

Carbohydrates expressed on *Aspergillus fumigatus* induce *in vivo* allergic Th2-type response

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Summary

Background The mould *Aspergillus fumigatus* (Af) causes several forms of Th2-biased diseases such as Type I respiratory allergies. This mould contains abundant carbohydrates including glucan, chitin and galactomannan. However, little is known on whether these carbohydrates directly drive Th2 responses *in vivo*.

Objective We sought to determine the relative role of carbohydrates expressed on crude extracts of Af in the induction of Th2-type antibody production and local eosinophilia in mice.

Methods Carbohydrates on native Af were destroyed by sodium metaperiodate under mild conditions. CBA/J mice were sensitized intranasally with native, periodate-treated or mock-treated Af. Histologic changes and production of Af-specific IgE, IgG1 and IgG2a, as well as serum total IgE were determined. Inhibition ELISA for Af-specific IgE was performed using periodate-treated Af as an inhibitor.

Results Mice sensitized with periodate-treated Af displayed significant decreases in both total and specific IgE levels in comparison to mice sensitized with native or mock-treated Af. Furthermore, sensitization and subsequent challenge with periodate-treated Af significantly reduced the degree of eosinophil recruitment into the nasal mucosa, compared to the controls. On the other hand, competitive inhibition showed that periodate-treated Af could inhibit binding between native Af and specific IgE in a similar manner to that of native and mock-treated Af.

Conclusion These results suggest that carbohydrates on Af play a key role as internal adjuvants in inducing the allergic Th2-type response and are not the targets of the induced IgE response.

Keywords *Aspergillus fumigatus*, mould, allergy, Th2, rhinitis, IgE, eosinophil, mouse

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Introduction

Carbohydrates expressed on pathogens have the potential to elicit several types of immune responses. They have been known to function as T/B cell epitopes and are involved in various types of acquired immune responses [1–4]. They also act as ligands for selectins, the toll-like receptor, CR3, and the mannose receptor, which can induce innate immunity [5–8]. In addition, carbohydrates activate the complement pathway via binding to a mannose-binding lectin [9]. However, little is known about whether the carbohydrates play a direct role in the selective activation of T cells, which leads to their differentiation into Th1 or Th2 cells [10].

We have focused on the role of carbohydrates expressed on antigens/allergens in the induction of Th2-type allergic inflammation in mice. We have so far investigated two glycoproteins, *Schistosoma mansoni* egg antigen (SEA) and phospholipase A₂ (PLA₂) from the venom of the honeybee *Apis mellifera* [11,12].

We have demonstrated that carbohydrates on SEA, but not PLA₂, are essential to induce an *in vivo* Th2 response including specific IgE production, local eosinophilia and Th2-type cytokine production.

Aspergillus fumigatus (Af) is a ubiquitous mould that is capable of causing a number of diseases, especially Th2-biased respiratory disorders and opportunistic infections, examples of which include allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, invasive aspergillosis and atopic respiratory diseases such as allergic rhinitis and asthma [13,14]. It is well known that extracts of Af contain abundant carbohydrates including glucan, chitin and galactomannan [15,16]. For example, Hearn and Sietsma have reported that 71.5% of the dry weight of the Af wall consists of anthrone-reacting carbohydrate material [15]. Studies have revealed that several carbohydrate moieties from the extract of Af react with IgE or IgG from allergic patients sensitized to Af [15,16]. On the other hand, carbohydrates isolated from another major allergic component of Af (gp55) were not found to be IgE epitopes in patients with ABPA [17]. In addition, there are no reports demonstrating that carbohydrates on Af function as T cell epitopes capable of inducing proliferation and cytokine production in Af-specific T cells.

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Recently, a murine model of allergic rhinitis was developed using the culture filtrate and mycelial extracts of Af [18]. In this model, CBA/J mice displayed nasal eosinophilia and an increase in total serum IgE after the repeated intranasal application of Af in the absence of adjuvant. In the present study, we sought to determine the role of carbohydrates expressed on Af in the initiating of Th2-type allergic inflammation *in vivo* using a slight modification of this model. We demonstrated that carbohydrates on Af play a key role in the production of Af-specific IgE, and in the appearance of nasal eosinophilia, both of which are hallmarks of Th2-type allergic inflammation. Furthermore, we also showed that carbohydrates on Af function solely as Th2-activating adjuvants and are not themselves epitopes involved in induced IgE responses *in vivo*.

