

Aspergillus oryzae Lectin Induces Anaphylactoid Oedema and Mast Cell Activation Through its Interaction With Fucose of Mast Cell-Bound Non-specific IgE

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Abstract

We investigated whether *Aspergillus oryzae* lectin (AOL), a fucose-specific lectin, induces anaphylactoid reactions and mast cell activation. The injection of AOL into footpads of mice produced a dose-related acute paw oedema. The AOL-induced oedema was attenuated by predose of histamine H1 receptor blocker or pretreatment of the lectin with fucose before injection and was not observed in SCID and mast cell-deficient WBB6F1-W/W^v mice. These results suggested that the AOL-induced anaphylactoid reaction was mediated by histamine released from mast cells. In addition, the activation of mast cells was seemed to be induced by the crosslinking of IgE on the cell surface following the binding of AOL to fucose residues in IgE. Consistent with the *in vivo* results, AOL induced the degranulation of the rat mast cell line RBL2H3 sensitized with monoclonal IgE. As AOL induced the increase in intracellular Ca²⁺ concentration of IgE-sensitized RBL2H3 cells as well as antigen stimulation, AOL could input signals from FcεRI. The degranulation of IgE-sensitized RBL2H3 cells by AOL was diminished by pretreatment of AOL with fucose. Defucosylated IgE did not induce degranulation of RBL2H3 cells in response to AOL stimulation, in spite of its ability to induce degranulation by antigen stimulation as intact IgE. These results indicated that AOL bound to fucose residue of IgE causing antigen-independent IgE-mediated mast cell activation and anaphylactoid reactions *in vitro* and *in vivo*, respectively. AOL bound to human IgE as well as to mouse IgE, suggesting the possible implication of AOL in the allergic response to *Aspergillus oryzae* in humans.

Introduction

Provocation to sensitized antigen induces crosslinking of IgE receptors (FcεRI) on the surface of IgE-sensitized mast cells and basophils, and then release of various mediators such as histamine, cysteinyl-leukotriens and enzymes including β-hexosaminidase from the cells occurs. In type I allergic diseases such as anaphylaxis, food allergy, asthma and allergic rhinitis, this event has an important role in the induction of symptoms including vasodilation, mucous secretion, itching and bronchoconstriction. As well as antigen, lectins can induce mast cell activation through interaction with either specific carbohydrates on cell-bound IgE, cell surface glycoproteins or glycolipids [1–3] including FcεRI [4, 5]. Thus,

the lectins induce the release of histamine and chemical mediators from basophils and mast cells in a variety of species [6–9]. The potential of various lectins in inducing anaphylactic-like reactions, alternatively called as anaphylactoid reactions, and their roles in allergic diseases need to be addressed.

Aspergillus oryzae is an important fermentation starter organism in manufacture of soy sauce and soybean paste. However, allergic bronchopulmonary aspergillosis (ABPA) caused by *A. oryzae* provocation was reported in persons who lived in near to fermentation workshops [10]. Although α-amylase of *A. oryzae* is defined as an important allergen for ABPA [11, 12], other allergens and lectins are considered to participate in eliciting ABPA. *Aspergillus oryzae* lectin (AOL) identified from the

iron-deficient cultures of *A. oryzae* has a unique binding capacity to the α 1,6-fucosyl residue, which is called 'core fucose' [13, 14]. AOL binds to core-fucosylation site of glycoproteins more specifically than *Aleuria aurantia* lectin and Lens culinaris agglutinin-A, which have been used commonly for detection of core-fucosylated oligosaccharides before AOL became available [14, 15]. From its greater specificity, AOL is useful for clarification of the importance of core fucosylation of glycoproteins and their functions in physiological and pathological reactions, such as antibody-dependent cytotoxicity [16]. In contrast to its wide use for clarifying the role of glycosylation in various proteins, the activity of AOL as an anaphylactoid remains to be elucidated.

In this study, we investigated whether a fucose-specific lectin, AOL, induces an anaphylactoid response, namely acute paw oedema formation, *in vivo* and IgE-dependent activation of the rat basophilic leukaemia RBL2H3 *in vitro*. To determine the importance of fucose-binding activity, the abilities of non-treated AOL and fucose-pre-treated AOL to induce oedema and IgE-dependent RBL2H3 cells activation were compared. In addition, activation of RBL2H3 cells sensitized with defucosylated IgE in response to AOL was examined. Finally, the binding abilities of AOL to human polyclonal and monoclonal IgE as well as to mouse IgE in lectin blot analysis were determined to clarify the possible implication of AOL in the allergic response to *A. oryzae* in human.

Materials and methods

Animals. Female DBA/1J mice (Charles River Laboratories Japan Inc., Yokohama, Japan), female SCID mice (CREA Japan Inc., Tokyo, Japan), genetically mast cell-deficient WBB6F1-W/W^v and congenic wild-type WBB6F1-+/+ mice (Japan SLC, Inc, Shizuoka, Japan) at 8–13 weeks of age were used in all experiments. The animals were raised in a temperature-controlled environment, with feeding standard rodent chow and water. All procedures were approved by the Animal Ethics Committee of Kobe Pharmaceutical University.

