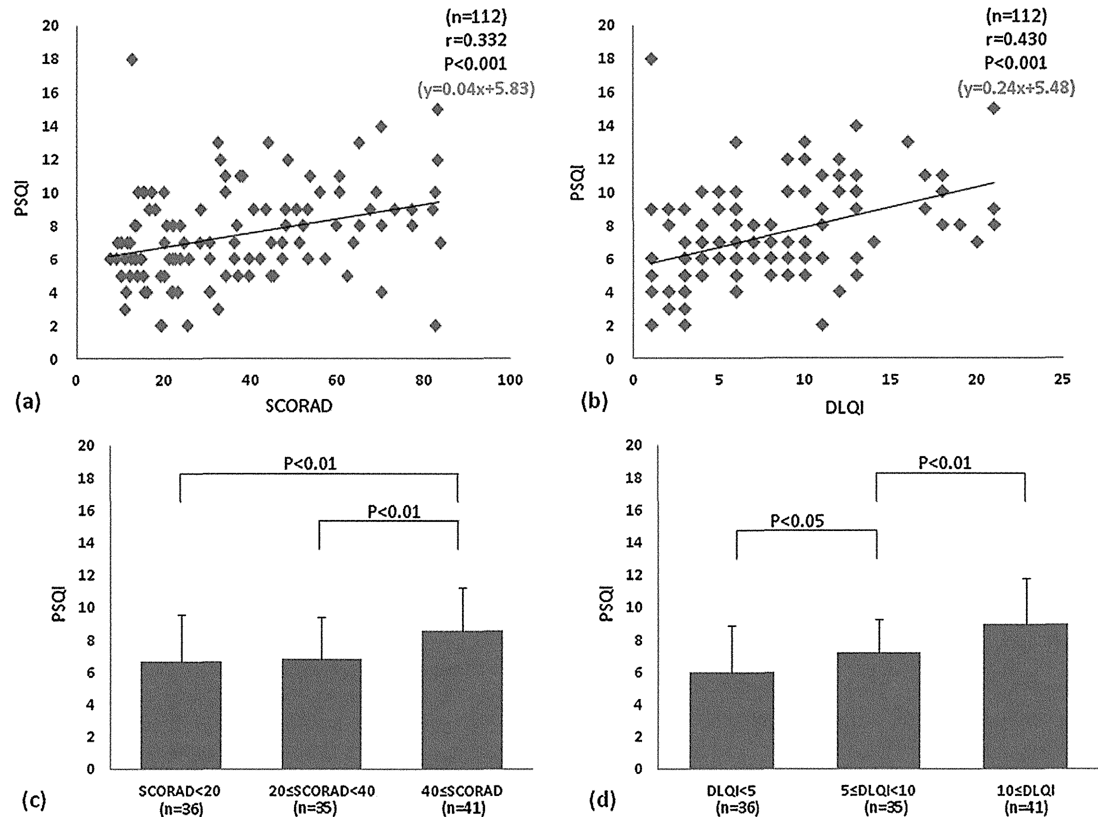

Fig. 1. Associations between PSQI and SCORAD/DLQI. (a) PSQI and SCORAD. (b) PSQI and DLQI. (c) PSQI and categorical SCORAD. (d) PSQI and categorical DLQI. (PSQI, Pittsburg Sleep Quality Index; SCORAD, severity scoring of AD; DLQI, dermatology life quality index).

There are several assessment tools for evaluating the degree of sleep disturbance other than PSQI: the Stanford Sleepiness Scale (SSS), Karolinska Sleepiness Scale (KSS), Epworth Sleepiness Scale (ESS), Oguri–Shirakawa–Azumi sleep inventory MA version (OSA-MA) and SleepMed Insomnia Index (SMI) [6–10]. The first three scales (SSS, KSS and ESS) are questions about subjective sleepiness in given situations, OSA-MA is a questionnaire assessing sleep state of the previous night and SMI is a diagnostic insomnia questionnaire. PSQI has been used widely for over 20 years in various clinical populations and seems to be the most suitable measure among those above-mentioned for evaluating sleep quality in chronic skin diseases including AD.

Table 1
Associations between PSQI component scores and SCORAD/DLQI.

PSQI and SCORAD	r	P
Subjective sleep quality	0.360	$P < 0.001$
Sleep latency	0.307	$P < 0.001$
Sleep duration	0.061	0.52
Habitual sleep efficiency	0.090	0.34
Sleep disturbance	0.077	0.41
Use of sleeping medication	0.159	0.09
Daytime dysfunction	0.123	0.19
PSQI and DLQI	r	P
Subjective sleep quality	0.403	$P < 0.001$
Sleep latency	0.321	$P < 0.001$
Sleep duration	0.162	0.08
Habitual sleep efficiency	0.198	$P < 0.05$
Sleep disturbance	0.250	$P < 0.01$
Use of sleeping medication	0.018	0.84
Daytime dysfunction	0.323	$P < 0.001$

PSQI, Pittsburg Sleep Quality Index; SCORAD, severity scoring of AD; DLQI, dermatology life quality index.

Mostaghimi showed the prevalence of sleep problems by PSQI in chronic skin diseases ($n = 16$) as a pilot study [1]. The mean PSQI score of his patients was 10.5 ± 4.0 and 15 out of the 16 (93.8%) had a PSQI score of more than 5, indicating poor sleep quality. In addition, 9 out of the 16 participants (56.3%) in his study reported mood problems such as depression or anxiety and participants with chronic skin diseases that did not cause itching, such as alopecia areata, also reported poor sleep quality. Ours is the first study to evaluate the sleep quality of Japanese adult AD patients using PSQI-J. The mean PSQI score of AD patients ($n = 112$) was 7.3 ± 2.8 and 70 out of the 112 (62.5%) had a PSQI score of more than 5, suggesting poor quality. We demonstrated that global and component PSQI scores such as subjective sleep quality and sleep latency were positively and significantly associated with SCORAD/DLQI scores, indicating that nocturnal itching and scratching behavior impair the sleep quality of AD patients. Furthermore, PSQI score was more strongly associated with DLQI than with SCORAD, and component PSQI scores such as habitual sleep efficiency, sleep disturbance and daytime dysfunction were associated with DLQI but not with SCORAD, suggesting the possibility that mood problems also impair sleep quality. Further studies are necessary to elucidate the relationship between mood problems and impaired sleep quality in Japanese AD patients.

Acknowledgments

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Letter to the Editor

Very mild lamellar ichthyosis with compound heterozygous TGM1 mutations including the novel missense mutation p.Leu693Phe

Keywords:
Autosomal recessive congenital ichthyosis; β-Barrel 2 domain; Collodion membrane; Lamellar ichthyosis; TGM1

The presence of a collodion membrane at birth represents a defect in skin barrier function and is usually the initial presentation of a congenital ichthyosis, most often autosomal recessive congenital ichthyosis (ARCI). However, in 10% of the neonates born with collodion membrane, spontaneous healing occurs [1]. ARCI includes a wide range of ichthyosis phenotypes, including harlequin ichthyosis, lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma [2,3], and seven causative genes for ARCI have been identified: *TGM1*, *ABCA12*, *ALOX12B*, *ALOXE3*, *NIPAL4*, *CYP4F22*, and *PNPLA1* [2–4]. The most common underlying gene defect is in transglutaminase-1 (TGase-1), with mutations in this gene found in 30–40% of cases of ARCI [2]. Most of these cases show an LI phenotype, i.e., large, brown, plate-like scales in a generalized distribution, although cases of ARCI with *TGM1* mutations can display considerable clinical and phenotypic diversity. Two such well-defined phenotypic variants are bathing suit ichthyosis and self-healing collodion baby (SHCB) [2]. Nearly complete resolution of scaling is observed within the first 3 months of life in SHCB [2]. Here we describe a patient who presented at birth with a collodion membrane and eventually developed very mild but continuing LI with compound heterozygous mutations, including a novel type of missense mutation in the β-barrel 2 domain of TGase-1.

In a Japanese boy born to nonconsanguineous parents at 40 weeks gestation, a collodion membrane with eclabium was noted at birth. A skin biopsy from the lesion on the trunk at 18 days after birth showed mild hyperkeratosis with normal granular layers (data not shown). He showed mild, fine white scaling without erythroderma on the trunk or palms at the age of 5 months (Fig. 1a and b).