Methods

Animals

Young adult (6–10 weeks old) CBA/J strain female mice were purchased from Charles River Japan (Yokohama, Japan). The mice were maintained in specific pathogen-free (SPF) conditions at Okayama University Medical School according to the guidelines set forth by the Okayama University Medical Area Research Committee.

Antigen

A crude extract of Af culture filtrate was provided by Torii Co. (Tokyo, Japan). Endotoxin contamination was considered to be negligible due to a negative Endospec™ ES test result (Seikagaku Kogyo Co., Tokyo, Japan). Periodate oxidation was performed under mild conditions as previously described [12]. In brief, two 1.8 mL tubes of Af (1.2 mg/mL) were processed simultaneously in an identical manner, except that one sample was not incubated in sodium metaperiodate. We described this sample as mock-treated Af. Af samples were initially dialysed overnight at 4°C against a 0.05-M acetate buffer, pH 4.5 (Sigma Chemical Co., St Louis, MO, USA). Then 200 mL of 100 mm sodium meta-periodate (Sigma) was added to 1.8 mL of the sample to yield a final concentration of 10 mm. The reaction vial was gently mixed for 1 h at 25°C in the dark. Following this, the samples were dialysed against an excessive amount of phosphate buffered saline (PBS) for 3 days at 4°C. Protein concentration was determined by a bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). These antigens were stored at –20°C until use.

Sensitization of mice

Mice ($n=6$ per group) were lightly anaesthetized by inhalation with diethyl ether (Ishizuka Iyaku Ltd, Osaka, Japan). The mice were then sensitized by intranasal administration of 100 µg protein of native, periodate-treated, or mock-treated Af in a total volume of 50 mL concentrated with Dulbecco's PBS (Gibco, Grand Island, NY, USA) by microsyringe (Hamilton Co., Reno, NV, USA) in the absence of an adjuvant. This procedure was performed three times in the first week. Thereafter, the mice were sensitized intranasally with 50 µg protein of their respective antigens in a total volume of 50 µL three times a week for the following 2 weeks. Peripheral blood was collected from the tail 12 h after the third (Day 7), sixth

(Day 14) and ninth (Day 21) sensitization in addition to prior to the first sensitization. The blood was centrifuged at 200 g, and the serum was collected and stored at –20°C until use. One week following the ninth sensitization, the mice were challenged daily with intranasal administration of 50 µg protein of their respective antigens in a total volume of 50 µL for 7 consecutive days.

Ab determination

The levels of Af-specific Th2-associated IgG1 Ab and Th1-associated IgG2a Ab were measured by ELISA, as described previously [12]. Briefly, each well of a 96-well ELISA plate (Corning Inc. Corning, NY, USA) was coated with 2 µg/mL of Af in 0.05 M carbonate buffer, pH 9.6 (Sigma), during an overnight incubation at 4°C. The plates were then washed with PBS containing 0.05% Tween 20 (PBS/Tween 20; Sigma) and were blocked with PBS containing 10% fetal calf serum (FCS; Gibco) for 2 h at 37°C. Each individual serum sample was then plated in duplicate in twofold serial dilution, beginning at 1:100, and incubated for 2 h at 37°C. Bound antibodies were detected by incubation with either goat anti-mouse IgG1 or goat anti-mouse IgG2a, diluted to 1/4000 (Boehringer-Mannheim, Indianapolis, IL, USA). After a further incubation period at 37°C for 1 h, tetramethylbenzidine (TMB) substrate solution (Kirkegaard and Perry, Gaithersburg, MD, USA) was added to the plates. The reaction was stopped by 5% phosphoric acid after approximately 10 min, and the OD 450 nm was read on a microplate reader (Bio-Rad, Hercules, CA, USA). Results were expressed as Unit/mL from a standard curve. A serum pool from CBA/J female mice hypersensitized intraperitoneally with Af adsorbed to alum was used as internal laboratory standard. This pool was arbitrarily determined as 1000 Unit/mL.