Induction of acute paw oedemas. The mice were injected subcutaneously with 25 μ l of phosphate-buffered saline (PBS) containing the indicated amounts of AOL (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) into the footpad of the left hind paw. To evaluate the acute paw oedema, the thickness of both footpads of the hind paws was measured by means of a dial gauge caliper calibrated with 0.01 mm graduations. The net increase in footpad thickness because of the injection of AOL or haemocyanin (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) was calculated by subtracting the thickness of the hind-paw footpad before the challenge. In some experiments, AOL was preincubated in PBS containing 1 mg/ml fucose (Sigma-Aldrich Fine Chemicals) to block its binding

ability to fucose residues in the glycoproteins. For blocking histamine H1 receptor, chlorphenylamine (Sigma-Aldrich Fine Chemicals) was dissolved in 250 μ l of PBS and orally administered 1 h before AOL injection.

To induce IgE-dependent anaphylactic oedema, the mice were passively sensitized by intravenous injection of 50 μ g of anti-ovalbumin (OVA) IgE (OE-1). After 24 h, 10 μ g of OVA (Sigma-Aldrich Fine Chemicals) was injected into the footpad of the left hind paw.

Cell culture. RBL2H3 cells were shared from the Cell Resource Center for Biomedical Research Institute of Development, Ageing and Cancer, Tohoku University, Sendai, Japan. Hybridoma producing OE-1 was previously established by using Polyethylene Glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol [17]. For production of anti-OVA IgE bearing non-fucosylated modified sugar chains (non-Fuc-OE-1), OE-1-producing hybridoma was cultured with 30 nM monensin, a monovalent ionophore antibiotic (Sigma-Aldrich Fine Chemicals).

Assay of degranulation of RBL2H3 cells. RBL2H3 cells were maintained in 5% CO₂ at 37 °C in minimum essential medium (Invitrogen Japan K.K., Tokyo, Japan) containing 10% (v/v) foetal bovine serum (Invitrogen Japan K.K.), 100 units of penicillin and 100 units of streptomycin (Invitrogen Japan K.K.). The cells were harvested by trypsin (0.25%, w/v) (Invitrogen Japan K.K.) and EDTA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (0.01%, w/v) in PBS and suspended at 0.25×10^6 cells/ml in medium in the presence or absence of the indicated concentrations of intact OE-1 or non-Fuc-OE-1. Five hundred microlitres of the cell suspension was added to each well of 24-well cluster dishes and cultured for 24 h. The cells were washed with PIPES buffer [25 mM PIPES (pH 7.2), 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 1 mM CaCl₂ and 0.1% (w/v) bovine serum albumin (BSA)] [18] and stimulated for 20 min with the indicated concentrations of AOL or OVA (Sigma-Aldrich Fine Chemicals). β -hexosaminidase in the cultured supernatant was determined by colorimetric assay using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich Fine Chemicals). Values for β -hexosaminidase released in the medium were expressed as percentages of the total β -hexosaminidase, which was determined from the cells lysed in 0.1% Triton X-100.

Intracellular Ca²⁺ measurement. Measurement of the levels of intracellular Ca²⁺ was performed by means of the calcium-reactive fluorescence probe, Fluo-3 [19]. Briefly, RBL2H3 cell suspensions (6×10^5 cells/ml) were incubated with minimum essential medium containing 10% (v/v) foetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10 ng/ml OE-1, and fluorescence probe 4 μ M Fluo-3-AM (Dojindo Laboratories, Kumamoto, Japan) for 30 min at 37 °C and then washed twice with and resuspended in the PIPES buffer. The

cells were then stimulated with AOL or OVA at 0 min and monitored for fluorescence (FL1) until 120 s by FACSCalibur (BD Bioscience, San Jose, CA, USA).

Coomassie staining, immunoblot and lectin blot analysis. The proteins including OE-1, non-Fuc-OE-1, human monoclonal IgE (BioPorto Diagnostics, Gentofte, Denmark), human polyclonal IgE (Nordic Immunological Laboratories, Tilburg, the Netherlands) and BSA were separated by SDS-PAGE and detected by coomassie staining (Wako Pure Chemical Industries, Ltd.). The separated proteins in the polyacrylamide gel were also transferred onto a PVDF membrane (ATTO, Tokyo, Japan). The total mouse IgE on the membrane was detected by alkaline phosphatase-labelled polyclonal antibodies for mouse IgE (Bethyl Laboratories, Inc., Montgomery, TX, USA). The level of fucosylation of proteins was detected by 1 µg/ml biotinylated AOL made by manufacturer's protocol using NHS-biotin (ThermoFisher Scientific Inc., Waltham, MA, USA) and streptavidin-conjugated horse radish peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA).

Statistics. To analyse the data statistically, the ANOVA (Tukey) test was used for multiple comparisons. For comparison between two groups, the Mann-Whitney *U*-test was also employed. *P* < 0.05 was considered significant.

Results

AOL injection into footpad induced acute paw oedema

Paw oedema induced by AOL injection into the footpad was significantly stronger than that by haemocyanin (Fig. 1A), used as the negative control. Although the AOL-induced oedema peaked at 15 min and decreased gradually thereafter, significant swelling was observed in the AOL-injected paw even at 3 h (Fig. 1A). Varying doses of AOL (5, 25 and 125 µg/paw) produced dose-related increases in paw thickness at 30 min after injection (Fig. 1B).