The present studies were conducted according to the Declaration of Helsinki Principles and approved of by the ethics committee of the Nagoya University Graduate School of Medicine. The participants gave written informed consent. The coding region of *TGM1* (GenBank Accession No. 359) was amplified from genomic DNA by PCR as described previously [5]. Direct sequencing of the patient's PCR products revealed that the patient had the compound heterozygous *TGM1* mutations p.Arg78X (c.232C>T) and p.Leu693Phe (c.2077C>T), which were not detected in the 110 control alleles (data not shown) (Fig. 1c). p.Arg78X was previously reported as an LI-causative mutation [6]. The leucine residue mutated by p.Leu693Phe is in the β-barrel 2 domain of TGase-1 (Fig. 2c). This leucine residue was confirmed to be highly conserved

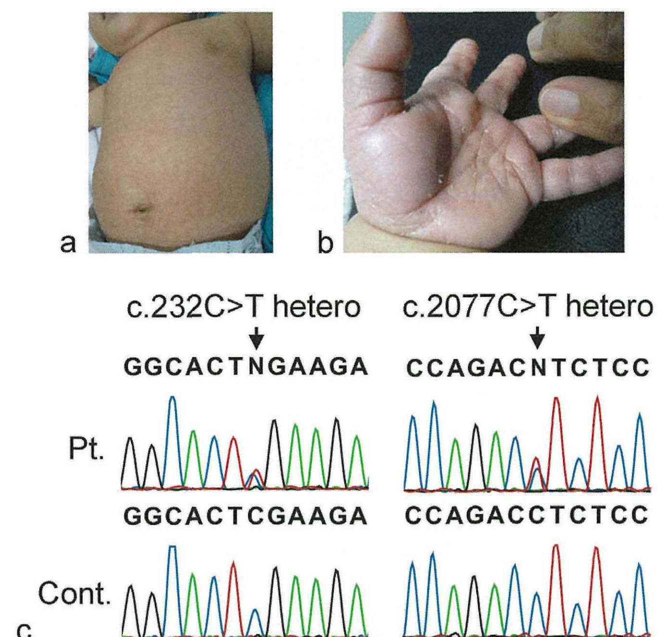

Fig. 1. Clinical features and *TGM1* sequence data of the patient. The patient shows very mild, fine white scaling without erythroderma on the trunk (a) or palms (b). Sequence data of *TGM1* in the patient and control in exon 2 (left) and exon 13 (right). Arrows indicate c.232C>T (heterozygous) and c.2077C>T (heterozygous).

Fungal protein MGL_1304 in sweat is an allergen for atopic dermatitis patients

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Background: Sweat is a major aggravating factor of atopic dermatitis (AD) and approximately 80% of patients with AD show type I hypersensitivity against sweat.
Objective: To identify and characterize an antigen in sweat that induces histamine release from basophils of patients with AD.
Methods: Basophil histamine-releasing activity in sweat was purified by a combination of chromatographies, and proteins were analyzed with mass spectrometry. Recombinant proteins of the sweat antigen were generated, and their biological characteristics were studied by immunoblots, histamine release tests, and neutralization assays.
Results: We identified a fungal protein, MGL_1304, derived from *Malassezia globosa* (*M globosa*) in the purified sweat antigen. Recombinant MGL_1304 induced histamine release from basophils of most of the patients with AD, in accordance with the semi-purified sweat antigen. Moreover, recombinant MGL_1304 abolished the binding of serum IgE of patients with AD to the semi-purified sweat antigen, or vice versa in immunoblot analysis, and attenuated the sensitization of RBL-48 mast cells expressing human FcεRI by serum IgE. Studies of truncated mutants of MGL_1304 indicated that IgE of patients with AD recognized the conformational structure of MGL_1304 rather than short peptide sequences. Western blot analysis of the whole lysate, the culture supernatant of *M globosa*, and the semi-purified sweat antigen showed that MGL_1304 was produced as a minor immunological antigen of *M globosa* with posttranslational modification, cleaved, and secreted as a 17-kDa major histamine-releasing sweat antigen.
Conclusion: MGL_1304 is a major allergen in human sweat and could cause type I allergy in patients with AD. (J Allergy Clin Immunol 2013;132:608-15.)

***Key words:** Sweat, atopic dermatitis,* Malassezia globosa, *histamine release, IgE*

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Atopic dermatitis (AD) is a chronic pruritic skin disease with characteristic distribution and morphology of skin lesions, associated with impaired skin barrier function and atopic background.[1] The 2003 national survey of children's health in the United States revealed that 10.7% of children younger than 18 years were reported to have had a diagnosis of AD in the past 12 months.[2] In a nationwide/Japanese study with age groups from 4 months old to university-aged students, the prevalence of AD was reported to be 12.8% to 8.2%.[3] Numerous triggering factors are known in AD, such as irritants, aeroallergens, food, microbial organisms, and sweating.[4] In hot and humid conditions, sweat increases and tends to be retained in fossae in the body. The characteristic distribution of skin lesions of AD, such as face, neck, cubital, and popliteal fossae, implies the association between exacerbation of AD and sweating. Moreover, we and other authors reported that skin test with autologous sweat was positive in the majority of patients with AD[5,6] and that clinical symptoms of children with AD significantly improved during the summer if they took showers at schools.[7-9] We previously revealed that semi-purified sweat antigen, which was obtained from human sweat by a combination of various chromatography steps, induced histamine release from the basophils of 77% of patients with AD[10] and 66% of patients with cholinergic urticaria.[11] The histamine release by sweat antigen was mediated by specific IgE.[10] The sensitivity of patients against sweat antigen may also be screened by histamine release-neutralization assay for sera of the patients, using basophils of other sweat-sensitive volunteers.[12] However, the antigen in human sweat itself has not yet been identified and the amount of IgE against sweat antigen has not been quantified by biochemical methods, such as ELISA.

In this study, we extensively purified the histamine-releasing activity of the semi-purified sweat antigen by a combination of various chromatographies and finally identified a putative protein, MGL_1304 of *Malassezia globosa* (*M globosa*), as a major

Abbreviations used

AD:	Atopic dermatitis
AR:	Allergic rhinitis
HRT:	Histamine release test
hMnSOD:	Human manganese superoxide dismutase
TF:	Trigger factor

allergen in human sweat, which induced histamine release of basophils obtained from the majority of patients with AD.

METHODS

Subjects

Seventy-four patients with AD (39 men/35 women; mean age ± SD, 28.8 ± 12.7 years; mean serum IgE ± SD, 7542.4 ± 10036.3 IU/mL) who were diagnosed by the criteria of Japanese Dermatological Association[13] were recruited and investigated by histamine release tests (HRTs), ImmunoCAP-specific IgE for mold allergens, Western blots, and experiments with human and rat mast cells. Seven patients with allergic rhinitis (AR) who met the diagnostic criteria of the Japanese Society of Allergology[14] were recruited and investigated by HRT.

Purification and mass-spectrometric analysis of the sweat antigen

The semi-purified sweat antigen described in the previous report,[12] QR, was further fractionated by a gel chromatography column (Superdex 75 PC 3.2/30; GE Healthcare, Buckinghamshire, United Kingdom), using the SMART system at a flow rate of 50 μL/min. The fractions with histamine-releasing activity were collected as QRX, and used for Western blot analysis and immunization to obtain mAbs against sweat antigen. The histamine-releasing activity in QRX was further purified into a single peaked protein by 2 reverse-phase chromatography columns, an Aqua 5μ-C18-200Å HPLC column (Phenomenex, Torrance, Calif) at a flow rate of 500 μL/min and a Jupiter 5μ-C18-300Å HPLC column (Phenomenex) at a flow rate of 100 μL/min. This fraction was gas-phase reduced, alkylated, and then digested with trypsin. Trypsin-digests were then analyzed by electrospray-ionization-mass spectrometry and MS/MS for partial amino acid sequence as previously performed for a protease inhibitor.[15]

Generation of an mAb against the semi-purified sweat antigen

The semi-purified sweat antigen (QRX) was applied to the column coupled with anti–cystatin A antibody (Biogenesis, Kingston, NH) to eliminate cystatin A, which is abundant in human sweat. The purified antigen, emulsified with complete Freund's adjuvant, was injected into hind footpads of female Balb/c mice of 8 weeks of age. Additional immunizations were performed after 7 days and 19 days with the mixture of the antigen and incomplete Freund's adjuvant. After 22 days from the date of the first immunization, popliteal lymph nodes were resected. Cells of the lymph nodes (9.9×10^7) were mixed with P3U1 myeloma cell line (1.98×10^7), and hybridomas were generated by electrofusion with SSH-2 Somatic Hybridizer (Shimazu, Kyoto, Japan). Clones of hybridomas were screened by the neutralization ability of their culture supernatants against the histamine-releasing activity from basophils of patients with AD in response to QRX. Positive hybridomas were cloned by limiting dilution, and a clone that produced IgG1-κ with the highest neutralizing ability was established as Smith-2.