Flat-bottomed ELISA plates were coated overnight at 4°C with 5 µg/mL of rat anti-mouse IgE mAb (clone LO-ME-3; BioSource, Camarillo, CA, USA) diluted in carbonate buffer to determine levels of Af-specific and total serum IgE antibody. The plates were washed four times with PBS/Tween 20 and were blocked as described above. After this, each individual serum samples in twofold dilution beginning at 1:2, and purified mouse IgE standards (2 µg/mL starting concentration, PharMingen, San Diego, CA, USA), were added to the plates in duplicate and the plates were incubated overnight at 4°C. The plates were washed three times, and 0.05 µg of biotinylated anti-mouse IgE mAb (clone R35-72, PharMingen) was added to each well in order to determine the total IgE level in the serum. Alternatively, 0.1 µg of biotinylated Af was added to each well to determine levels of Af-specific IgE. After 2 h of incubation at 37°C with the appropriate secondary reagents, the plates were incubated with 100 µL of streptavidin-peroxidase conjugate (1/1000 dilution in PBS/10% FCS; Sigma) for 1 h at 37°C. Finally, the plates were washed three times, and TMB substrate solution was added. The reaction was stopped in approximately 10 min with the addition of 5% phosphoric acid, and OD 450 nm was read on a microplate reader. In Af-specific IgE, results were expressed as Unit/mL as described above. For biotinylation, Af (2 mg/mL) in sodium bicarbonate buffer, pH 8.5, was incubated with biotin (long arm) N-hydroxy succinimide ester (Vector, Burlingame, CA, USA) for 2 h at room temperature. The reaction was stopped by the addition of 5 µL of ethanolamine, and the complex was dialysed overnight with PBS/0.05% sodium azide as previously described [12].

Histological examination

Mice were killed 12h after the final nasal challenge. They were decapitated and the heads were fixed overnight at 4°C with 10% formalin. Following this, the samples were decalcified with 2.5% EDTA-2Na solution for 7 days at 4°C. Then coronal nasal sections (6µm thick) were stained by both haematoxylin/eosin and Luna staining, and the number of eosinophils in nasal mucosa was determined microscopically in a high power field (10 × 40).

Competitive inhibition ELISA for Af-specific IgE

The competitive inhibition ELISA protocol that we described previously was followed [11]. In brief, ELISA plates were coated with rat anti-mouse IgE mAb (5µg/mL) in carbonate buffer overnight at 4°C. Plates were washed and blocked as described above. Then 100µL of 1:5 diluted pooled sera from native Af-sensitized mice was added to triplicate wells and incubated for 2h at 37°C, after which the plates were washed in PBS/Tween 20. In a separate plate, biotinylated Af (1µg/mL) was mixed with serial concentrations (0, 0.005, 0.05, 0.5, 5.0 and 50 µg/mL) of native, periodate-treated, or mock-treated Af, after which it was added to the IgE-bound ELISA plates for 1h at 37°C. Thereafter, extravidin-peroxidase was added, followed by the addition of TMB substrate and phosphoric acid as described above. Absorbance at 450nm was measured with a microplate reader. Percent inhibition was calculated as follows: $(1 - (\text{OD}_{450 \text{ nm with inhibitor}} / \text{OD}_{450 \text{ nm without inhibitor}})) \times 100$.

Statistical analysis

Student's unpaired *t*-test was used to determine the statistical significance of the values obtained. A *P*-value of 0.05 was

considered statistically significant. Values are given as means ± SEM.

Results

Carbohydrates on *Aspergillus fumigatus* induce Th2-Ab production

An increase in serum total IgE was seen in CBA/J female mice following six intranasal sensitizations with 100µg protein of native Af. A further elevation in this increase was noted after the nine sensitizations compared to the levels seen in mice sensitized with saline (Fig. 1a). Production of Af-specific IgE was also detected after six sensitizations in mice treated with native Af, and this was further increased after nine sensitizations as determined by captured ELISA (Fig. 1b). This signal diminished when we used sera heated at 56°C for 2h (99.2%, data not shown), which suggests that this signal indeed reflects reagenic IgE activity. In addition, mice treated with native Af also produced Af-specific IgG1 (Fig. 1c). On the contrary, signals indicating the productions of Af-specific IgG2a were not detected in these mice (Fig. 1d). Mice treated with either PBS or 10µg protein of native Af did not produce any of the specific Abs tested. These results suggest that CBA/J female mice produce Af-specific Th2-associated IgG1 and IgE Abs after repeated intranasal sensitization with native 100µg protein of Af in the absence of an adjuvant. Therefore, this protein dose was used to administer native, mock-treated, or periodate-treated Af as sensitizing antigens in order to determine the effects of carbohydrates on Af in the *in vivo* induction of Th2 allergic response