AOL-induced acute paw oedema was because of histamine release from mast cells, which was possibly triggered by crosslinking of FcεRI following the binding of AOL to core fucose of IgE on the receptors

Especially, in tissues such as ears, mast cells, but not basophils [20], have an indispensable role in inducing anaphylactic [21] and anaphylactoid oedema [22]. To clarify whether AOL induced the anaphylactoid reaction observed as paw swelling via activating mast cells and histamine release, we tested the effect of histamine H1 receptor antagonist, chlorphenylamine, on AOL-induced paw oedema. Significant inhibition of the AOL-induced paw oedema was observed when chlorphenylamine was orally administered to mice at 30 and 100 mg/kg (Fig. 2A). AOL injection induced acute paw swelling in

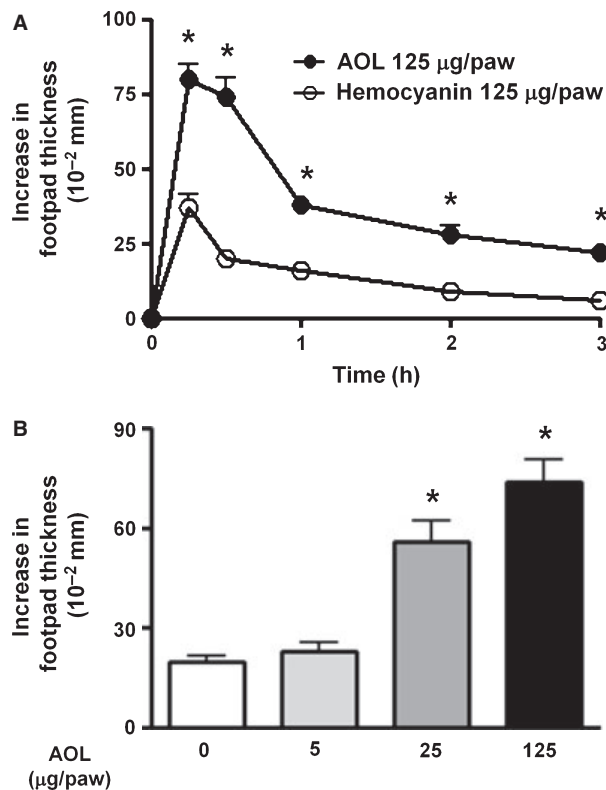


Figure 1 Acute paw oedema elicited by *Aspergillus oryzae* lectin (AOL) injection. Mice were injected with the indicated concentrations of AOL into the footpad. (A) Time course of footpad swelling induced by AOL injection. **P* < 0.05 versus haemocyanin. (B) The concentration-dependent increase of footpad thickness by AOL injection. **P* < 0.05 versus without AOL. Data show mean + SEM of 5–7 mice. Data are representative of three independent experiments.

wild-type WBB6F1-+/+ mice but not in mast cell-deficient WBB6F1-W/W^v mice (Fig. 2B).

Lectins bind to sugar moieties of IgE and induce crosslinking of IgE on mast cells followed by histamine release and anaphylactoid reactions [8, 9]. Moreover, as human and mouse IgE have relatively high contents of carbohydrates (10–13%) including fucose residue [23, 24], among the molecules involved in anaphylactic and anaphylactoid reactions such as IgG1 [25], it is supposed that AOL might bind to fucose residues of IgE and thus induce crosslinking of IgE causing anaphylactoid reactions. To this end, we examined the possible participation of IgE in AOL-induced anaphylactoid reaction by means of SCID mice, which do not have IgE [26]. In contrast to the results from DBA/1J mice, AOL failed to induce acute paw oedema in SCID mice (Fig. 2C), whereas SCID mice developed anaphylactic paw oedema similar to DBA/1J mice by intravenous OE-1 sensitization and subsequent OVA injection into footpad (Fig. 2D). To determine whether the action of AOL as an anaphylactoid was dependent on the fucose-binding activity of the lectin, the oedema-inducing effect of AOL

