A human monoclonal IgE antibody that binds to MGL_1304, a major allergen in human sweat, without activation of mast cells and basophils

Kaori Ishii, Makiko Hiragun, Takaaki Hiragun, Takanobu Kan, Tomoko Kawaguchi, Yuhki Yanase, Akio Tanaka, Shunsuke Takahagi, Michihiro Hide*

Department of Dermatology, Institute of Biochemical and Health Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8851, Japan

Abstract

MGL_1304, a major allergen in human sweat for patients with atopic dermatitis and/or cholinergic urticaria, is secreted from Malassezia globosa on human skin. The amounts of MGL_1304 and IgE against MGL_1304 are evaluated by the histamine release test using basophils or mast cells sensitized with serum containing IgE against MGL_1304, and enzyme linked sorbent assay (ELISA) using MGL_1304 and anti-MGL_1304 antibodies. Here, we identified a human monoclonal IgE (ABS-IgE) that binds to the high affinity IgE receptor (FcεRI) and MGL_1304 with high affinity (KD = 1.99 nM) but does not release histamine from basophils and mast cells. An ELISA using ABS-IgE as a standard IgE revealed that the amount of IgE against MGL_1304 (1000 U/ml) in the standard sera of patients with AD, employed in our previous report, is 32 ng/ml. A sandwich ELISA using ABS-IgE as a detection antibody showed approximately 10 times lower detection limit for MGL_1304 than ELISA in which MGL_1304 is directly bound to an ELISA plate. Moreover, ABS-IgE prevented histamine release from mast cells and basophils by neutralizing MGL_1304 not only in a free form in solution, but also on FcεRI expressed on the cell surface without cell activation. ABS-IgE may be used both to quantify the amount of MGL_1304 and anti-MGL_1304 IgE, and possibly for the treatment of diseases caused/aggravated by type I allergy to MGL_1304.

1. Introduction

Atopic dermatitis (AD) is a chronic pruritic skin disease occurring in an atopic background associated with altered skin barrier and immune dysregulation [1]. The impairment of the innate immune system in AD is augmented by numerous triggering factors, such as irritants, aeroallergens, food, microbial organisms and sweating [2]. We previously reported that skin test with autologous sweat was positive in approximately 80% of the patients with AD and that the semi-purified sweat antigen induced histamine release from the basophils of 77% of patients with AD [3,4] and 66% of patients with cholinergic urticaria [5]. We finally have identified a fungal protein, MGL_1304, derived from Malassezia globosa (M. globosa), which belongs to the normal human cutaneous flora, as an antigen in sweat for patients with AD [6]. Recently, it has been revealed that human skin surface is colonized by a wide range of fungi, such as M. globosa, Malassezia restricta and Malassezia sympodialis [7]. However, the major antigens of M. globosa recognized by serum IgE of the patients [8] were different from MGL_1304, and not detected in sweat [6]. Moreover, the pathogenic role of commensal Malassezia yeasts in skin diseases has been a matter of controversy for a long time [9]. Therefore, quantification of specific IgE against MGL_1304 is critical in the assessment and effective skin care for AD. We previously established an ELISA for specific immunoglobulins against MGL_1304 in sera of patients with AD, using a mouse monoclonal antibody Smith2, raised against purified sweat antigen [10]. By that assay, the amount of MGL_1304-specific IgE may be quantified in comparison with a bulk stock of sera collected from patients with AD, but not measured as weight/volume concentrations. In this study, we examined several human monoclonal IgE antibodies and have identified a clone that specifically binds to MGL_1304. Unexpectedly, it causes histamine release in response to anti-IgE, but not by MGL_1304 when bound to mast cells and basophils. We revealed the binding characteristics of this IgE antibody, and incorporated it as a standard IgE in ELISA to...
measure human IgE against MGL_1304, and as a capture antibody in ELISA to detect MGL_1304 at high sensitivity, as well.

2. Materials and methods

2.1. Purification of MGL_1304 from culture of M. globosa

MGL_1304 was purified from culture supernatant of M. globosa as previously described [6]. The purified MGL_1304 from M. globosa was named as “sMGL_1304”. The amount of sMGL_1304 was measured as the amount of protein using a protein assay kit, Micro BCA™ protein assay (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Recombinant proteins

Each recombinant MGL_1304 fused with the Triggering Factor (TF) and tagged with polyhistidine (His-tag) were expressed by Escherichia coli and purified as previously described (TF-MGL_1304) [6].