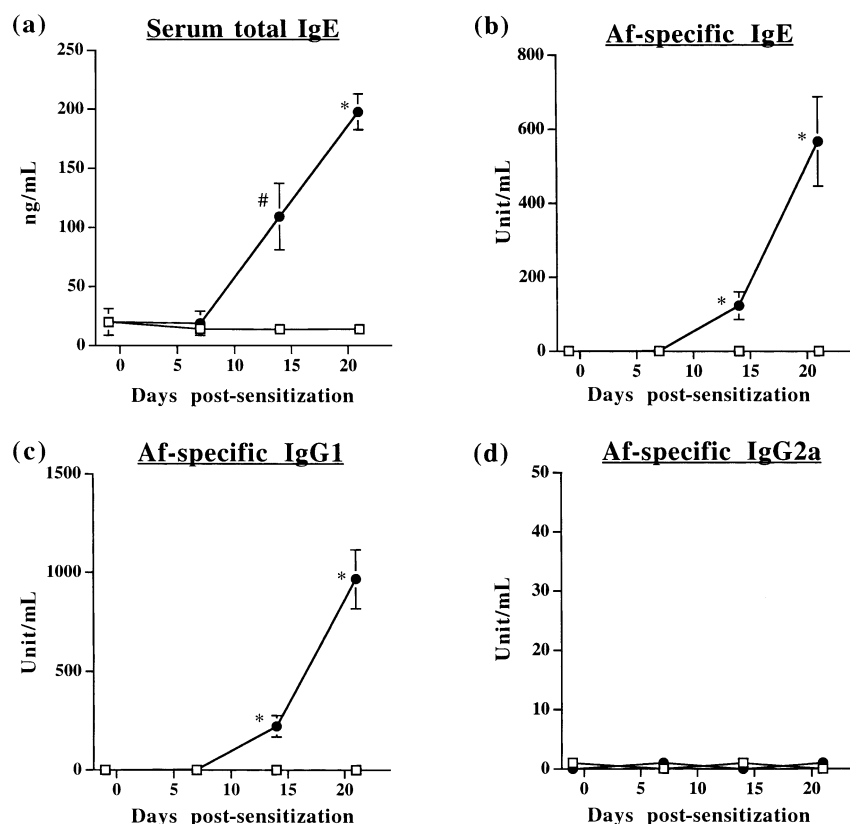


Fig. 1. Levels of serum total IgE (a), Af-specific IgE (b), Af-specific IgG1 (c), and Af-specific IgG2a (d) following intranasal sensitization with native Af. CBA/J female mice (*n* = 6 per group) were sensitized by intranasal administration of 100µg protein of native Af in a total volume of 50µL concentrated with Dulbecco's PBS. This procedure was performed three times in the first week. Thereafter, the mice were sensitized intranasally with 50µg protein of native Af in a total volume of 50µL three times a week for the following 2 weeks. Control mice were treated with PBS alone in an identical manner. Peripheral blood was collected from the tail 12h after the third, sixth and ninth sensitization in addition to prior to the first sensitization. Results show (a) the mean ng/mL ± SEM of six serum samples from each group, and (b, c and d) the mean Unit/mL ± SEM of six serum samples from each group. Data are representative of two separate experiments. #*P* < 0.05; **P* < 0.01.

After nine sensitizations, serum total IgE levels were significantly lower in mice sensitized with periodate-treated Af compared to those sensitized with native or mock-treated Af ($P < 0.05$). The mean $\pm$ SEM levels (ng/mL) were 240.36 ± 44.52 , 88.29 ± 24.67 and 293.16 ± 69.11 for mice sensitized with native, periodate-treated, and mock-treated Af, respectively (Fig. 2a). Mice sensitized with periodate-treated Af also produced lower amounts of Af-specific IgE compared to the control mice sensitized with native ($P < 0.01$) or mock-treated ($P < 0.05$) Af (Fig. 2b). In addition, a significant decrease in specific IgG1 production was seen in mice sensitized with periodate-treated Af compared to those mice treated with native or mock-treated Af ($P < 0.01$, Fig. 2c). Levels of serum total IgE, Af-specific IgE and specific IgG1 were similar between native and mock-treated Af-sensitized mice.