Plasmids

M globosa (CBS 7966) was obtained from American type culture collection (Manassas, Va) and ground by a Coolmil (Tokken, Chiba, Japan). Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, Calif) and converted to cDNA with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA fragment of MGL_1304 was amplified with PfuUltra DNA polymerase (Agilent Technologies, Santa Clara, Calif) and specific primer pairs (see Table E1 in this article's Online Repository at www.jacionline.org). The DNA fragments were double-digested with *Kpn* I/*Eco*R V or *Kpn* I/*Hind* III, and then ligated into pSecTag2/Hygro (Invitrogen) or pCold trigger factor (TF) (Takara Bio, Shiga, Japan), respectively. pCold TF vectors expressing truncated MGL_1304 were generated with the specific primers (Table E1) and the full length cDNA of MGL_1304 as the PCR template. The cDNA of Der f 1 was kindly provided by Dr Tsunehiro Aki (Graduate School of Advanced Sciences of Matter, Hiroshima University, Hiroshima, Japan), and amplified with PfuUltra DNA polymerase and a primer pair (Table E1). The DNA fragment was double-digested with *Bam*H I/*Xho* I and then ligated into pSecTag2/Hygro. The cDNA of human manganese superoxide dismutase (hMnSOD) was amplified with the specific primer pairs (Table E1) and human skin cDNA library, which was constructed by mRNA of human skin (Agilent Technologies) and CloneMiner cDNA library construction kit (Invitrogen), as the PCR template. The DNA fragment was digested with *Kpn* I/*Xba* I and then ligated into pCold TF vector.

Generation of recombinant proteins

For mammalian expression, pSecTag2 vector coding MGL_1304 or Der f 1 was introduced to COS7 cells by electroporation at 250 V/250 μF with Gene Pulser II (Biorad, Hercules, Calif). After 24 to 48 hours of incubation, the culture supernatants were collected. For bacterial expression by using *Escherichia coli* (JM109), the cultures from single clones of JM109 that contained pCold TF vectors were lysed with xTractor buffer kit (Takara Bio), and the polyhistidine tagged- and TF-fused recombinant MGL_1304 (rMGL_1304) and rhMnSOD were purified by HisTARON gravity columns purification kit (Takara Bio), according to the manufacturer's instructions. In some experiments, the TF-rMGL_1304 protein was digested with HRV 3C protease (EMD Chemicals, San Diego, Calif) and rotated with TARON resin at 4°C for 2 hours to remove TF and the protease.

HRT with human peripheral basophils

HRTs with basophils obtained from peripheral blood were performed as described previously.[12] Cells were stimulated with 1 μg/mL of goat antihuman IgE antibody (Bethyl Laboratories, Montgomery, Tex), 33 ng/mL of QR, various concentrations of TF-rMGL_1304, or 10-fold–diluted culture supernatant of COS7 cells transfected with pSecTag2 vector coding MGL_1304.

Measurement of β-hexosaminidase release of RBL-48 cells

RBL-48 cells, which express the α subunit of the human high-affinity IgE receptor (FcεRI) on the cell surface,[16] were provided by Dr John Hakimi (F. Hoffmann-La Roche, Nutley, NJ). Sera (250 μL) of patients with AD were preincubated with 10 μg of TF alone or TF-rMGL_1304 resolved in 2 μL of tris buffer (50 mM tris-HCl, pH 7.5; 150 mM NaCl) overnight at 4°C, and IgE bound to the recombinant proteins was absorbed and removed by 50 μL of TARON resin at 4°C for 2 hours. RBL-48 cells were sensitized with 10 times diluted pretreated sera at 37°C overnight, washed twice with glucose/PIPES buffer,[17] and incubated with the same buffer for 10 minutes. The cells were then stimulated with 1 μg/mL of antihuman IgE antibody, 165 ng/mL of QR, or 3.23 μg/mL of Mite-Df (extract of *Dermatophagoides farinae* from LSL, Tokyo, Japan) for 15 minutes. The exocytosis of cells was quantified by measuring the hexosaminidase activity of the supernatants as previously described.[18]

Western blot analysis

Samples were loaded into a SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane as reported elsewhere.[17] The membrane was incubated with anti-Penta-His antibody (Qiagen), anti-Myc-tag antibody (Merck Millipore), 1:100 diluted sera of patients with AD, or 10 μg/mL of Smith-2 at 4°C overnight. The membrane-bound primary antibodies were

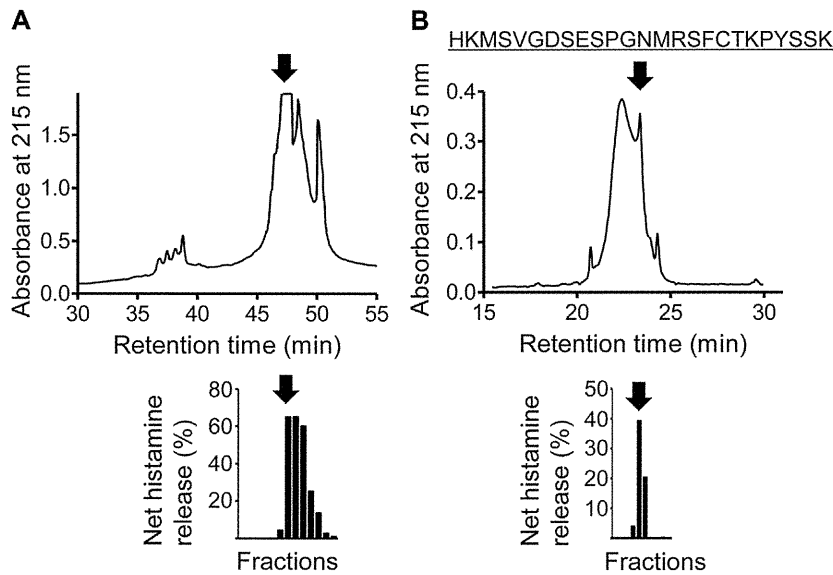

FIG 1. Identification of MGL_1304 in human sweat. QRX was purified with an Aqua 5μ-C18-200Å column **(A)** and with a Jupiter 5μ-C18-300Å HPLC column **(B)** based on histamine-releasing activity *(black bars in lower panels)*. The fraction indicated by *arrows* in Fig 1, *A* was further purified in Fig 1, *B*. The peptide sequence identified by mass spectrometry is shown in Fig 1, *B*.

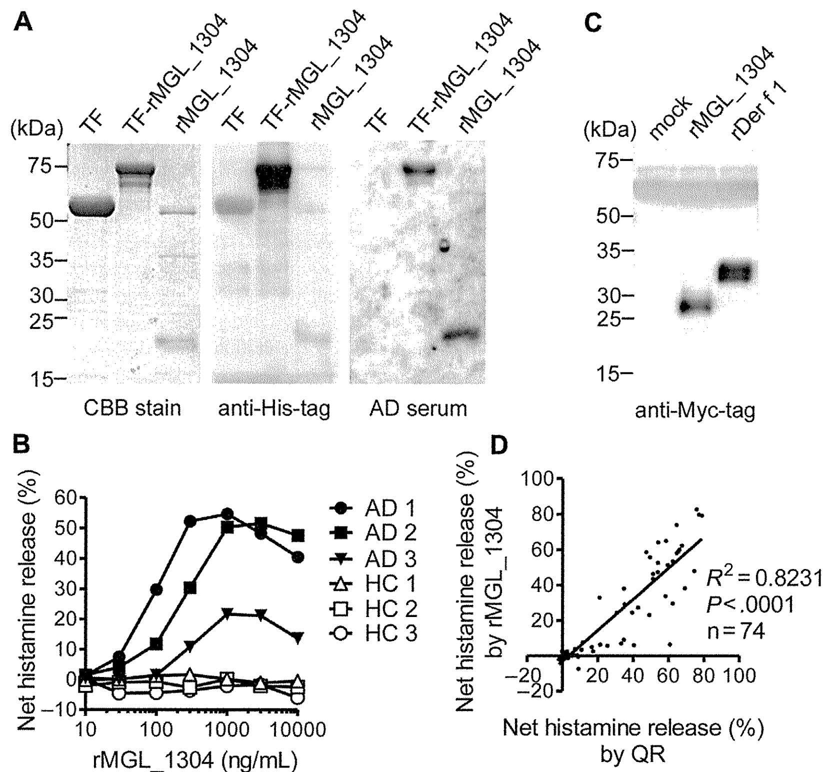

FIG 2. rMGL_1304 induces histamine release from basophils of patients with AD. **A,** TF, TF-rMGL_1304, and rMGL_1304 were stained by CBB *(left)*, or blotted with anti-His *(center)* and AD serum *(right)*. **B,** Histamine release in response to rMGL_1304 from basophils of patients with AD and of healthy controls *(HC)*. **C,** Supernatants of COS7 cells transfected with mock, MGL_1304, or Der f 1 were blotted with anti-Myc. **D,** Plot of histamine release from basophils of each patient with AD induced by QR and rMGL_1304. *CBB,* Coomassie brilliant blue.

visualized with horseradish peroxidase–conjugated secondary antibodies and chemiluminescence. In some experiments, 30 μL of sera of patients with AD was mixed with PBS and 10 μg of QRX or 1 μg of rMGL_1304 into 300 μL of reaction mixture, and preincubated at 4°C overnight. All images were adjusted by using "auto levels" in Adobe Photoshop (Adobe Systems, San Jose, Calif).