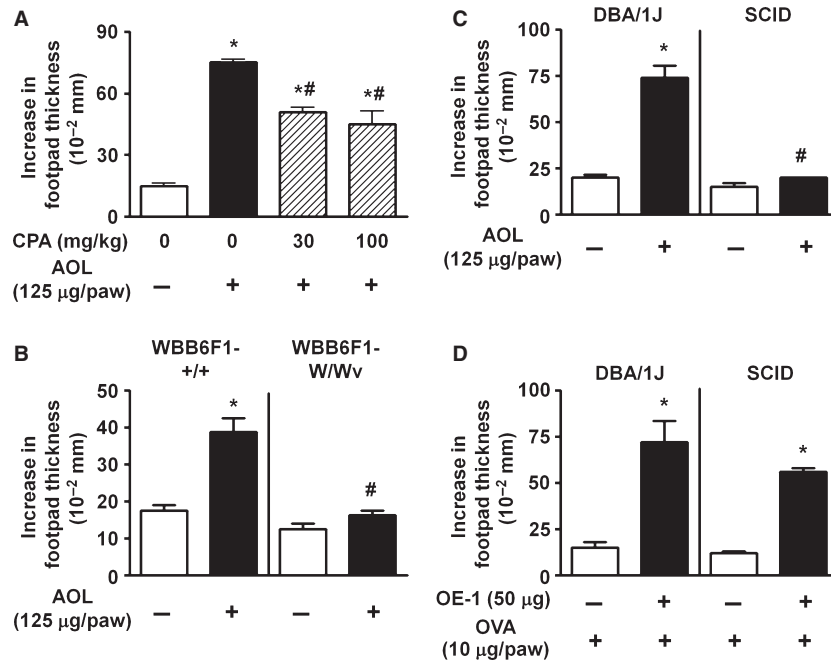


Figure 2 *Aspergillus oryzae* lectin (AOL)-induced paw oedema was mediated by histamine release from mast cells and not observed in SCID mice. (A) Mice were orally administered the indicated concentrations of chlorphenylamine (CPA). After 1 h, the mice were injected with 125 µg of AOL into the footpad. * $P < 0.05$ versus without AOL. # $P < 0.05$ versus AOL without CPA. (B) WBB6F1-+/+ and WBB6F1-W/W^v mice were injected with the indicated concentrations of AOL into the footpad. * $P < 0.05$ versus phosphate-buffered saline (PBS), # $P < 0.05$ versus WBB6F1-+/+. (C) DBA/1J and SCID mice were injected with 125 µg of AOL into the footpad. * $P < 0.05$ versus PBS, # $P < 0.05$ versus DBA/1J. (D) DBA/1J and SCID mice were sensitized by intravenous injection of 50 µg of anti-ovalbumin (OVA) IgE (OE-1). After 24 h, the mice were injected with 10 µg of OVA into the footpad. * $P < 0.05$ versus without OE-1. Data show mean + SEM of 5–7 mice. Data are representative of two or three independent experiments.

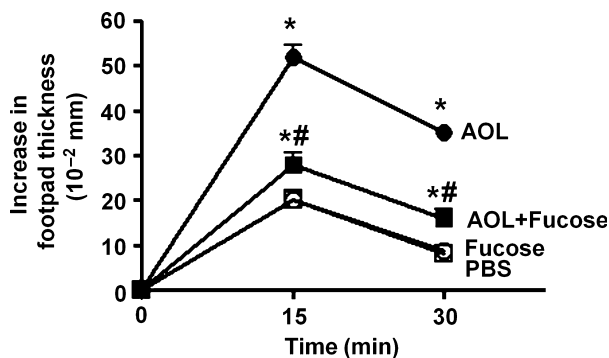


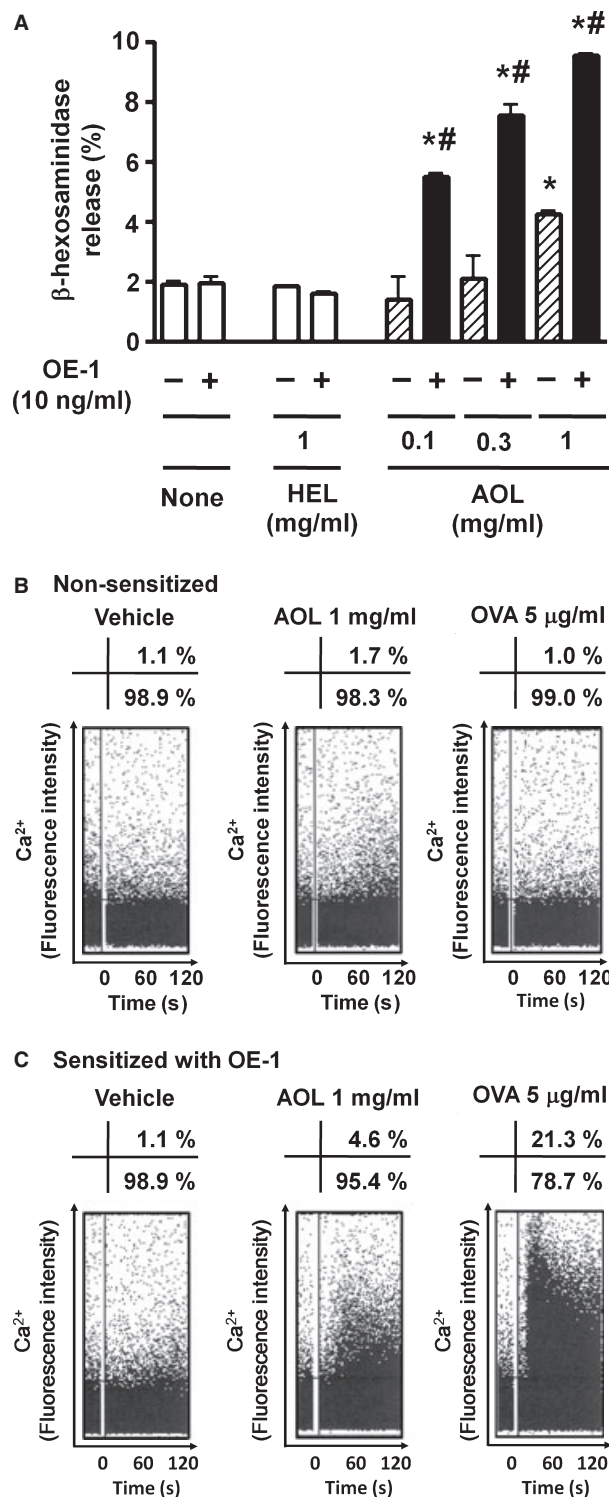
Figure 3 *Aspergillus oryzae* lectin (AOL) pretreated with fucose failed to elicit paw oedema. Mice were injected with 125 µg of AOL pretreated with or without fucose (1 mg/ml) into the footpad. Data show mean + SEM of 5–7 mice. * $P < 0.05$ versus phosphate-buffered saline, # $P < 0.05$ versus AOL. Data are representative of three independent experiments.

preincubated with fucose was assayed. The AOL-induced acute paw oedema was greatly diminished by pre-incubation of the lectin with fucose (Fig. 3).