Carbohydrates on Af induce nasal eosinophilia

Next we investigated the local accumulation of eosinophils after repeated intranasal applications of native Af, which is one of the hallmarks of the Th2 response. Nasal eosinophilia was not detected in CBA/J mice 12h after nine sensitizations (data not shown). However, 12h after the last seven consecutive nasal

challenges, Af-sensitized mice displayed remarkable eosinophilia in the nasal septum (Fig. 3a). In addition, the degree of eosinophilia in mice sensitized and subsequently challenged with mock-treated Af was similar (Fig. 3c). In contrast, the degree of eosinophilia was significantly reduced in mice sensitized and subsequently challenged with periodate-treated Af compare to that observed in mice that had received native ($P < 0.05$) or mock-treated ($P < 0.01$) Af (Fig. 3b). The mean number $\pm$ SEM of eosinophils infiltrating into the nasal septum per field (10×40) was 41.75 ± 12.52 , 4.25 ± 2.72 , and 52.00 ± 2.48 in mice sensitized and subsequently challenged with native, periodate-treated and mock-treated Af, respectively ($n = 5$).

Carbohydrates on Af are not epitopes for the induced IgE

Finally, in order to determine whether carbohydrates on Af function as epitopes for the induced Af-specific IgE, inhibition ELISA was performed using native, periodate-treated or mock-treated Af as inhibitors. Native Af inhibited the binding of $1 \mu\text{g/mL}$ biotinylated native Af to plate-bound IgE in a dose-dependent manner. In contrast to all the proceeding data