2.3. Enzyme-linked immunosorbent assay (ELISA)

For the ELISA with direct-coated antigen, microtiter 96-well plates (Greiner, Frickenhausen, Germany) were coated with 1 μg/ml or a titrated concentrations of sMGL_1304, cedar pollen extract (LSL, Tokyo, Japan), or Der f1 (Asahi Beer, Tokyo, Japan) in phosphate-buffered saline (PBS) at 4 °C overnight. After aspiration, the plates were blocked with 5% skim milk in PBS at room temperature for 1hr. After washing three times with wash buffer (0.05% Tween 20 in PBS), four commercially available human IgE clones (ANTIBODYSHOP in BioPorto Diagnostics A/S, Gentofte, Denmark; Merck Millipore, Darmstadt Germany; GenWay Biotech, San Diego, CA, USA; AbD Serotec, Oxford, UK) or Smith2, a mouse monoclonal antibody against MGL_1304, obtained by immunization with semi-purified human sweat antigen [6], at indicated concentrations in 0.5% skim milk in PBS, were incubated at room temperature for 1hr. After washing four times, each well was incubated with horse-radish peroxidase-conjugated goat anti-human IgE (KPL, Gaithersburg, MD, USA) or horse-radish peroxidase-conjugated horse anti-mouse IgG (Cell Signaling Technology, Boston, MA, USA) diluted at 1/2000 in 0.5% skim milk at room temperature for 1 h. Color development was achieved by substrate solution (TMB Microwell Peroxidase Substrate System; KPL) and stopped by 1% hydrochloric acid based solution. The absorbance at 450 nm was measured using a microplate spectrophotometer (Benchmark plus®; BIO-RAD, Berkeley, CA, USA). For the inhibition of IgE binding to TF-MGL_1304 or sMGL_1304, the human monoclonal IgE obtained from ANTIBODYSHOP (ABS-IgE) was pre-incubated at 50 ng/ml with indicated concentrations of sMGL_1304 or TF-MGL_1304 at 4 °C overnight and used as detection antibody after blocking with 5% skim milk as described above. For sandwich ELISA, 50 μl of Smith2 at 5 μg/ml in PBS was incubated in a 96-well plate at 4 °C overnight. The blocking and washing methods were the same as those for the direct-coat antigen assay. After blocking and washing, 100 μl of various concentrations of sMGL_1304 or pre-filtered sweat were added and incubated in each well at room temperature for 2 h. After washing four times, 100 μl of ABS-IgE at 0.5 μg/ml in 0.5% skim milk in PBS was incubated in each well at room temperature for 1 h. Subsequent reactions of HRP-conjugated antibody and detection were performed as same as the direct-coat antigen assay. All assays were performed in triplicate.

2.4. Surface plasmon resonance experiments

The binding characteristics between sMGL_1304 and ABS-IgE were studied by surface plasmon resonance (SPR) using the Biacore X™ instrument (GE Healthcare, Buckinghamshire, UK), and HEPES buffered saline (150 mM NaCl, 3.4 mM EDTA pH 7.4) for sample perfusion. Diluted sMGL_1304 (29.4, 58.8, 294, 588 nM) were injected serially at flow rate of 20 μl/min to ABS-IgE immobilized on a sensor chip with amino-coupling method according to the manufacturer’s instructions.

2.5. Western blot analysis

Samples were subjected into 5–20% gradient SDS-PAGE or 15% Native-PAGE gel (ATTO, Tokyo, Japan) and transferred to a polyvinylidene fluoride membrane as described previously [6]. Immunoblotting was performed with 1 μg/ml of ABS-IgE, Smith2, or anti-His-tag antibody (Merck Millipore, Darmstadt, Germany) at 4 °C overnight. For visualization, horseradish peroxidase-conjugated secondary antibodies and chemiluminescence were used. All images were adjusted by using “auto levels” function of Adobe Photoshop® Ver. 13.0 (Adobe System, San Jose, CA, USA).