AOL activated RBL2H3 cells via IgE-dependent mechanism

To clarify whether AOL induces mast cell activation *in vitro*, we tested the effect of AOL on degranulation

response of RBL2H3 cells. For the determination of degranulation of RBL2H3 cells, β -hexosaminidase release from the cells was measured. When AOL was added to non-sensitized RBL2H3 cells, the lectin weakly induced β -hexosaminidase release (Fig. 4A). Only at 1 mg/ml, AOL could induce significant release of β -hexosaminidase (Fig. 4A). In contrast, when AOL was added to OE-1-sensitized RBL2H3 cells, the lectin induced β -hexosaminidase release significantly at 0.1–1 mg/ml (Fig. 4A). These results indicated that the mast cell activation by AOL depended on IgE, which was consistent with the results from *in vivo* study (Fig. 2C). The negative control, hen egg lysozyme (HEL), failed to affect β -hexosaminidase release by RBL2H3 cells (Fig. 4A). To confirm the activating effect of AOL on IgE-sensitized RBL2H3 cells, the levels of intracellular Ca^{2+} in the cells were measured as an activation marker. As shown in plots in Fig. 4B, in non-sensitized cells, intracellular Ca^{2+} levels were increased in only 1.7% (upper right quadrant) of total cells (upper right and lower right quadrants) upon AOL stimulation, whereas 1.1% of total cells were detected in the upper right quadrant in vehicle-treated control cells. Antigen stimulation also did not increase the levels of intracellular Ca^{2+} in non-sensitized RBL2H3 cells (1.0% in upper right quadrant). In contrast to



non-sensitized RBL2H3 cells, AOL and OVA stimulations to OE-1-sensitized RBL2H3 cells moderately and strongly increased the intracellular Ca^{2+} levels in 4.6 and 21.3% of total cells, respectively (Fig. 4C), as well as the β -hexosaminidase releases in 9.5 (Fig. 4A) and 21.8% [17].

Figure 4 *Aspergillus oryzae* lectin (AOL) induced IgE-dependent activation of RBL2H3 cells. To investigate the ability of AOL to induce degranulation response (A), RBL2H3 cells (2.5×10^5 cells/well) were incubated with or without anti-ovalbumin (OVA) IgE (OE-1) for 24 h. The cells were washed and then incubated with the indicated concentrations of AOL or HEL. After 20 min, the releases of β -hexosaminidase from the cells were measured. $*P < 0.05$ versus none, $\#P < 0.05$ versus without OE-1. Data show mean $\pm$ SEM of six cultures of the cells. Data are representative of three independent experiments. For measurement of intracellular Ca^{2+} concentration, RBL2H3 cells (6×10^5 cells) were incubated without (B) or with (C) anti-OVA IgE (OE-1) and 4 μ M Fluo-3-AM at 37 $^{\circ}\text{C}$ for 30 min. The cells were washed and stimulated for 120 s in PIPES buffer containing AOL or OVA. Data are representative of three independent experiments.

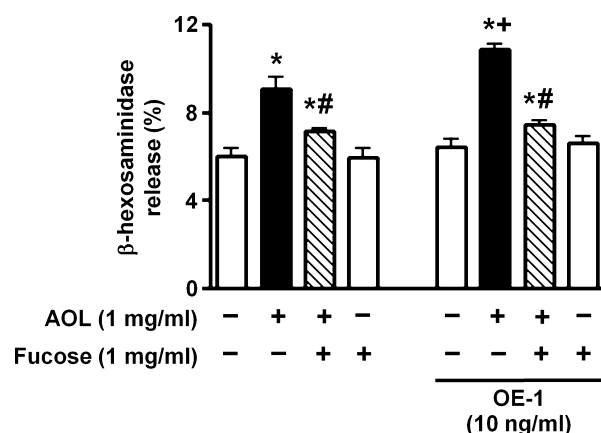


Figure 5 Pretreatment of *Aspergillus oryzae* lectin (AOL) with fucose diminished its ability to induce the release of β -hexosaminidase from RBL2H3 cells. RBL2H3 cells (2.5×10^5 cells/well) were incubated with or without anti-ovalbumin IgE (OE-1) for 24 h. The cells were washed and then incubated with AOL treated with or without fucose. After 20 min, the releases of β -hexosaminidase from the cells were measured. $*P < 0.05$ versus without AOL and fucose, $\#P < 0.05$ versus AOL, $++P < 0.05$ versus without OE-1. Bars show mean $\pm$ SEM of six cultures of the cells. Data are representative of three independent experiments.