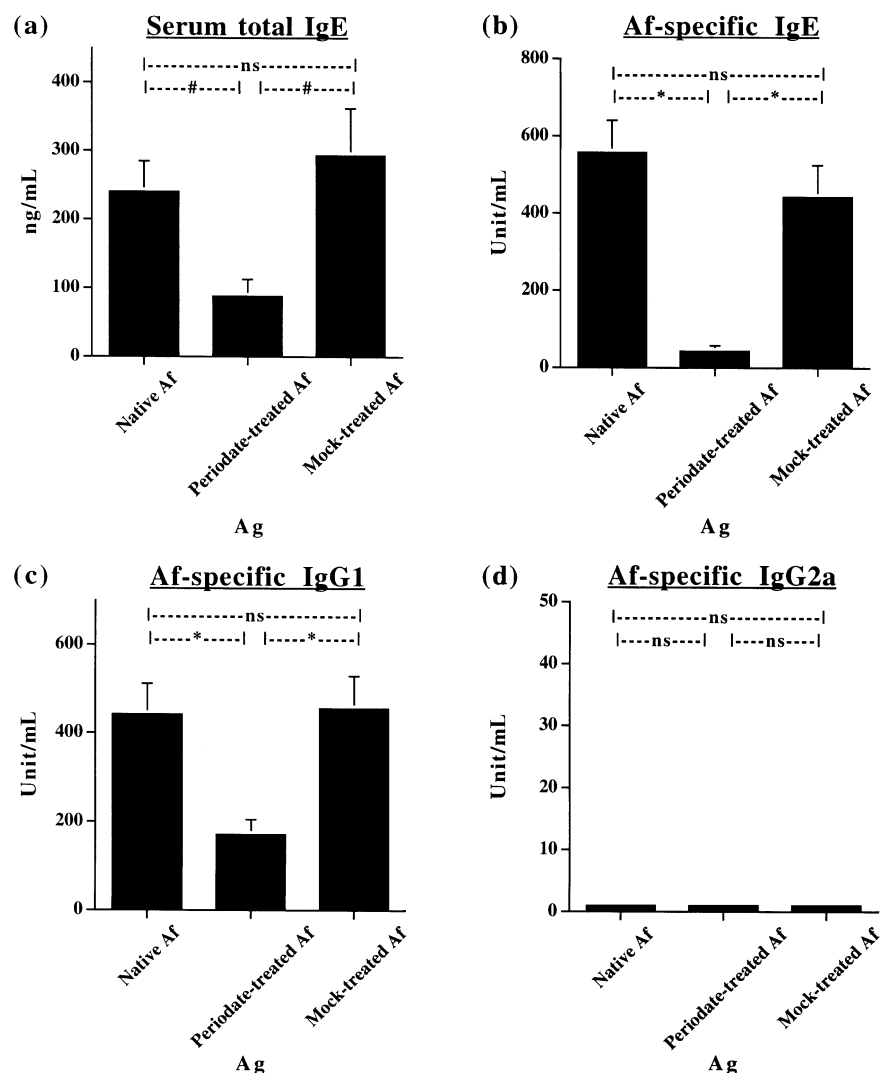


Fig. 2. Antibody production in CBA/J mice after repeated intranasal sensitization with native, periodate-treated, or mock-treated Af. CBA/J mice were sensitized intranasally with native, periodate-treated, or mock-treated Af as described in Materials and Methods. After the ninth sensitization, blood was sampled, and levels of serum total IgE (a), Af-specific IgE (b), Af-specific IgG1 (c), and Af-specific IgG2a (d) were determined by ELISA. Results show (a) the mean ng/mL $\pm$ SEM of six serum samples from each group, and (b, c and d) the mean Unit/mL $\pm$ SEM of six serum samples from each group. Data are representative of two separate experiments. # $P < 0.05$; * $P < 0.01$.

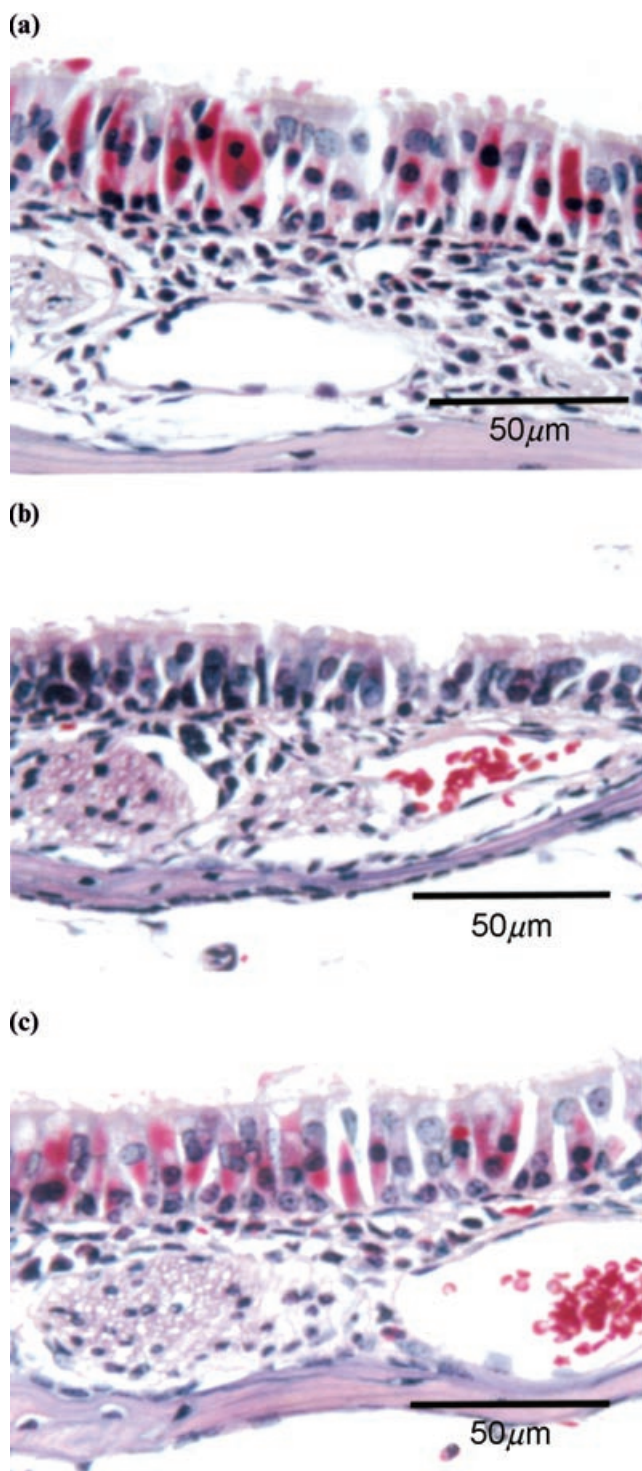


Fig. 3. Nasal eosinophilia in CBA/J mice intranasally sensitized with native Af (a), periodate-treated Af (b), or mock-treated Af (c). 12 h after the final nasal challenge, as described in Materials and Methods, the mice were killed. Nasal sections were fixed followed by decalcification, and Luna staining was performed to detect eosinophils in the nasal mucosa. Typical sections from the tip of the nasal septum are shown.