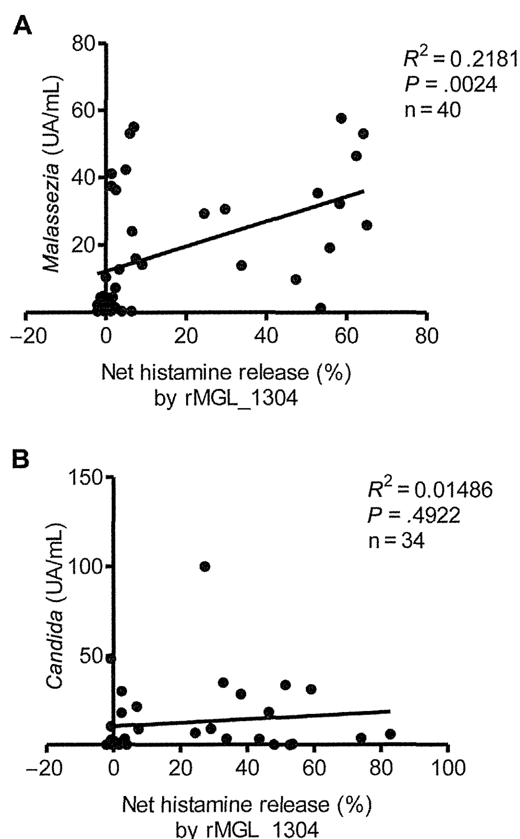

FIG 3. Correlation between histamine release against MGL_1304 and specific IgE against *Malassezia* and *Candida*. Plot of histamine release from basophils of each patient with AD induced by rMGL_1304 and specific IgE against *Malassezia* (**A**) and *Candida* (**B**) quantified by ImmunoCAP.

Purification of MGL_1304 from *M globosa*

M globosa was cultured in modified Dixon medium (ATCC medium 2693) in a shaking incubator at 32°C. The culture supernatants and cell pellets were collected by centrifugation. The pellets of *Malassezia* yeasts were suspended in PBS supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, Ill) and lysed by sonication. MGL_1304 was purified from the culture supernatant and lysate by the same procedure used for human sweat purification.

Measurement of the release of β-hexosaminidase from LAD2 human mast cells

The LAD2 human mast cell line was kindly provided by Dr Cem Akin (National Institutes of Health, Bethesda, Md) and maintained with StemPro-34 (Invitrogen) supplemented with 100 ng/mL of human stem cell factor (Wako Pure Chemical Industries, Osaka, Japan) as described previously.[19] Cells were incubated with 50 times diluted sera from a healthy control or a patient with AD, and then stimulated with semi-purified sweat antigen for 30 minutes. The percent release of β-hexosaminidase was calculated as described above.

Measurement of the release of IL-4 from human peripheral basophils

The peripheral blood basophils of patients with AD were purified with Basophil Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The basophils (2.3×10^4/well) were stimulated with 1 μg/mL of antihuman IgE, TF, TF-rMGL_1304, MGL_1304 purified from supernatant of *M globosa*, and 50 ng/mL of MGL_1304 purified from human sweat, and cultured for 5 days. The concentration of IL-4 in the culture supernatants was measured by using Human IL-4 Quantikine HS ELISA Kit (R&D systems, Minneapolis, Minn) according to the manufacturer's instructions.

Statistical analysis

The coefficient of determination and the *P* value were calculated in GraphPad Prism 5 (GraphPad Software, La Jolla, Calif) by linear regression.

Study approval

Blood samples from patients with AD and healthy volunteers were obtained with written informed consent, and the studies were approved by the ethics committee of Hiroshima University, Faculty of Medicine.

RESULTS

Identification of MGL_1304 in human sweat

The semi-purified product from human sweat, named QRX, was produced as reported previously[10,12] followed by a slight modification described in the Methods section. Further fractionations and purifications of the histamine-releasing activity on basophils of patients with AD were performed to obtain a single peak of substance (Fig 1). Mass spectrometric amino acid sequence analysis of the protein corresponding to the histamine-releasing activity containing a single UV-absorbing peak revealed a partial peptide sequence, HKMSVGDSESPGNMRSFCTKPYSSK, which by Blast search was revealed to be identical to 51-75 of the amino acid sequence of MGL_1304 from *M globosa* (Fig 1 and Fig E1 in this article's Online Repository at www.jacionline.org).

Recombinant MGL_1304 evoked histamine release from basophils of patients with AD

Recombinant MGL_1304-proteins were generated from *E coli* and COS7 cells. Western blotting revealed that TF-fused rMGL_1304 and rMGL_1304 whose TF was removed with HRV 3C protease were both immunoreactive to IgE antibodies of patients with AD (Fig 2, *A*). HRTs using peripheral blood basophil-enriched leukocytes showed that rMGL_1304, derived from *E coli*, induced histamine release from basophils of patients with AD but not from those of a healthy control (Fig 2, *B*). The histamine release induced by rMGL_1304 was dose-dependent and highest at a concentration around 1 μg/mL.

The COS7 cell-derived rMGL_1304 and rDer f 1 were also detected in the culture supernatants with their Myc tag (Fig 2, *C*). A good correlation coefficient was observed between histamine release induced by semi-purified sweat antigen, QR, and that induced by rMGL_1304, derived from COS7 cells, in 74 patients with AD (Fig 2, *D*) ($R^2 = 0.8231$, $P < .0001$). In addition, we performed HRTs of patients with AR against QR and rMGL_1304. Two out of the 7 patients with AR showed positive response against QR and rMGL_1304. The correlation between HRTs by rMGL_1304 and specific IgE against *Malassezia* was significant but not strong, although higher than that between HRTs by rMGL_1304 and specific IgE against Candida (Fig 3, *A* and *B*).

MGL_1304 is a critical allergen in the semi-purified sweat antigen

The cross-reactivity of patients' sera against semi-purified sweat antigen and those against rMGL_1304 was studied with immunoblot analysis and hexosaminidase release assay with RBL-48 cells, which express the human FcεRI α subunit, to which human IgE binds.[16] As shown in Fig 4, *A*, sera of all

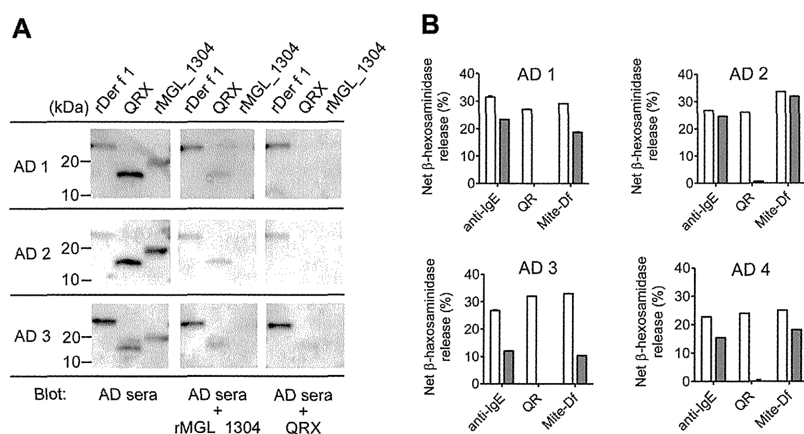

FIG 4. rMGL_1304 captures immunoreactive IgE against the semi-purified sweat antigen from sera of patients with AD. **A,** Antigens (rDer f 1, QRX, and rMGL_1304) were blotted with sera of patients with AD, sera pretreated with rMGL_1304, or sera pretreated with QRX. **B,** RBL-48 cells were sensitized by sera treated with TF or TF-MGL_1304, and then stimulated with anti-IgE, QR, or the mite extract (Mite-Df). Data were expressed as mean ± SEM of net release of β-hexosaminidase (%) in 3 independent experiments done in duplicate.

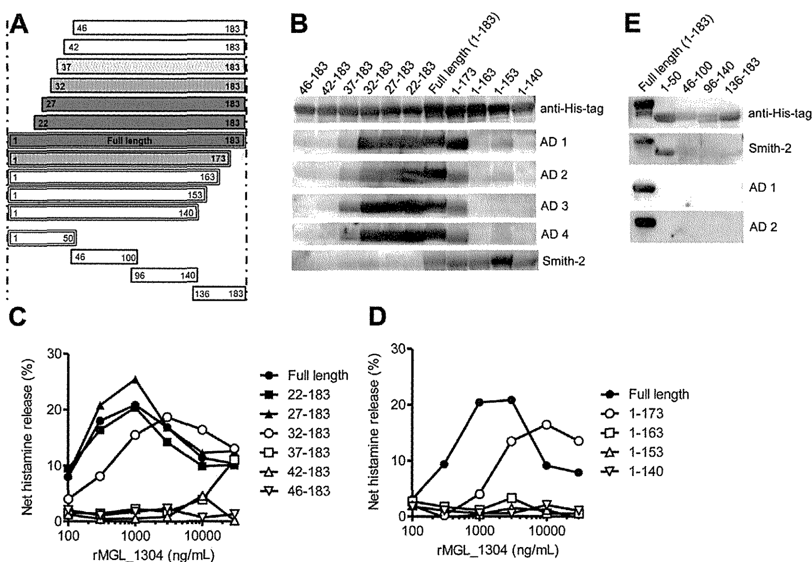

FIG 5. IgE of patients with AD recognizes the conformational structure of MGL_1304. **A,** A schematic figure of MGL_1304 mutants. The density of *dots of bars* indicated the histamine-releasing activity and IgE-binding ability. The *bars highlighted by double lines* represent immunoreactivity to Smith-2. **B** and **E,** The truncated proteins were blotted with anti-His, sera from patients with AD, or Smith-2. **C** and **D,** Histamine release from basophils with rMGL_1304 mutants that lack amino acids in the N-terminus (Fig 5, *C*) or the C-terminus (Fig 5, *D*).

