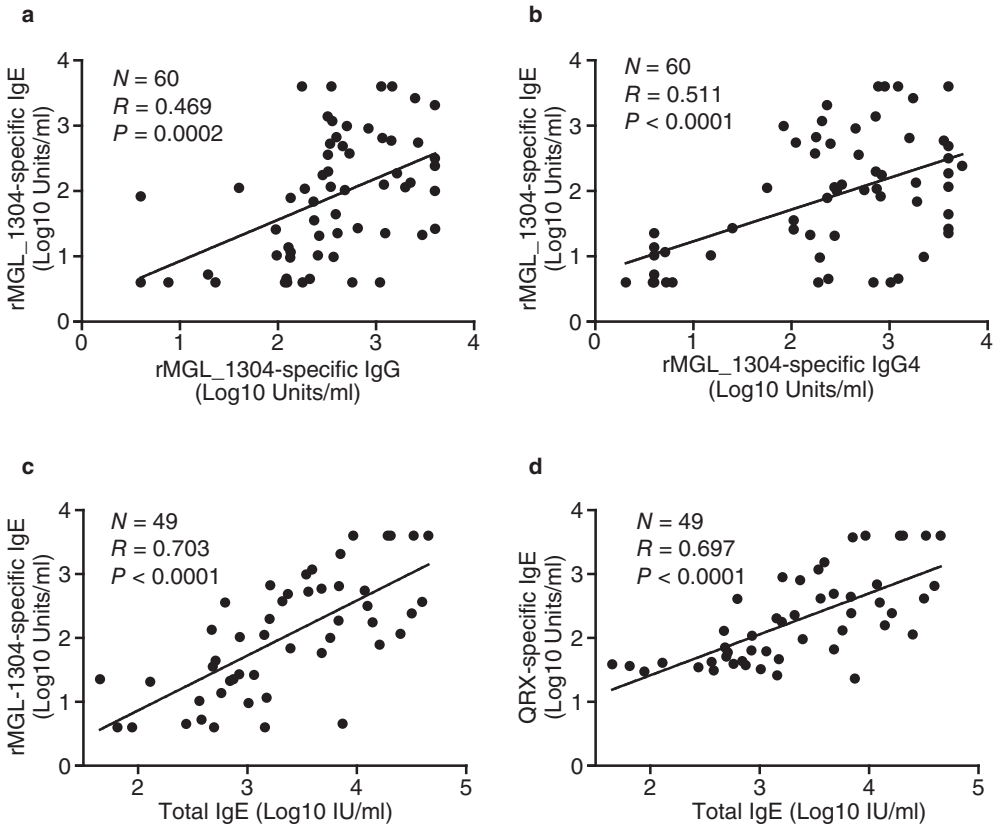

Fig. 5 Correlations among rMGL_1304-specific immunoglobulins in sera of patients with AD. Significant correlations were observed between serum levels of rMGL_1304-specific IgE and rMGL_1304-specific IgG (**a**), rMGL_1304-specific IgG4 (**b**), or total IgE (**c**) in patients with AD. The levels of serum total IgE were also significantly correlated with QRX-specific IgE (**d**) in patients with AD.

severities rather than short-term disease activities of AD. The presence of serum IgG against sweat antigens, were suggested by our previous study using histamine release-neutralization assay.[17] However, there were no significant differences between the levels of rMGL_1304-specific IgG or IgG4 in patients with AD and those of NC. These results suggest that both patients with AD and healthy individuals may be sensitized with MGL_1304, but IgE class switching against MGL_1304 occurs much more strongly in patients with AD than in NC, although the opportunity of sensitization with MGL_1304 exists even in normal controls. Thus, for the patients with AD, the measurement of IgE against MGL_1304 is more important than that of IgG or IgG4 against rMGL_1304.

