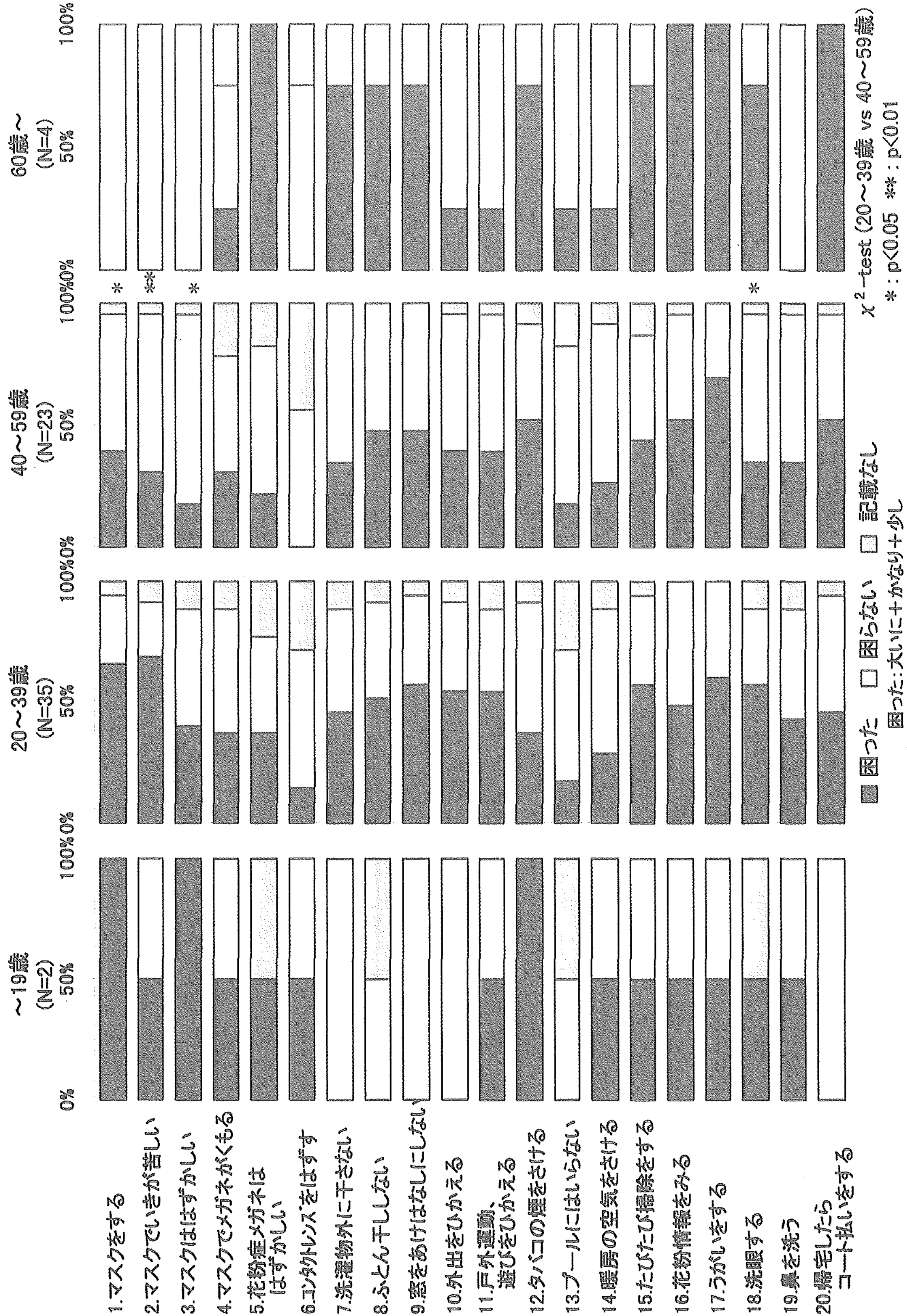

図 28.　予防行為のQOL項目影響率の年齢別分布

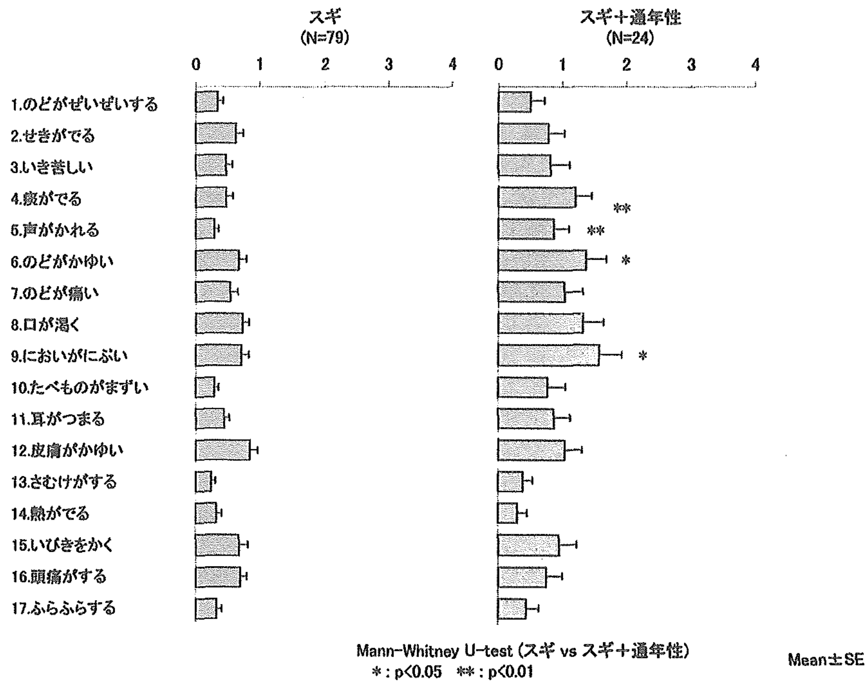

図 29.　鼻，目以外の症状の QOL 各項目スコア
―弁別感受性―

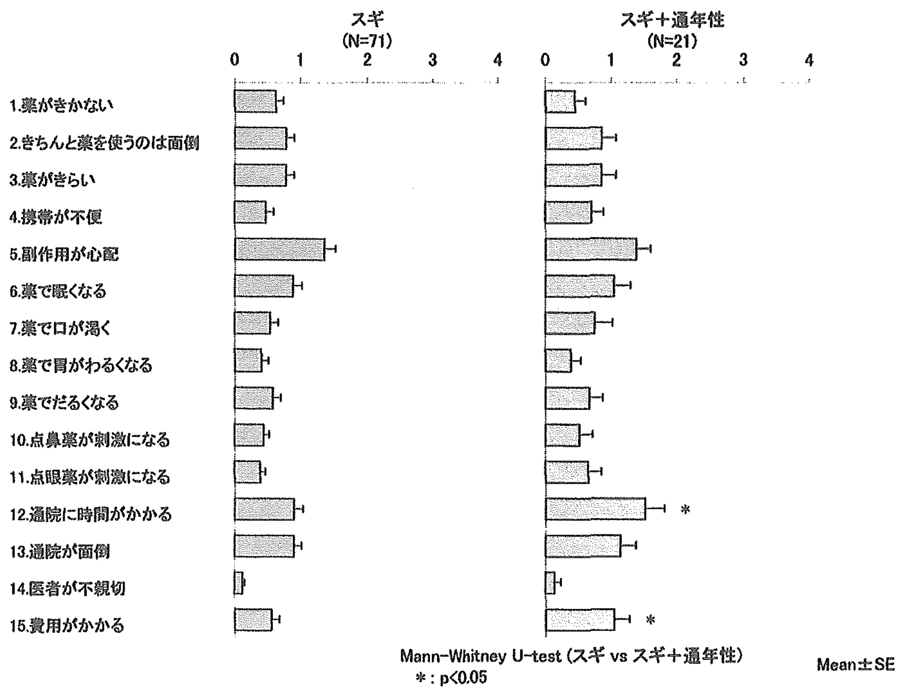

図 30.　治療の QOL 項目スコア
―弁別感受性―

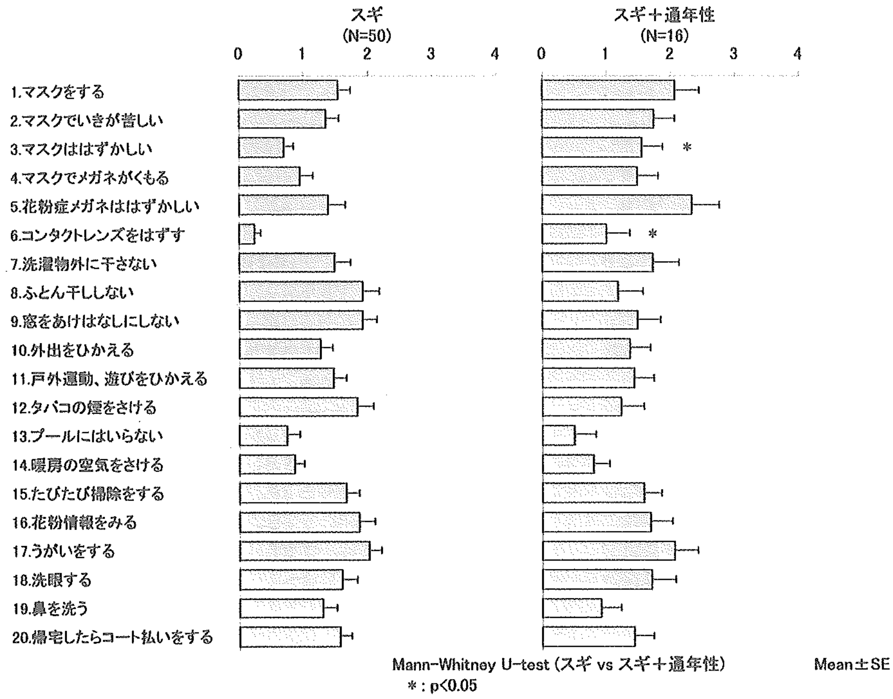

図31．予防行為のQOL項目スコア
―弁別感受性―