AOL-induced degranulation is mediated by crosslinking of IgE on the surface of mast cells following the binding of AOL to core fucose of IgE

The indispensable role of fucose residue of IgE in AOL-induced mast cell activation was clarified in two ways. First, as is in the case of *in vivo*, the degranulation-inducing activity of AOL was assayed following pre-incubation with fucose. The degranulation response induced by fucose-pretreated AOL was significantly lower than that by non-treated AOL in both OE-1-sensitized and non-sensitized RBL2H3 cells (Fig. 5). Second, the lack of ability of AOL to induce degranulation of defucosylated modified sugar chain bearing OE-1 (non-Fuc-OE-1)-sensitized RBL2H3 cells was investigated. To obtain non-Fuc-OE-1, hybridoma was cultured in the presence of a trimming inhibitor monensin [27, 28]. In this condition,

OE-1 hybridoma produced non-Fuc-OE-1 detected at lower molecular weight than intact OE-1 in anti-IgE Western blot analysis (Fig. 6A). The absence of core fucose in non-Fuc-OE-1 in contrast to intact OE-1 was proven by the absence of AOL binding to non-Fuc-OE-1 in AOL lectin blot (Fig. 6A). OE-1 and non-Fuc-OE-1 showed similar activities to bind to antigen in ELISA (data not shown) and to induce degranulation of RBL2H3 cells in response to antigen (Fig. 6B). In contrast to antigen stimulation, AOL-induced degranulation of non-Fuc-OE-1-sensitized RBL2H3 cells was significantly lower than that of intact OE-1-sensitized RBL2H3 cells (Fig. 6C). These results clearly indicated that AOL induces crosslinking of IgE by binding to their core fucose residue. And thus, AOL induced the mast cell activation and anaphylactoid reactions *in vitro* and *in vivo*.

AOL bound to human IgE *in vitro*

Next, we analysed the binding activity of AOL to human IgE, to clarify its possible implications in ABPA in humans. To this end, lectin blot and coomassie staining for human monoclonal IgE and human polyclonal IgE were carried out as well as BSA, which bears no carbohydrates. As shown in the lectin blot in Fig. 7, AOL clearly bound to human polyclonal IgE and monoclonal IgE, but not to BSA, while coomassie staining detected all three proteins equally. The binding ability of AOL to human IgE indicated the potential of AOL to induce mast cell activation and to participate in allergic responses in humans as well as in mice.

Discussion

In this study, we demonstrated for the first time that AOL induced acute paw oedema through IgE-mediated mast cell activation *in vivo*. Our *in vitro* results also showed that AOL induced the release of β -hexosaminidase from the rat basophilic leukaemia RBL2H3 sensitized with IgE and the mechanism of activation by the lectin was binding to core fucose residue of IgE causing Fc ϵ RI crosslinking. Furthermore, we revealed the binding ability of AOL to human IgE *in vitro* and the potential of lectin to participate in ABPA in humans.

Release of chemical mediators such as histamine and serotonin by degranulation of mast cells is an important step in type I allergic reactions and immune responses. Our results showed that AOL injection into footpad induced paw oedema that peaked at 15 min after injection (Fig. 1), and thus, this lectin may be implicated in immune, allergic and anaphylactic reactions to *A. oryzae*. This is the first study to reveal that fucose-binding lectin elicits the anaphylactic reaction, complementing reports on other lectins such as phytohaemagglutinin-P and concanavalin A, which bind to sugars other than fucose [29].