The influence of *Malassezia* species on the pathogenesis of AD has attracted attention in recent years. Many studies have shown the effectiveness of antifungal therapy on AD with *Malassezia* allergy,[19] and the association of high levels of *Malassezia*-specific IgE and severe AD.[20,21] Ishibashi *et al*. identified 42 kDa protein (MGp42) as a major antigen of *M. globosa* for serum IgE of patients with AD.[22] Moreover, Darabi *et al*.[19] reported that the peripheral blood mononuclear cells of patients with AD who have specific IgE against *Malassezia* showed an increased proliferation and an increased production of inflammatory cytokines upon the exposure to *Malassezia* compared with cells from healthy control subjects.

The identification of MGL_1304 as a histamine releasing antigen in human sweat has revealed the involvement of *M. globosa* in type 1 allergy against sweat observed in patients with AD and/or CU. However, western blotting by using sera of patients with AD for lysates or culture supernatant of *M. globosa* shows many bands including MGp42,[15,22] whereas patients with AD react to the fractions of sweat eluted between molecular markers of 17 kDa and 1.35 kDa by gel chromatography.[9] Moreover, in the present study, serum levels of MGL_1304-specific IgE and *Malassezia*-specific IgE were not completely matched, suggesting that the importance of MGL_1304-specific IgE rather than IgE against the whole *Malassezia* antigens in patients whose symptoms are exacerbated by sweating.

The relationship between MGL_1304 and CU is in-

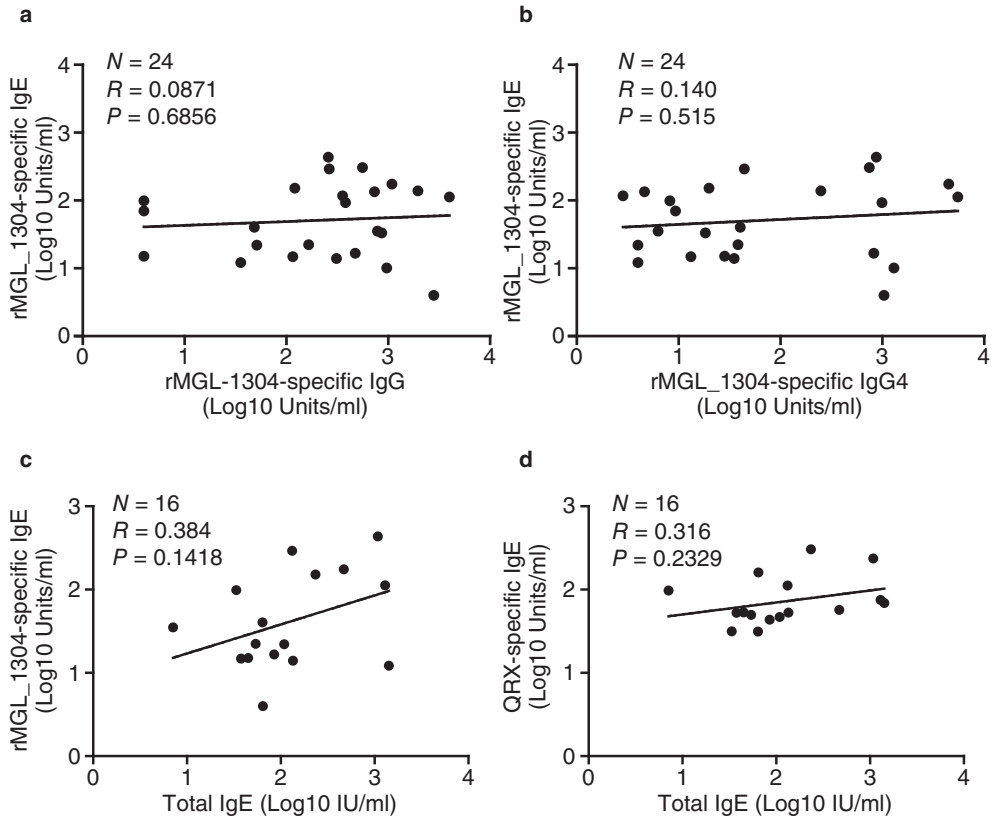

Fig. 6 Correlations among rMGL_1304-specific immunoglobulins in sera of patients with CU. No significant correlations was observed between serum levels of rMGL_1304-specific IgE and IgG (**a**), IgE and IgG4 (**b**) or the specific IgE and total serum IgE (**c**). Likewise, no apparent correlation was observed between serum levels of QRX-specific IgE and total serum IgE in patients with CU (**d**).