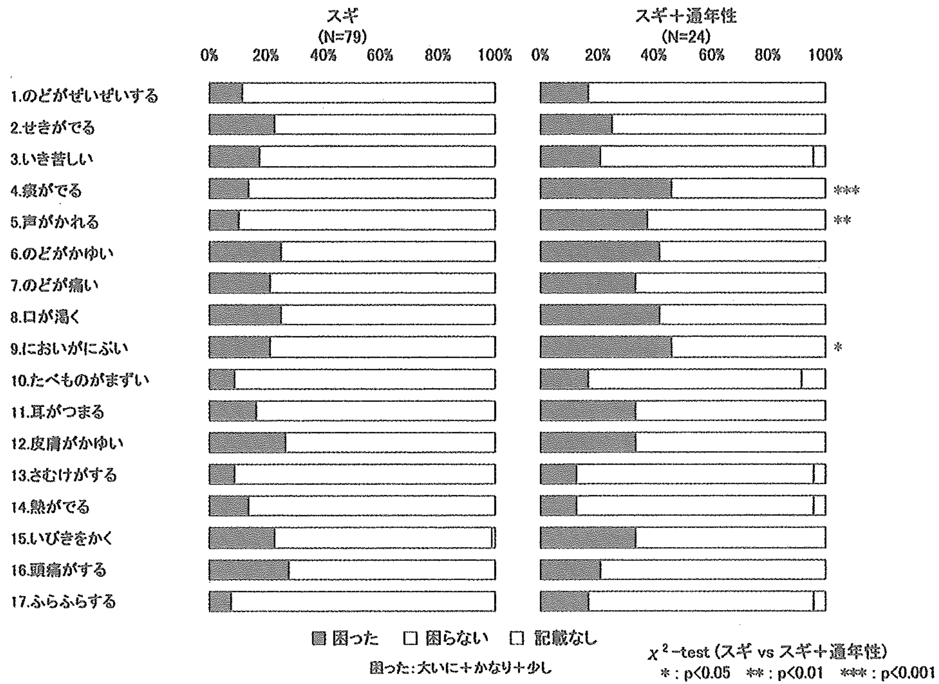

図32．鼻，目以外の症状のQOL項目影響率
―弁別感受性―

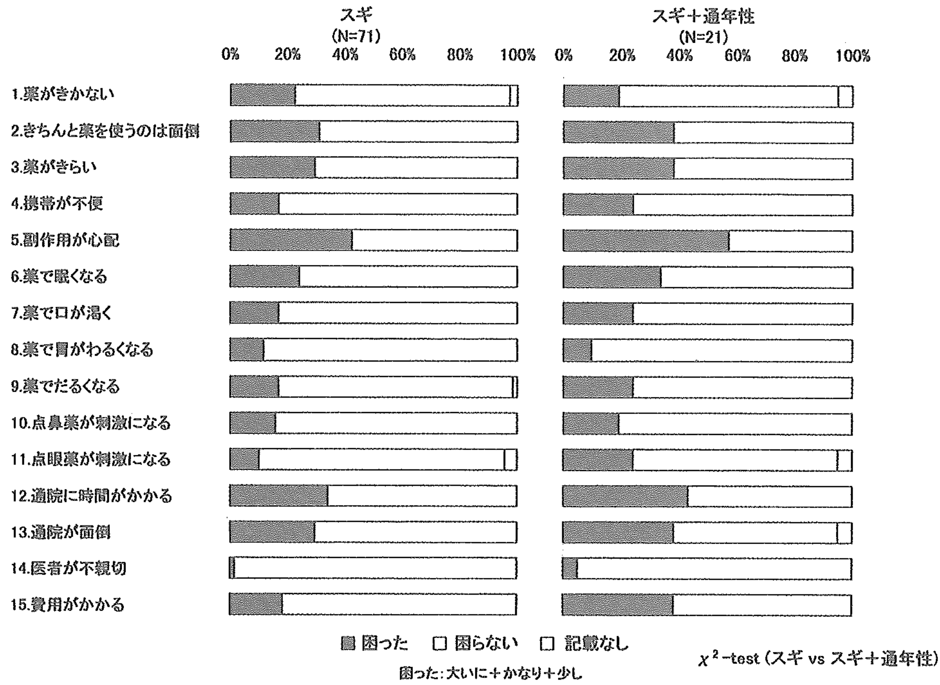

図 33.　治療の QOL 項目影響率
—弁別感受性—

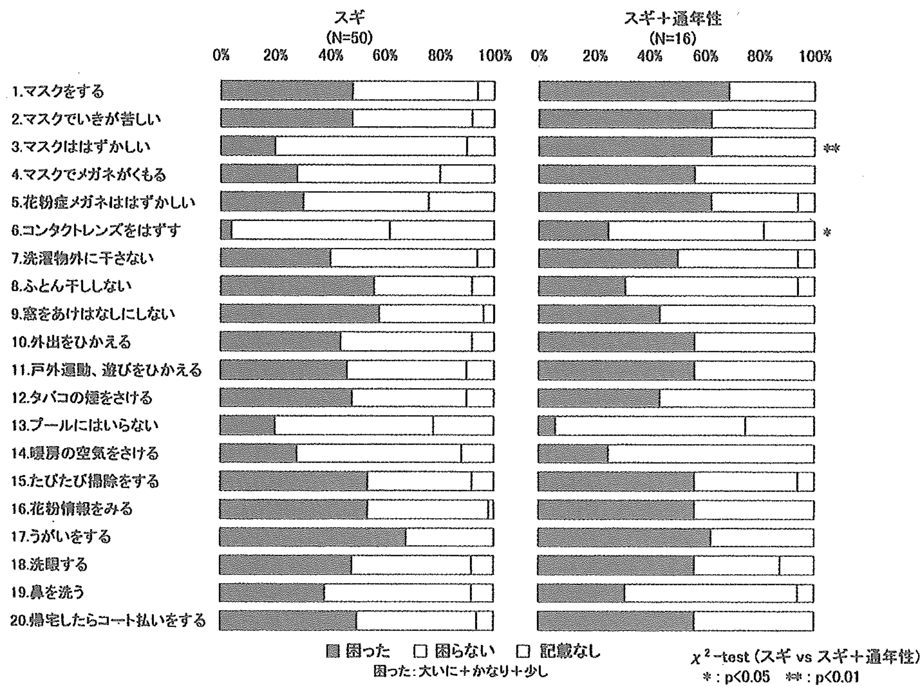

図 34.　予防行為の QOL 項目影響率
—弁別感受性—

プール，暖房，鼻洗を除き，スギ花粉症と通年性AR合併に生活支障率が単独より高かった(図32～34)．すなわち弁別感受性が示された．

まとめ