2.6. Histamine release test (HRT)

The histamine release test (HRT) with human basophils or RBL-48 cells, a rat mast cell line expressing the α-subunit of the human high-affinity IgE receptor (FcεRI), was performed as described previously [3]. Sweat samples were collected from nine healthy volunteers and filtered with a 0.2 μm filter (PALL, Port Washington, NY, USA). In HRT, basophils were stimulated with five-times-diluted sweat or MGL_1304 pre-incubated with or without ABS-IgE (Fig. 3B, Fig. 4A and B). For HRT using basophils re-sensitized with IgE, basophils of a non-atomic volunteer were treated with lactic acid solution (pH 3.9) to remove endogenous IgE, and incubated with twice-diluted serum of a patient with AD or 100 ng/ml of ABS-IgE. RBL-48 cells were incubated with ABS-IgE at 50 ng/ml or twenty-times diluted serum of a patient with AD or a normal control at 37 °C overnight. The cells were stimulated with 1 μg/ml of goat anti human IgE antibody (Bethyl Laboratories, TX, USA) or 1 μg/ml of 6F7, monoclonal antibody against FcεRI [11], 10 ng/ml of sMGL_1304 or 1 μg/ml of mite antigen (Mite-Df, LSL).

2.7. Statistical analysis

All data were obtained from three or more independent experiments in triplicate and shown as average ± standard deviation, or otherwise as described in figure legends. P values were calculated in Graph-Pad Prism 6 (GraphPad Software, La Jolla, CA, USA). Differences were considered as significant at values of P < 0.01 using one-way ANOVA and Dunnett’s multiple comparison test.

2.8. Study approval

Sweat samples from healthy volunteers and blood samples from patients with AD and healthy volunteers were obtained with written informed consent. All studies were approved by the ethics committee of Hiroshima University.

3. Result

3.1. ABS-IgE recognizes MGL_1304

In a study to develop ELISA for IgE against MGL_1304, an IgE clone obtained from ANTIBODYSHOP which was employed as negative controls showed unexpected binding to MGL_1304 (Fig. 1A). We named this IgE as “ABS-IgE” and assessed its binding to MGL_1304, Der f1 (mite antigen) and cedar extract at various concentrations of
ABs-IgE. Neither Der f1 nor cedar extract was detected by ABs-IgE (Fig. 1B), whereas ABs-IgE bound to MGL_1304 in a dose-dependent manner of MGL_1304 coated on an ELISA plate. Moreover, all ABs-IgE binding at concentrations from 1 ng/ml to 1 μg/ml to sMGL_1304 that coated an ELISA plate at 1 μg/ml was abolished by the pre-incubation with or without 100 μg/ml TF-MGL_1304. Conversely, the binding of ABs-IgE to TF-MGL_1304 bound to an ELISA plate at 3 μg/ml was completely inhibited by pre-incubation with 10 μg/ml SMGL_1304. The binding of ABs-IgE (1 μg/ml) to TF-MGL_1304 or SMGL_1304 coating an ELISA plate at 3 μg/ml or 0.1 μg/ml was inhibited, respectively, by the other antigens in a dose-dependent manner. Western blotting of SMGL_1304 (0.5 μg/lane) in native condition using ABs-IgE and Smith2. Western blotting of TF-MGL_1304 (2 μg/lane) in reduced condition using ABs-IgE and anti-His-tag antibody. SPR analysis of the binding affinity between ABs-IgE and SMGL_1304. Diluted SMGL_1304 (29.4, 58.8, 294, 588 nM) was injected to immobilized ABs-IgE on a sensor chip. K<sub>a</sub>: association rate constant, K<sub>d</sub>: dissociation rate constant, KD: dissociation constant.

To identify the epitope of MGL_1304 recognized by ABs-IgE, we performed blot analyses with recombinant fragments of MGL_1304, regardless of the glycosylation. In western blot analyses under non-reduced condition without SDS (native gel), SMGL_1304 was detected by ABs-IgE as a smear band, as by Smith2 (Fig. 1G). In reduced condition, TF-MGL_1304 was detected as a solid band by either ABs-IgE or anti-His-tag antibody (Fig. 1H). We further analyzed the binding characteristics of ABs-IgE for MGL_1304 by SPR. The analysis verified that the affinity of ABs-IgE to MGL_1304 by the dissociation constant of 1.99 × 10<sup>-9</sup> M, which is similar to that for standard antigen–antibody reactions (10<sup>-6</sup> M > K<sub>D</sub> > 10<sup>-9</sup> M) (Fig. 1I).