3 patients contained IgE antibodies against rDer f 1, QRX, and rMGL_1304. However, the preincubation of patients' sera with rMGL_1304 or QRX resulted in the loss of IgE binding to QRX and rMGL_1304, respectively, whereas IgE binding to rDer f 1 was not affected.

We further treated sera of patients with polyhistidine-tagged and TF-fused rMGL_1304 followed by incubation with TARON resin to eliminate MGL_1304-specific IgE. RBL-48 cells were then sensitized with this treated sera at 37°C overnight, and stimulated with QR or Mite-DF. As shown in Fig 4, *B*, the removal of MGL_1304-specific IgE resulted in the loss of hexosaminidase release from RBL-48 cells in response to QR, whereas the hexosaminidase release against mite extract remained the same. These results indicated that rMGL_1304 contains the epitope of the semi-purified sweat antigen.

Identification of the core, immunoreactive structure of MGL_1304

A set of proteins lacking various length of amino acid sequences from the N-terminus or the C-terminus were generated as shown in Fig 5, *A*. IgE from patients with AD did not bind to rMGL_1304 proteins lacking more than 32 amino acids from the N-terminal end, or proteins lacking more than 10 amino acids from the C-terminal end (Fig 5, *B*). Histamine-releasing activities of these recombinant proteins (Fig 5, *C* and *D*, and Fig E2 in the Online Repository at www.jacionline.org) were almost identically confined to the IgE immunoreactivity shown in the immunoblots (Fig 5, *B*). None of N-terminally truncated proteins were immunoreactive to Smith-2 (Fig 5, *B*). We also generated several other mutant proteins that contain 1-50, 46-100, 96-140, or 136-183 amino acids (Fig 5, *A*). The binding of Smith-2 antibody was

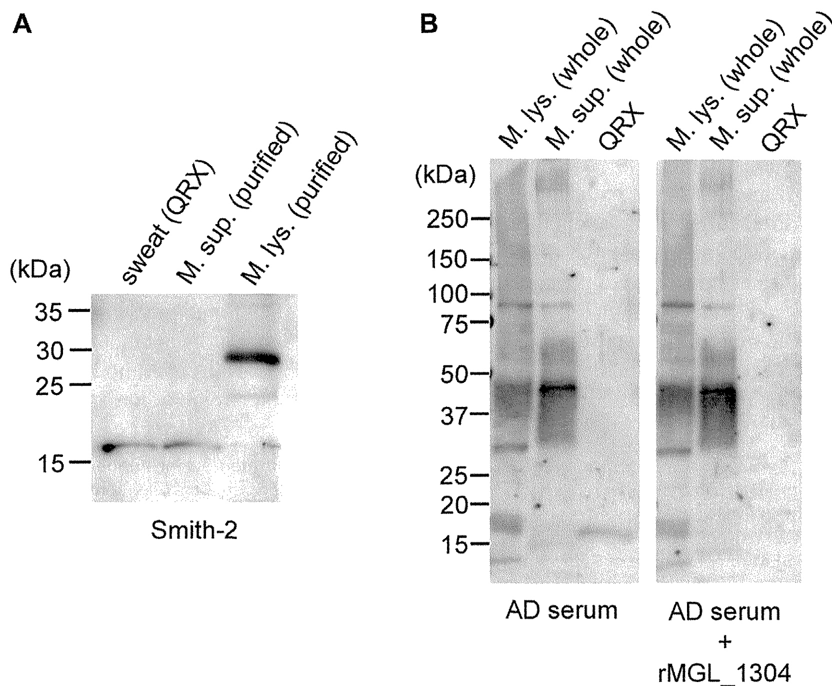

FIG 6. MGL_1304 is cleaved and secreted into sweat from *M globosa*. **A,** Histamine-releasing activity was purified from sweat (QRX), culture supernatants (M. sup. [purified]), and lysates (M. lys. [purified]) of *M globosa*. The purified proteins were blotted with Smith-2. **B,** The whole lysate (M. lys. [whole]) and whole culture supernatant (M. sup. [whole]) of *M globosa*, and QRX were blotted by sera of patients with AD or sera pretreated with rMGL_1304.

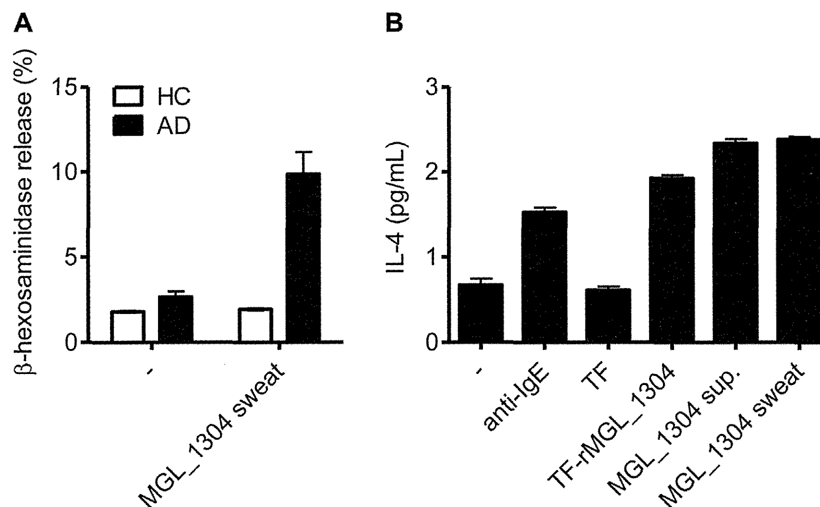

FIG 7. MGL_1304 induces degranulation of LAD-2 cells and cytokine production of human basophils. **A,** LAD-2 cells were sensitized with sera of HC and patients with AD, and then stimulated with MGL_1304 purified from sweat. The release percent of β-hexosaminidase was calculated from 3 independent experiments (mean ± SEM). **B,** Purified human basophils from AD were stimulated with MGL_1304 from different sources. The released IL-4 level was measured in triplicate (mean ± SEM). Similar results were obtained in 2 independent experiments with basophils of different donors. *HC,* Healthy controls.

detected by using the mutant that contained 1 to 50 amino acids of MGL_1304. However, none of the 4 slightly overlapping recombinant proteins was detected by immunoblotting using sera of patients with AD (Fig 5, *E*) and did not induce histamine release from basophils of patients with AD (data not shown). These results indicated that IgE from patients with AD bind to the conformational structure of MGL_1304 protein but not short peptide sequences.

MGL_1304 is cleaved and secreted into sweat from *M globosa*

We applied the method of purification for the semi-purified sweat antigen on culture supernatant and lysate of *M globosa*. Their histamine-releasing activity and molecular size were examined by basophil HRT and Western blotting, respectively. Both culture supernatant and lysate showed histamine-releasing

activity. However, MGL_1304-like immunoreactivity against Smith-2 antibody in the lysate appeared at approximately 29 kDa in the immunoblot analysis, whereas those in sweat and in the culture supernatant were 17 kDa (Fig 6, *A*). Western blot analysis of whole lysate and whole culture supernatant of *M globosa* showed that IgE from patients with AD dominantly binds a 42-kDa protein (Fig 6, *B*) in accordance with a previous report of a *M globosa* antigen.[20] Preincubation of patients' sera with rMGL_1304 resulted in the loss of IgE binding to QRX, whereas the IgE-binding patterns to whole lysate and whole culture supernatant of *M globosa* were not affected. These results indicate that MGL_1304 was generated in *M globosa* as a posttranslationally modified 29-kDa protein, and cleaved into a smaller fragment, so as to be secreted and dissolved in human sweat.

MGL_1304 induces degranulation of human mast cells and cytokine production of human basophils

To determine whether MGL_1304 could induce degranulation of human mast cells, we utilized the human mast cell line, LAD-2. LAD-2 cells sensitized with serum of a patient with AD, but not with serum of healthy control, released β-hexosaminidase in response to the semi-purified sweat antigen (MGL_1304 purified from sweat) (Fig 7, *A*). Moreover, purified basophils of patients with AD released IL-4 in response to rMGL_1304, and MGL_1304 purified from supernatant of *M globosa* and sweat (Fig 7, *B*).