presented in this study, periodate-treated Af was also able to inhibit binding between biotinylated Af and plate-bound IgE in a dose-dependent manner (Fig. 4). The inhibition by

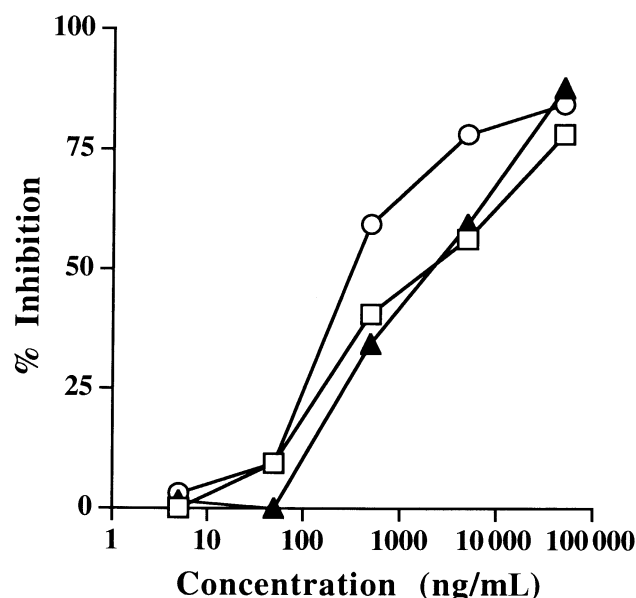


Fig. 4. Inhibition of binding between Af and serum IgE. CBA/J mice were bled following the ninth intranasal sensitization with native Af. The binding between biotinylated Af and serum IgE was inhibited by adding serial concentrations of inhibitors; native Af (open square), periodate-treated Af (closed triangle), or mock-treated Af (open circle). Results show the mean percentage inhibition $\pm$ SEM of triplicate wells. Data are representative of two separate experiments.

periodate-treated Af was similar to that observed for native or mock-treated Af at a concentration of 50 μg/mL (>80.0%).

Discussion

In the present study, we demonstrated that CBA/J mice displayed Af-specific IgE production and nasal eosinophilia after the repeated intranasal application of Af in the absence of adjuvant. Production of antigen-specific IgE and nasal eosinophilia are two of the hallmarks of allergic rhinitis [19]. Thus this model appears to reflect the initiation of allergic rhinitis caused by Af in humans. The present study reveals that carbohydrates on Af play a major role in the induction of Af-specific IgE production and nasal eosinophilia. In addition, the carbohydrates also lead to the production of specific IgG1 but not IgG2a. It is known that IgG1 is regarded as an indicator of Th2 response and IgG2a an indicator of Th1 type response in mice [20]. To our knowledge, this is the first report to show the role of carbohydrates on not only Af, but also on mould antigens in general, in the induction of allergic inflammation *in vivo*. We have recently investigated this effect of carbohydrates on the induction of allergic Th2 responses using two different antigens, SEA from *S. mansoni* and PLA₂ from honeybee venom. Carbohydrates on PLA₂ had no inductive effect on the Th2 response since CBA/J mice sensitized intranasally with deglycosylated PLA₂ produced large amounts of PLA₂-specific IgE and IgG1 as did those treated with native PLA₂ [11]. On the other hand, carbohydrates on SEA were found to be essential to the induction of a Th2 response since BALB/c mice sensitized to periodate-treated SEA produced significantly lower amounts of SEA-specific IgE and exhibited less nasal eosinophilia than mice sensitized with native or mock-treated

SEA [12]. The results presented here are consistent with the latter results and confirm the effect of carbohydrates on the induction of the allergic Th2 response.

Glucan, chitin and galactomannan are known to be contained in the extract of Af [15,16]. On the other hand, the most popular and well-characterized carbohydrate on SEA is lacto-N-fucopentaose III (LNFIII) [21]. Thus the major carbohydrates expressed on Af and SEA appear to be different, although it can not be ruled out that these two antigens share unknown minor carbohydrates. These results suggest that the effect of carbohydrates expressed on the pathogen/antigen driving the Th2 response may depend on the particular composition and/or structure of sugar residues, or on the type of interaction between the sugars and their protein moiety.