To identify the epitope of MGL_1304 recognized by ABs-IgE, we performed blot analyses with recombinant fragments of MGL_1304 (Fig. 1J,K,L). Most binding to ABs-IgE was observed in the fragments of TF-MGL_1304 that preserve amino acids from the 46th to the end of C-terminal. In contrast, 10 or more amino acid truncations at C-terminal abolished the binding (Fig. 1K). The binding of ABs-IgE to the fragment of 46th amino acid to the C-terminal was detected by western blotting at the expected position of molecular weight. However, ABs-IgE hardly detected 4 overlapped fragments of MGL_1304 consisting of 1-50th, 51-100th, 101-150th, and 151-183th amino acids, respectively (Fig. 1L). These results suggest that ABs-IgE recognizes a conformational structure made by the sequence of 46th to 183th amino acid of MGL_1304, regardless of the glycosylation.
3.3. The measurement of MGL_1304 in human sweat

We next measured the amount of MGL_1304 in sweat collected from nine healthy donors by the sandwich ELISA using ABS-IgE as a capture antibody. The histamine release activity of the same sweat samples were also evaluated by using basophils of a patient with AD. Sweat samples subjected to HRT were diluted five times, taking into account that the minimal and the maximum release of histamine induced by human sweat [12]. The concentrations of MGL_1304 were determined as 0.6–8.0 ng/ml (3.76 ± 2.64 ng/ml, mean ± SD) (Fig. 3A), showing a strong correlation to the degree of histamine release by sweat samples (Fig. 3B). The concentration of IgE against MGL_1304 defined as 1 unit using the sera collected and pooled as a standard from 20 patients with AD in our previous study [10] was determined as 32 pg.

3.4. Neutralization of histamine release activity of MGL_1304 by ABS-IgE

The pretreatment of sMGL_1304 with 1 μg/ml ABS-IgE shifted the dose-dependent curve of MGL_1304 for histamine release to the right by close to one log (Fig. 4A). The histamine release induced by 3 ng/ml sMGL_1304, which induces the maximal release of histamine, was neutralized by ABS-IgE in a dose-dependent manner at 1–30 ng/ml. No further effect of ABS-IgE was observed at concentrations higher than 100 ng/ml (Fig. 4B).

3.5. ABS-IgE does not induce histamine release in response to MGL_1304

Since ABS-IgE is a human monoclonal IgE, it was predicted to sensitize human basophils and induce histamine release in response to MGL_1304. Indeed, basophils sensitized with ABS-IgE released histamine in response to anti-IgE, endorsing the occupation of FcεRI with IgE (Fig. 4C, D). The removal/decrease of endogenous IgE and re-sensitization with AD serum IgE was also verified by the reaction to 6F7, a monoclonal antibody that binds to the α-subunit of FcεRI in competition with IgE [11]. Nevertheless, basophils obtained from a patient with AD and those from a healthy control re-sensitized with ABS-IgE showed no release of histamine in response to sMGL_1304. Likewise, when RBL-48 cells, a rat mast cell line, expressing the α-subunit of human FcεRI, did not release histamine upon sMGL_1304 stimulation (Fig. 4E). These results indicate that ABS-IgE binds to MGL_1304 and FcεRI, but does not induce histamine release when they sensitize mast cells and basophils.

4. Discussion

The ABS-IgE clone has been established from a human B cell of a healthy donor and used as a non-specific IgE monoclonal antibody [13,14]. In this study, we demonstrated that ABS-IgE specifically binds to MGL_1304 with a Kd value of 1.99 nM and FcεRI, without histamine release from basophils and mast cells. Because of these unique characteristics, ABS-IgE has great advantages in both clinical diagnosis and treatment of diseases mediated by MGL_1304. In general, the amount of antigen-specific IgE is expressed using a unit of “International Unit (IU)” or “Ua”. The amount of 1 IU, equivalent to 2.4 ng IgE [15] was established as the amount of total IgE that binds to anti-IgE antibody in standard serum used as a reference in each assay [10]. The amount of antigen-specific IgE, however, was detected as the amount of IgE that specifically binds to the antigen. Thus, it largely depends on the binding characteristics of IgE to the antigens and may vary among different assay systems [16]. Therefore, it is expected to set standard IgE, which is antigen-specific, ideally monoclonal antibody for each antigen. Such a standard IgE provides data as weight (g)/volume (L), which is comparable among assays. ABS-IgE is suitable as a standard IgE, not only because of its human origin, but also because of its epitope. ABS-IgE was suggested to recognize a conformational structure of MGL_1304, as IgE in all four sera of the patients with AD examined in our previous study [6]. While the serum IgE recognized a structure mostly consisting of the amino acid sequence from the 46th to the C-terminal.