DISCUSSION

Numerous kinds of microbes (eg, bacteria, fungi, and viruses) colonize the surface of human skin.[21] Several reports provided evidence that *Malassezia* species could induce skin inflammations related to diseases, such as pityriasis vesicolor, seborrheic dermatitis, *Malassezia* folliculitis, and even AD.[22] The presence of anti-*Malassezia* IgE in patients with AD[23-26] and the effectiveness of antimycotic treatments on dermatitis in the area of seborrhea (head and neck)[27-29] and the flexural area[30] all suggested the involvement of *Malassezia* species in the pathogenesis of AD. Moreover, a major therapeutic agent for AD, tacrolimus, was reported to have antifungal activities against *Malassezia*.[31] In this study, we have identified the molecule, MGL_1304, that is produced by *Malassezia* and secreted into human sweat.

The genus *Malassezia* consists of 14 species,[32] including *Malassezia furfur*, *Malassezia sympodialis*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia slooffiae*, *Malassezia obtuse*, and *Malassezia pachydermatis*. A homology search showed that Mala s 8, an allergen from *M sympodialis*,[33,34] shares 68.5% of the amino acid sequence with MGL_1304. Among them, *M globosa* tends to grow rapidly at higher temperature (37°C), higher concentration of sodium chloride, and lactic acid *in vitro*.[35] In fact, it is a major pathogen of *Malassezia* folliculitis occurring in summer. Therefore, it may grow better than other *Malassezia* species in areas with sweat. Moreover, both *M globosa* and *M restricta* are dominant in *Malassezia* microflora on the skin of patients with AD.[36] These observations imply the importance of *M globosa* and MGL_1304 secreted from *M globosa*, but not Mala s 8 from *M sympodialis*, in the pathogenesis of AD. In fact, the histamine-releasing ability of recombinant Mala s 8 in basophils from patients with AD was much weaker than that of MGL_1304 (unpublished data). Furthermore, Ishibashi et al[20] reported that a 42-kDa protein (MGp42) was the major allergen of *M globosa* relevant for AD by using immunoblot analysis of crude lysates of *M globosa*. We also detected an approximately 42-kDa protein as a major antigen in both lysates and culture supernatants of *M globosa* by Western blotting using sera of patients with AD. Nevertheless, both histamine-releasing activity in sweat and that in culture supernatants coincided with the 17-kDa form of MGL_1304, but not the other antigens detected by Western blotting. It is noteworthy that conventional Western blotting of *M globosa* by sera of patients with AD barely detects MGL_1304 among the other antigens that bind to IgE, but do not induce histamine release. Moreover, no MGL_1304-derived peptides were detected by electrospray ionization-mass spectrometry under ordinary conditions to detect cationic peptides without alkylation.

The reason why MGL_1304, but not other numerous antigens derived from *Mglobosa*, is detected as a major allergen in human sweat is not clear. However, MGL_1304 is likely a major allergen with histamine-releasing activity in the secreted proteins of *M globosa*, because the chromatographic profile of histamine-releasing activity of the culture supernatant of *M globosa* was very similar to that of human sweat (data not shown). The mechanism for MGL_1304 to enter skin is also a matter of discussion. It may penetrate either via sweat glands or through defects in the skin barrier due to microinjuries. The localization of MGL_1304 in skin is now under investigation. Alternatively, it is feasible that certain endogenous substances may cross-react with IgE against MGL_1304. Such cross-reactivity of IgE has already been observed between an antigen of *M sympodialis* (Mala s 11) and hMnSOD.[24,37] No homologous protein of MGL_1304 has been reported to date in mammalians including *Homo sapiens*. Moreover, the cross-reactivity between MGL_1304 and hMnSOD was not observed (see Fig E3 in this article's Online Repository at www.jacionline.org). Further studies of the tertiary structure of the epitope may help in the identification of a corresponding endogenous substance in humans.

In conclusion, MGL_1304 is a major allergen in human sweat that elicits histamine release via specific IgE. Further studies of MGL_1304 characteristics in sweat and skin tissues would be beneficial to develop new treatments for patients with AD and remedies to prevent the onset of this recalcitrant disease.

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Key messages

- **Sweat is a major aggravating factor of atopic dermatitis (AD), and known to cause allergic reactions in patients with AD, but the antigen in sweat has not been determined.**
- **Basophil histamine-releasing activity in sweat was purified into a single peaked protein and identified as MGL_1304 produced by *Malassezia globosa*.**
- **MGL_1304 is a major allergen in human sweat and could cause type I allergy in patients with AD.**

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Match to: **gi|164661827** Score: **100**
hypothetical protein MGL_1304 [Malassezia globosa CBS 7966]
Found in search of T868_B2-B5_merge.mgf

Nominal mass (M_r): **20547;** Calculated pI value: **5.12**

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  1 MVSLNIFSAA FVASLASAVF AAPSALERRA APDNTVWVTS VADHCLILPR
 51 HKMSVGDSES PGNMRSFCTK PYSSKQGQLA SDFWTKAHFK KTDKYVQITG
101 CINPNVQSTL LSNDEGGQYD SNGGEGGRGN PAGSVCLGYS SYVELVEPAG
151 NRACIRCCYD PSDCDVSQDE AGCETVIPGK YDC
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Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
51 - 65	549.8653	1646.5742	1646.7141	-0.1399	1	R.HKMSVGDSESPGNMR.S Oxidation (M) (Ions score 26)
51 - 65	555.1953	1662.5642	1662.7090	-0.1448	1	R.HKMSVGDSESPGNMR.S 2 Oxidation (M) (Ions score 18)
53 - 65	691.7440	1381.4734	1381.5602	-0.0868	0	K.MSVGDSESPGNMR.S Oxidation (M) (Ions score 31)
53 - 65	699.7040	1397.3934	1397.5551	-0.1617	0	K.MSVGDSESPGNMR.S 2 Oxidation (M) (Ions score 52)
53 - 65	699.7140	1397.4134	1397.5551	-0.1417	0	K.MSVGDSESPGNMR.S 2 Oxidation (M) (Ions score 31)
53 - 65	699.7240	1397.4334	1397.5551	-0.1217	0	K.MSVGDSESPGNMR.S 2 Oxidation (M) (Ions score 50)
66 - 75	418.1553	1251.4442	1251.5958	-0.1516	0	R.SFCTKPYSSK.Q (Ions score 20)
66 - 75	418.1653	1251.4742	1251.5958	-0.1216	0	R.SFCTKPYSSK.Q (Ions score 22)
66 - 75	418.1653	1251.4742	1251.5958	-0.1216	0	R.SFCTKPYSSK.Q (Ions score 21)

FIG E1. Summary of mass spectrometric analysis. Mass spectrometric amino acid sequence analysis of the protein corresponding to the histamine-release activity containing UV-absorbing peak (Fig 1) revealed a partial peptide sequence, HKMSVGDSESPGNMRSFCTKPYSSK, which by Blast search was revealed to be identical to 51-75 of amino acid sequence of MGL_1304 from *Malassezia globosa*.

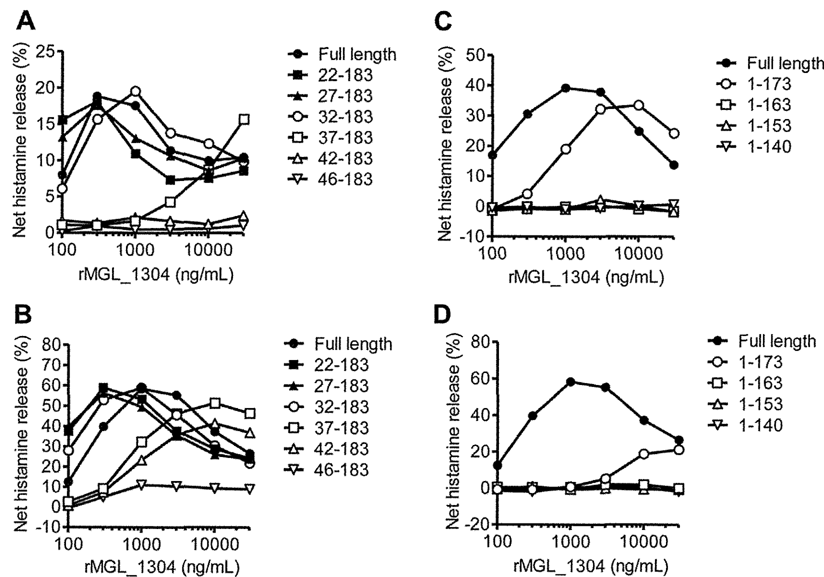

FIG E2. HRTs by the use of rMGL_1304 mutants. Histamine release with mutant rMGL_1304 proteins that lack amino acids in the N-terminus **(A** and **B)** or the C-terminus **(C** and **D)**. Each panel shows independent data for a patient with AD performed in duplicate.