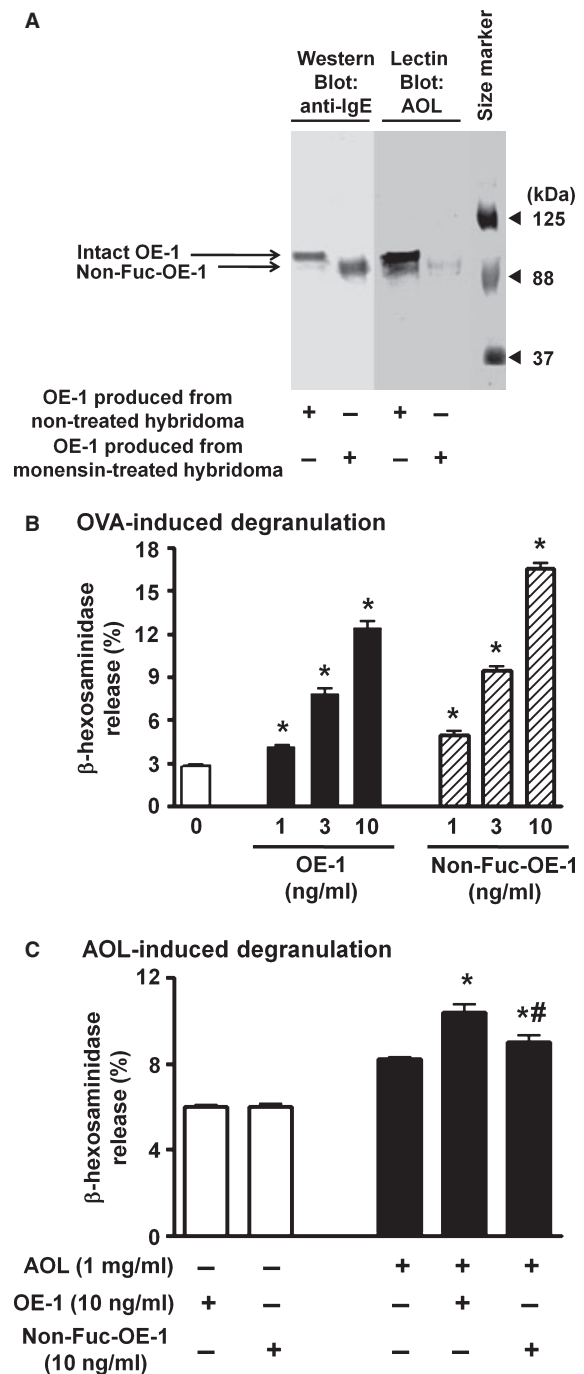


Figure 6 Non-Fuc-OE-1 could induce degranulation in response to ovalbumin (OVA), but not in response to *Aspergillus oryzae* lectin (AOL). (A) Purified OE-1 produced from non-treated and monensin-treated hybridoma were separated by SDS-PAGE in reducing condition and detected by Western blot by means of anti-IgE and lectin blot by means of AOL-biotin. (B) Degranulation was equally induced by 5 μ g of OVA in OE-1-sensitized and non-Fuc-OE-1-sensitized RBL2H3 cells at the indicated concentrations. * P < 0.05 versus without sensitization. (C) Compared with OE-1-sensitized RBL2H3 cells, the lectin induced weaker degranulation of non-Fuc-OE-1-sensitized RBL2H3 cells. * P < 0.05 versus without sensitization, # P < 0.05 versus OE-1-sensitized with AOL stimulation. Bars show mean $\pm$ SEM of six cultures of the cells. Data are representative of three independent experiments.

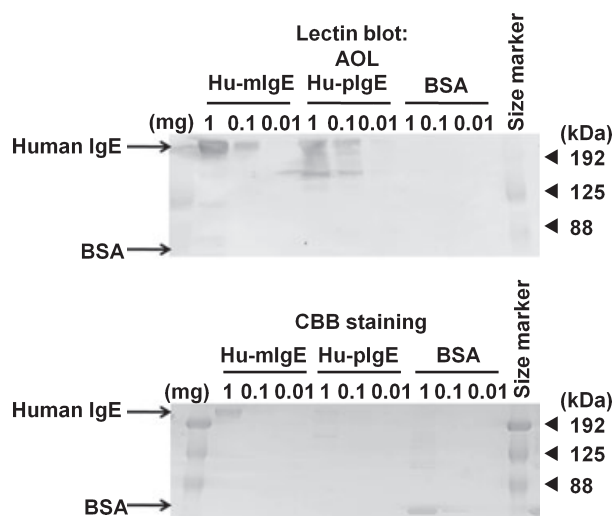


Figure 7 *Aspergillus oryzae* lectin (AOL) bound to human monoclonal IgE and polyclonal IgE. Purified human monoclonal IgE (Hu-mIgE), human polyclonal IgE (Hu-pIgE) and bovine serum albumin were separated by SDS-PAGE in non-reducing condition. The binding capacity of AOL to human IgE and loaded protein amounts were detected by lectin blot by means of AOL-biotin and CBB staining, respectively. Data are representative of three independent experiments.

The time-course change of paw swelling induced by AOL injection was quite similar to that induced by OVA injection into footpads of mice sensitized by OE-1, anti-OVA IgE (data not shown). The histamine H1 blocker, chlorphenylamine, attenuated AOL-induced oedema (Fig. 2A) as well as OVA-induced oedema in OE-1 sensitized mice (data not shown). AOL-induced acute paw swelling was observed in wild-type WBB6F1-*+/+* mice, but not in mast cell-deficient WBB6F1-*W/W^v* mice (Fig. 2B), suggesting that mast cells had an indispensable role in eliciting acute oedema by AOL injection. On the other hand, phytohaemagglutinin-P injection into rat paw induced biphasic swelling peaked at 0.5 and 5 h, which is not diminished by the H1 blocker mepyramine treatment [29]. Concanavalin A injection causes a gradual increase in paw oedema reaching a maximum by 24 h [29]. Thus, in contrast to other lectins such as phytohaemagglutinin-P and concanavalin A, AOL might induce selective activation of mast cells to elicit anaphylactoid reactions similar to anaphylactic reactions by IgE and antigen-mediated type I allergy. Moreover, the following two facts suggested that AOL crosslinked IgE/FcεRI and other receptors such as FcγRs and complement receptors by binding to their fucose residues, and thus induced mast cell activation. AOL could not induce an anaphylactoid reaction in SCID mice (Fig. 2C), which have a sufficient number of mast cells expressing functional FcεRI to induce anaphylactic oedema (Fig. 2D), but not have functional IgE as well as T and B cells. Pretreatment of AOL with excess fucose blunted the ability of the lectin to induce oedema (Fig. 3).