There are several explanations of how carbohydrates on Af might induce a Th2 response. The most considerable explanation is that Af is easily internalized into antigen-presenting cells (APCs) via the binding of carbohydrates on Af with carbohydrate-specific receptors on APCs, which may lead to efficient antigen presentation [7,8,22–24]. APCs such as macrophages and dendritic cells express the mannose receptor [8,22]. For example, macrophages can uptake *Pneumocystis carinii* via the mannose receptor, a process which is inhibited by the addition of mannan [23]. In fact, 93 kDa glycoprotein purified from the water-soluble extract of Af contains a non-reducing terminal α 1–3 and α 1–6 mannose, suggesting that this glycoprotein can be taken up by APCs via mannose receptor [24]. In addition, CR3 (Mac-1, CD11b) expressed on macrophages and neutrophils mediates the phagocytosis of pathogens via its interaction with β -glucan expressed on pathogens [7]. It is well known that Af contains large amounts of β -glucan, thus this carbohydrate may be involved in the adjuvant effect via the ligation with CR3 [15,16].

Another possible explanation is that carbohydrates can alter the conformation of a peptide, which may lead to a superagonist response [25]. Harding et al. reported that several T cell hybridomas obtained after immunization of mice with glycopeptide showed a glycopeptide-specific T cell response but gave no response to non-glycosylated peptide [26]. Ishioka et al. also demonstrated that T cells from animals immunized with GlcNAc-Asn-substituted peptides failed to recognize the non-glycosylated analogue [27].

The route of sensitization performed in this study has to be considered. Dendritic cells, which express carbohydrate-binding receptors as described above, are likely to accumulate in the respiratory tract, including the nasal turbinates and nasal-associated lymphoid tissue [22,28]. In addition, B-1 cells can exist in effector sites on the respiratory tract, such as nasal passages in mice [29]. It is known that SEA and bacteria-derived carbohydrates such as lipopolysaccharide (LPS) can activate B-1 cells [30,31]. Thus carbohydrates on Af may activate B-1 cells in the nasal passage, which may induce Ab production [32].

Competitive inhibition ELISA demonstrated that periodate-treated Af could inhibit binding between biotinylated Af and plate-bound IgE in a dose-dependent manner. The role of the carbohydrates on allergen as a target of specific IgE remains a controversial issue [33–36]. Several carbohydrates expressed on allergens, such as Bermuda grass pollen and celery, are essential epitopes for allergen-specific IgE, and function as cross-reactive carbohydrate determinants in humans [33,34]. On the other hand, studies suggest that the carbohydrate moiety is not

necessarily IgE epitopes in several pollens, such as ryegrass and Japanese cedar, in humans [35,36]. In the case of Af, fractions of 90 kDa and 38 kDa react with IgE from allergic patients sensitized to Af [16]. However, carbohydrates from another major allergic component of Af (gp55) were not IgE epitopes in patients with ABPA [17]. Our present data is consistent with the latter finding, and suggest that carbohydrates on Af are not major epitopes for induced IgE in mice, although they are largely involved in the induction of IgE production. In addition, these results are consistent with our previous result in which we used SEA as a sensitizing antigen, and clearly demonstrated that carbohydrates on Af act as internal adjuvants that lead to the production of IgE against the protein portion [12].

Treatment with sodium metaperiodate, especially in strong conditions, may render the antigen more susceptible to degradation or clearance. However, we performed the treatment under mild conditions in the present study [12]. In addition, we have recently demonstrated that periodate oxidation under mild conditions does not affect the antigenicity of peptides representing major T cell epitopes in Cry j1, the major allergen molecule of Japanese cedar pollen, since mAbs specific for major T cell epitopes could bind to both periodate-treated and mock-treated Cry j1 with the same magnitude [37]. Because the treatment with sodium metaperiodate under mild conditions is completely identical between Cry j1 and Af, the treatment does not appear to render Af more susceptible to degradation or clearance as compared to mock treatment.

In summary, we have demonstrated the functional role of carbohydrates expressed on Af in terms of the induction of Th2 allergic inflammation. Carbohydrates on Af act as internal adjuvants leading to nasal eosinophilia and specific IgE production against the protein portion. Although the composition and structure of the specific carbohydrate that acts as an adjuvant needs to be clarified in the future, these results provide an insight into the pathogenesis of Af-associated IgE-mediated diseases. In addition, it is known that mucosal application with Th2-inducing antigens such as SEA can alter cellular responses in Th1 type autoimmune diseases [38]. Thus these findings may contribute to the development of novel mucosal adjuvants for therapeutic purpose.

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