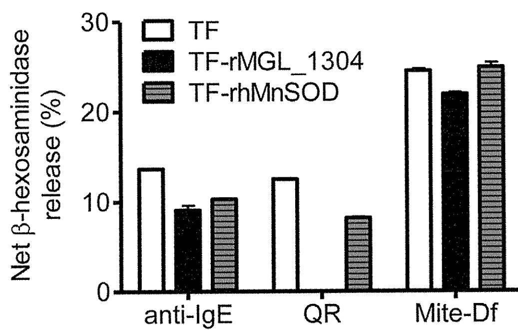

FIG E3. Immunoreactive IgE against the semi-purified sweat antigen does not cross-react with hMnSOD. RBL-48 cells were sensitized with serum of a patient with AD that was pretreated with TF, TF-rMGL_1304, or TF-rhMnSOD, and then stimulated with anti-IgE, QR, or the mite extract (Mite-Df), respectively. Data were expressed as mean ± SEM of net release of β-hexosaminidase (%) performed in triplicate.

TABLE E1. Cloning primers

Cloning primers
Cloning primers for MGL_1304 expression vectors
pSecTag2/Hygro-MGL_1304:
sense: 5′-GGGGTACCGTATCCCTCAACATTTTCTCAGCTGC-3′
antisense: 5′-GCAGATATCCAGTCGTACTTGCCGGGGATGAC-3′
pCold TF-MGL_1304:
sense: 5′-GGGGTACCGTATCCCTCAACATTTTCTCAGCTGC-3′
antisense: 5′-CCCAAGCTTTTAGCAGTCGTACTTGCCGGGGATG-3′
Cloning primers for Der f 1 and hMnSOD
pSecTag2/Hygro-Der f 1:
sense: 5′-CGGGATCCAAATTCGTTTTGGCCATTGCCTC-3′
antisense: 5′-CCGCTCGAGACATGATTACAACATATGGATATTG-3′
pCold TF-hMnSOD
sense: 5′-GGGGTACCAGCACTAGCAGCATGTTGAGC-3′
antisense: 5′-GCTCTAGACAGCATAACGATCGTGGTTTAC-3′
Cloning primers for the truncated MGL_1304 mutants with pCold TF
22-183 sense: 5′-GGAATTCCATATGGCCCCATCTGCTCTCGAGCGACG-3′
27-183 sense: 5′-GGAATTCCATATGGAGCGACGGGCTGCCCCTGAC-3′
32-183 sense: 5′-GGAATTCCATATGCCTGACAACACGGTTTGGGTGACC-3′
37-183 sense: 5′-GGAATTCCATATGTGGGTGACCAGTGTAGCGGACCAC-3′
42-183 sense: 5′-GGAATTCCATATGGCGGACCACTGCCTTATTCTTCC-3′
46-183 sense: 5′-GGGGTACCCTTATTCTTCCCCGCCATAAGATG-3′
1-173 antisense: 5′-CCCAAGCTTCTAACAGCCAGCCTCGTCTTGACTGAC-3′
1-163 antisense: 5′-CCCAAGCTTCTAATCGCTTGGGTCATAGCAGCAACG-3′
1-153 antisense: 5′-CCCAAGCTTCTAGGCACGGTTACCCGCAGGCTCAAC-3′
1-140 antisense: 5′-CCCAAGCTTCTATGAATAACCCAGACACACGGAGCC-3′
1-50 antisense: 5′-CCCAAGCTTCTAGCGGGGAAGAATAAGGCAGTGGTC-3′
46-100 antisense: 5′-CCCAAGCTTCTAGCCCGTAATCTGGACATACTTGTC-3′
96-140 sense: 5′-GGGGTACCGTCCAGATTACGGGCTGCATCAAC-3′
136-183 sense: 5′-GGGGTACCTGTCTGGGTTATTCATCTTATGTCGAG-3′

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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 (UA/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-

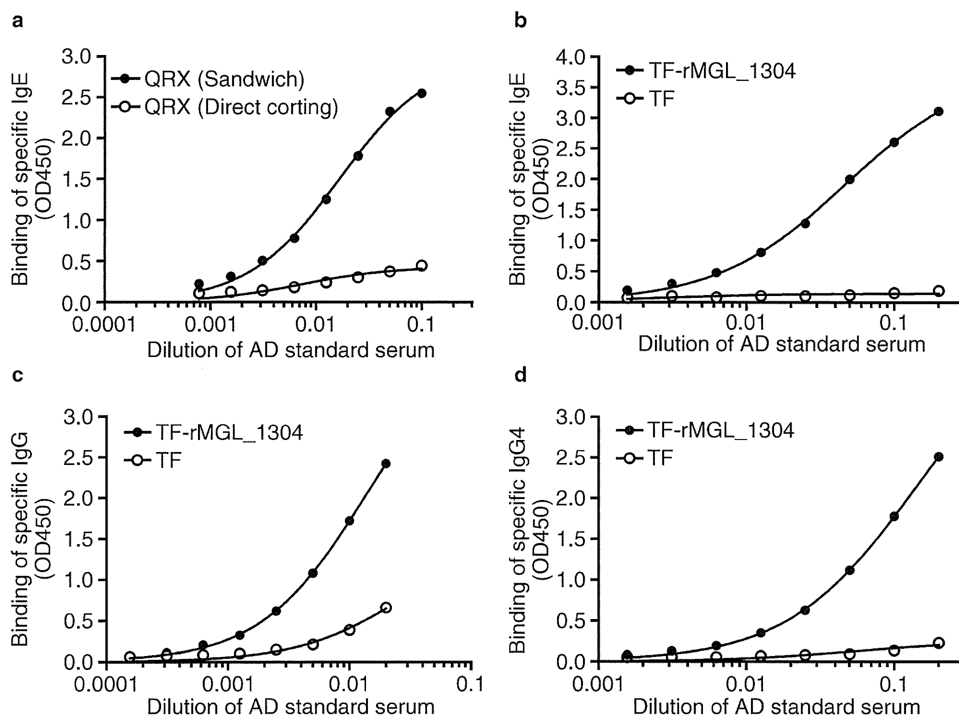

Fig. 1 Standard curves of MGL_1304-specific immunoglobulins plotted by using AD standard serum in ELISA. QRX-specific IgE in the serial diluted AD standard serum was measured by direct and sandwich ELISA (**a**). rMGL_1304-specific IgE (**b**), rMGL_1304-specific IgG (**c**), and rMGL_1304-specific IgG4 (**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-

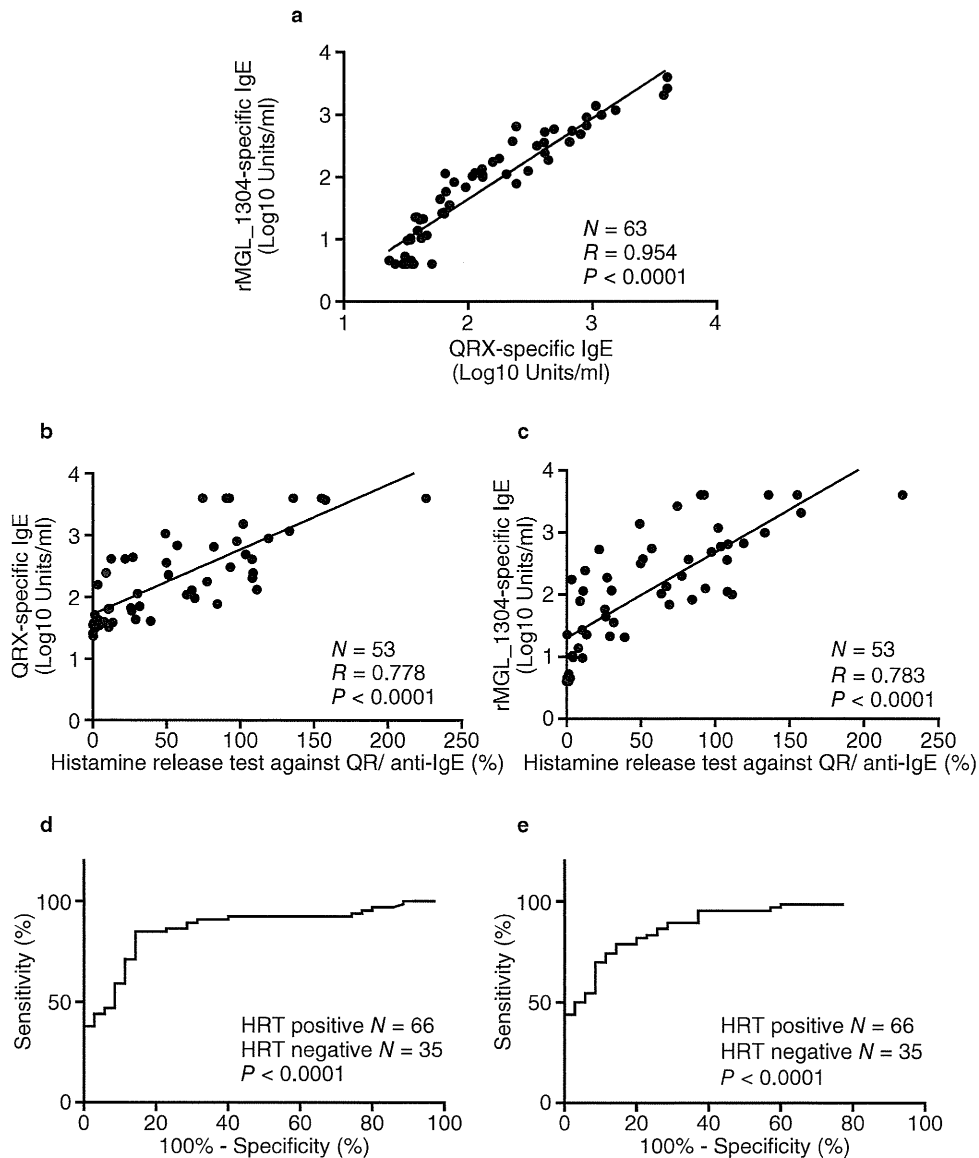

Fig. 2 Correlation between QRX-specific IgE, rMGL_1304-specific IgE, and basophil histamine release against QR. Levels of QRX- and rMGL_1304-specific IgE in sera of patients with AD (**a**). Histamine releases of basophils induced by QR are correlated with serum levels of QRX-specific IgE (**b**) and 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) x 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