The second advantage of ABS-IgE is that it captures MGL_1304 preserving the epitopes for Smith2 mouse IgG monoclonal antibody. Based on this characteristic, we constructed a sandwich ELISA to measure the amount of MGL_1304 with high sensitivity. In
this sandwich ELISA, Smith2 coating of an ELISA plate captures MGL_1304 in sample solutions, and ABS-IgE detects the amount of MGL_1304 captured by Smith2 on the plate. By this system, the concentration of MGL_1304 in sweat of nine healthy volunteers was revealed as 3.76 ± 2.64 ng/ml, and showed a good correlation with the histamine release activity in sweat (r = 0.9197, p < 0.0001, n = 9, Fig. 3). The y-intercept shown in Fig. 3B at zero concentration of MGL_1304 suggests the presence of unidentified substances in sweat in addition to MGL_1304. Homologous proteins produced and released by the other Malassezia species, such as M. restricta, and M. sympodialis on human skin, is a candidate of such activities, and needed to be elucidated by a panel of specific antibodies and proteins produced by these Malassezia species. The high correlation of MGL_1304 concentration to the histamine release activity in human sweat suggests that MGL_1304 is the major antigen for histamine release, or ABS-IgE cross-reacts with MGL_1304 homologous antigens released from the other Malassezia species on the patients. As compared with sera, sweat is not easy to collect, and likely to vary in terms of MGL_1304 concentration by temperature, location of the body, seasons and exercise of the subjects. Thus, measuring the amount of MGL_1304 in local sweat on the surface of various sites of the body on various occasions may elucidate a detailed mechanism for sweat allergy. The assay developed in this study is suitable for such studies, since it requires only tens or less microliters of sweat to quantitate the amount of MGL_1304. Moreover, ABS-IgE may also be used as a standard for assays to measure the amount of anti-MGL_1304 IgE in human sera, since it is human IgE. By this assay, 1 unit of anti-MGL_1304 IgE, defined in our previous study, has been revealed as 32 pg, and the titer of serum IgE against MGL_1304 can be compared with those against other antigens in weight/volume basis.

The third unique and advantageous feature of ABS-IgE is that it
neutralizes the histamine release activity of MGL_1304, but does not activate basophils and mast cells in response to MGL_1304 on the cell surface. The binding of ABS-IgE to FcεRI appears normal in that both basophils and RBL-48 mast cells acquired the reactivity to anti-IgE by the sensitization with ABS-IgE. Moreover, the dissociation constant (Kd) of ABS-IgE for MGL_1304 (1.99 x 10^{-9} M) is within an ordinal or somewhat low range as compared with general antigen–antibody interactions, indicating the strong binding of ABS-IgE to MGL_1304. Since the epitope of MGL_1304 for ABS-IgE consists of a conformational structure, one molecule of MGL_1304 may expose only one epitope to bind one molecule of ABS-IgE, and not cross-link multiple FcεRI on the cells. In any case, this feature should be a big advantage of ABS-IgE in prevention and treatment of sweat allergy. Recently, it has been revealed that numerous kinds of microbiotas (e.g., bacteria, fungi and viruses) stably construct the normal cutaneous flora [7]. Antimycotic treatments could suppress the excess growth of microbiotas. However, the amounts of histamine release activities in sweat of patients with AD were not different from those of healthy controls [3], suggesting that the increase of M. globosa itself is not critical, despite the increase of sensitivity of patients in sweat allergy. Moreover, a disturbance of normal flora by non-specific antimycotic treatments may cause unexpected adverse events in the patients. Thus, specific removal and/or inactivation of only pathologic antigen is preferred to non-specific antimycotic treatments.

To date, a variety of antigen-specific human IgG and non-specific human immunoglobulins have been used for the treatment of various infectious and/or immunogenic inflammatory diseases [17]. IgE has never been used for these purposes, because of its potential to activate mast cells and basophilies by crosslinking FcεRs. Moreover, IgE is even targeted by a pharmaceutical IgG (omalizumab) in the treatment of bronchial asthma and refractory urticaria [18,19]. Nevertheless, antigen-specific IgE that occupies FcεRI without cell activation, such as ABS-IgE, may be even more beneficial than IgG with the same specificity, because it does not only neutralized causative antigens, but also prevents the sensitization of FcεRI with pathological IgE that activates mast cells and basophilies.

In conclusion, ABS-IgE, a human monoclonal IgE against MGL_1304 may be used to quantify the amount of MGL_1304 and anti-MGL_1304 IgE, and possibly for the treatment of diseases caused/agravated by type I allergy to MGL_1304, such as AD and cholinergic urticaria. Further studies using this antibody should elucidate detailed mechanisms of Malassezia species in sweat allergy and its potential for therapeutic purposes.

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