The ability of AOL to induce anaphylactoid reaction through IgE and mast cells *in vivo* was strongly confirmed by *in vitro* analysis. RBL2H3 cells are a useful tool for investigating the direct effects of drugs [30], environmental factors [31] and natural products including lectins [3] on mast cell functions. AOL induced a higher rate of degranulation of IgE-sensitized RBL2H3 cells than that of non-sensitized RBL2H3 cells (Fig. 4A), suggesting that AOL and IgE cooperate to activate the mast cells. Intracellular Ca^{2+} increase is indispensable for IgE/FcεRI-mediated degranulation, and AOL stimulation via IgE clearly increased the intracellular Ca^{2+} concentration (1.1–4.6%) as well as antigen OVA (1.1–21.3%) (Fig. 4C). We could not exclude the possibility that other receptors for IgE contributed to activation of RBL2H3 cells by AOL. However, RBL2H3 cells do not express FcεRII [32] and FcγRIII [33]. In addition, FcγRIV is not expressed in mast cells and basophils [34]. From these observations, AOL, as well as antigen, was thought to activate RBL2H3 cells through IgE bound to FcεRI. In contrast to the strong activation by specific antigen OVA, the mild activation of anti-OVA IgE-sensitized RBL2H3 cells by AOL was observed. There are explanations why AOL could not induce degranulation as strong as antigen. A binding ability of AOL to IgE is considered to be lower than that of antigen. In fact, association constant (K_a) for antigen-antibody interaction ($K_a = 10^7$ – 10^{11}) [35] is quite higher than that for the interaction between AOL and fucose residues ($K_a = 10^4$ – 10^6) [15]. Different types of FcεRI-mediated activation are induced by different stimulations, namely AOL and antigen, as their presumed binding sites of IgE are different. Supporting this hypothesis, at least two patterns of activation via FcεRI induced by monomeric IgE bound and by crosslinking with antigen/IgE were reported [36]. For instance, crosslinking of the receptor by antigen and IgE, but not monomeric IgE bound to the receptor, induces degranulation and leukotriene C secretion strongly, while interleukin-6 mRNA expression is preferentially induced by monomeric IgE rather than antigen and IgE stimulation [36]. Considered from its binding to many other fucosylated proteins, the efficiency of AOL to bind to IgE might be low, whereas antigens specifically bind to IgE. The reason how AOL could induce degranulation of non-sensitized RBL2H3 cells is thought to be because of crosslinking of FcεRI by direct binding of AOL to fucose residue of the receptors.

In parallel to *in vivo* result (Fig. 3), pre-incubation of AOL with excess fucose almost completely attenuated the AOL-induced degranulation of RBL2H3 cells (Fig. 5). To confirm the role of fucose residue of IgE, defucosylated IgE, which could not bind to AOL was produced by means of monensin treatment and used for the experiments with RBL2H3 cells. When human skin fibroblasts

were incubated with monensin, the cells produce abnormal sugar chain-bearing fibronectin [28]. The abnormal fibronectin has a higher amount of mannose and lower amounts of galactose and fucose compared with that from the cells in the normal condition. Monensin treatment of hybridoma prevents the labelling of sugar chains of secreting IgM with [^3H] galactose or [^3H] fucose but not with [^{35}S] methionine or [^3H] mannose [27]. Consistent with these reports, in this study, monensin treatment led the hybridoma to produce non-Fuc-OE-1, which was more rapidly electrophoresed than intact OE-1, probably because of its lowered molecular weight by deglycosylation (Fig. 6A; Western blot). The lectin blot analysis result shown in Fig. 6A indicates that the rapidly electrophoresed non-Fuc-OE-1 produced by monensin-treated hybridoma was not detected by labelled-AOL in contrast to intact OE-1. Thus, OE-1 produced by monensin-treated hybridoma, namely non-Fuc-OE-1, lacks the sugars with fucose residue that are bound by AOL. The role of sugar chains of IgE in its mast cell-activating function is not completely understood. No differences in antigen-binding, mast cell-binding and passive cutaneous anaphylaxis-inducing activities were observed between intact antibody and the abnormal sugar-bearing IgE produced by addition of the glycosidase I inhibitors, N-methyl-1-deoxynojirimycin and catanospermine, and golgi mannosidase II inhibitor, swainosine, to hybridoma culture [37, 38]. In contrast, non-sugar-bearing antibodies produced by enzymatic digestion and culture of hybridoma with tunicamycin do not have the ability to induce passive cutaneous anaphylaxis and systemic anaphylaxis, in spite of their intact binding activity to antigen [38, 39]. We showed that non-Fuc-OE-1 IgE produced by monensin-treated cells had comparable antigen-binding activity (data not shown) and antigen-dependent degranulation-inducing activity (Fig. 6B) to the intact one, similar to deoxynojirimycin, catanospermine and swainosine. These results indicated that minor modification of sugar moieties in IgE does not affect the functions of IgE, although aglycosylation of IgE abolishes its allergenic ability. It should be noted that our results suggested that the sugar chains of IgE are important for IgE-dependent degranulation with lectin stimulation in contrast to antigen stimulation (Fig. 6B, C), as the degranulation by AOL stimulation of non-Fuc-OE-1-sensitized RBL2H3 cells was clearly lower than that of intact OE-1-sensitized cells (Fig. 6C). This fact also indicated that differences in rate and pattern of glycosylation in IgE might affect the anaphylactic and anaphylactoid responses upon exposure to various lectins. Altogether, AOL is suggested to bind to fucose residues of IgE and induce mast cell activation via crosslinking of IgE.

Aspergillus oryzae lectin binds to human polyclonal IgE and monoclonal IgE (Fig. 7) as well as mouse IgE (Fig. 6A). As both human IgE and mouse IgE bear fucose

residue, AOL might bind to fucose residue of human IgE and induce allergic reactions in humans via mast cell activation. In fact, some lectins induce mast cell activation and allergic reactions in humans [1, 8]. In conclusion, AOL had a potential to induce mast cell activation and anaphylactic-like anaphylactoid reactions by cross-linking of IgE via binding to its fucose residues in mice. As well as to IgE of mice, the lectin binds to human IgE *in vitro*. AOL might be involved in *A. oryzae*-induced allergic responses, and thus clarification of the roles of AOL in ABPA might lead to resolution of this disease.

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Authors' contribution

Kouya Yamaki performed the all experiments described herein and drafted the manuscript. Shin Yoshino acted as a laboratory supervisor to Kouya Yamaki and assisted in the preparation and proofing of this manuscript.

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