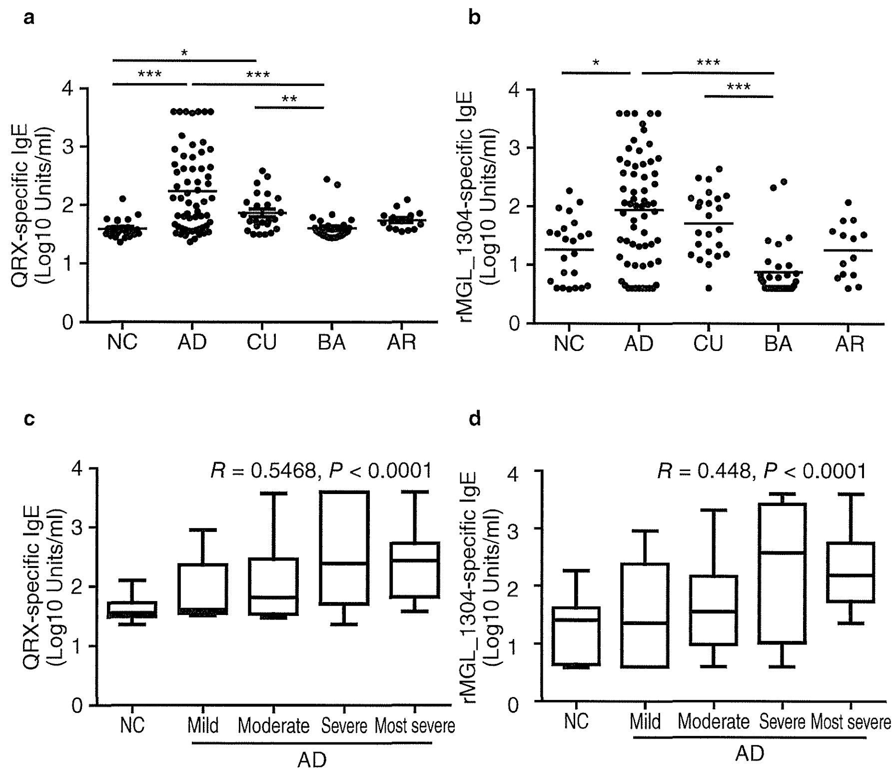

Fig. 3 Comparisons of serum levels of QRX- and rMGL_1304-specific IgE among diseases and correlations with severities of AD. Serum levels of QRX-specific IgE (**a**) and rMGL_1304-specific IgE (**b**) in patients with each disease were plotted and analyzed by Kruskal-Wallis test. The serum levels of 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

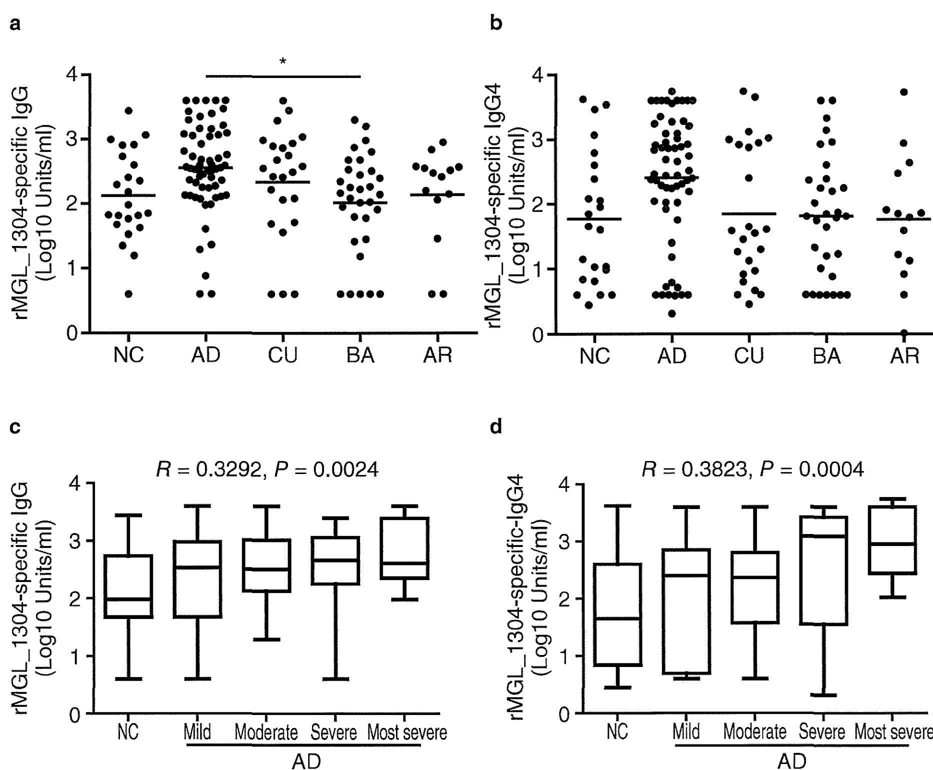

Fig. 4 Serum levels of rMGL_1304-specific IgG and IgG4 in diseases and their correlations with severities of AD. Serum levels of rMGL_1304-specific IgG (**a**) and IgG4 (**b**) in each disease were plotted and analyzed by Kruskal-Wallis test. The serum levels of rMGL_1304-specific IgG (**c**) and IgG4 (**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

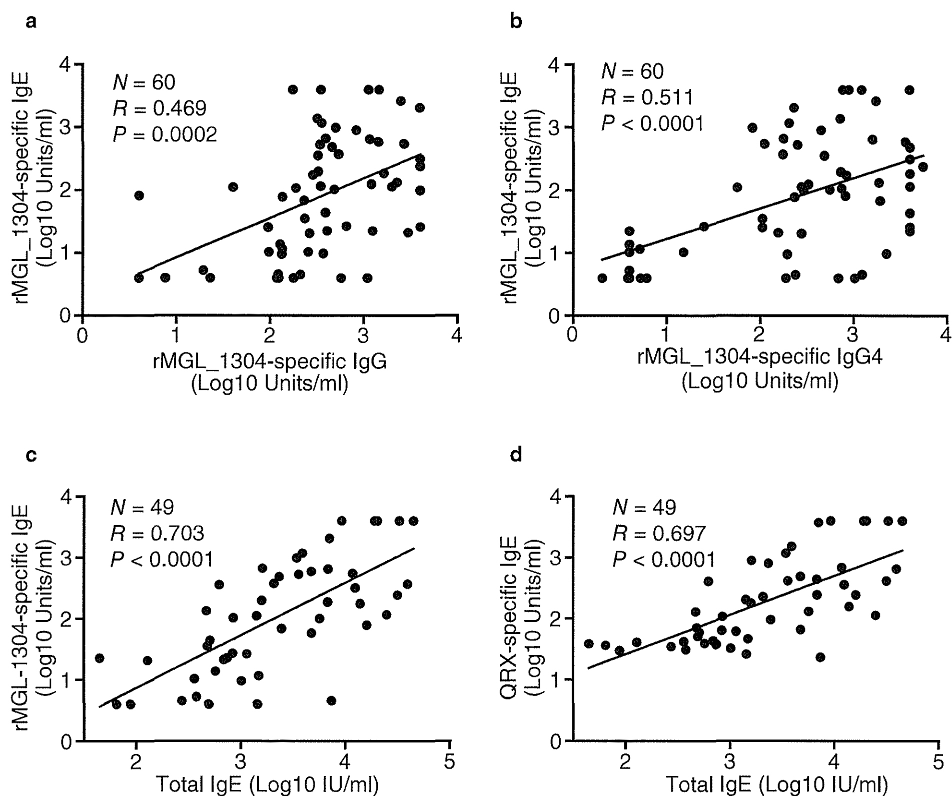

Fig. 5 Correlations among rMGL_1304-specific immunoglobulins in sera of patients with AD. Significant correlations were observed between serum levels of rMGL_1304-specific IgE and rMGL_1304-specific IgG (**a**), rMGL_1304-specific IgG4 (**b**), or total IgE (**c**) in patients with AD. The levels of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