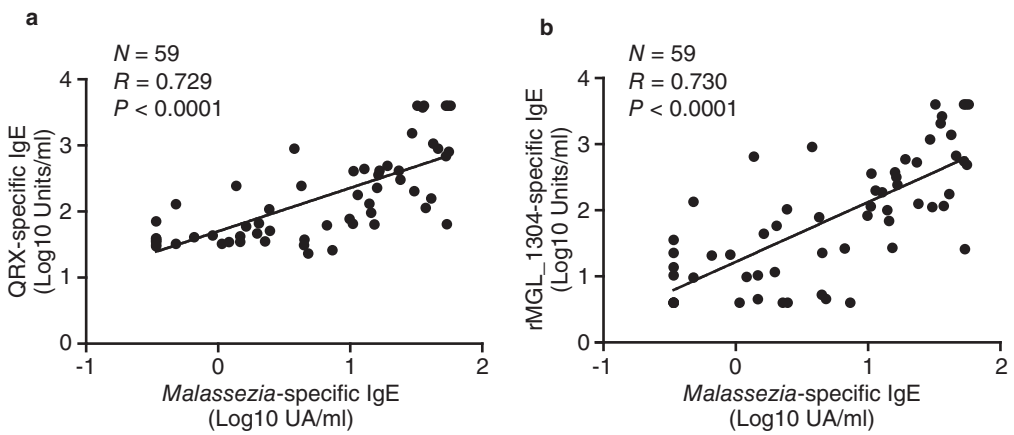

Fig. 7 Correlation between serum levels of MGL_1304-specific IgE and *Malassezia*-specific IgE. Serum levels of *Malassezia*-specific IgE and QRX-specific IgE (**a**) or rMGL_1304-specific IgE (**b**) in patients with AD were significantly correlated.

triguing. In the present study, we revealed that the levels of QRX specific-IgE in patients with CU were also higher than that of NC and BA. Takahagi *et al*.[14] also reported that the levels of basophils histamine release against QR in patients with CU were significantly higher than that of NC. On the other hand, the levels of rMGL_1304 specific-IgG or IgG4 in patients with CU were not correlated with their levels of QRX-

or rMGL_1304-specific IgE. Taking into account that both IgG and IgG4 against MGL_1304 likely neutralize the histamine release activity of MGL_1304, the presence of IgE over IgG and IgG4 in their binding activity to MGL_1304 may be critical in the pathogenesis of CU. Several groups have already performed clinical trials of hyposensitization therapy for CU by the use of autologous sweat.[23-25] Moreover, it is known that antigen-specific immunotherapy for allergic rhinitis induces the increase of antigen-specific IgG4.[26,27] Therefore, the methods to evaluate levels of serum IgG or IgG4 against MGL_1304 might be useful to monitor the effectiveness of immunotherapy on patients with CU.

The levels of QRX specific-IgE in patients with AR tended to be higher than that of NC, although this difference was not statistically significant. It might be due to the colonization of *M. globosa* in nasal cavity,[28] and suggests that MGL_1304 might also be a causative antigen for a certain population of patients with AR.

In conclusion, MGL_1304 is an important antigen in sweat and the methods to quantify the specific IgE against MGL_1304 in sera by ELISA were a useful means to diagnose allergy to MGL_1304 contained in sweat and estimate long term disease severities of AD. The measurement of QRX-specific IgE in patients with CU was also useful for the diagnosis and could be a clue to clarify the mechanism of CU. Further studies on the relationship between AD or CU and MGL_1304 in sweat are needed.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1-4 are available online.

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