各部門における生活支障度，不便度は予防を除き強くないが，調査票の応答性は良好で，各部門の項目間相関性，因子妥当性，臨床妥当性，信頼性，敏感性も満足すべきものと推定された．症状ではさむけ，ふらふら，治療では医師不親切，予防ではコンタクトレンズ，プールの項目の削除，質問文の文言を修正して，一応の成案を得た〈付2〉．疾患の程度，治療法，予防法にもよるが，予防を除き生活支障，不便度は強くなかった．JQLQ No2の鼻眼以外の症状はRQLQのそれとはのどの乾き，頭痛を除きまったく異なり，RQLQではむしろ日常生活，身体，精神生活に混入されている．治療，予防行為のQOLへの影響に関する項目はRQLQにはない．治療効果，予防効果の評価にQOLの重要性が唱えられているので，必要に応じJQLQ No2とともに，使用すれば便利である．なお調査票はスギ花粉症を主な対象に作成されたので，他の花粉症，通年性ARにも使用可能か今後の検討の必要があるが，第2編で通年性ARへの使用の可能性が検証されている．

小野薬品工業株式会社の本研究への協力を感謝する．調査票利用希望者は作成委員会代表奥田　稔に連絡し，所定の手続をとれば，使用の便宜を計らう．

文　献

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2) 萬代　隆：QOL評価質問表の開発と応用．万代隆監修，QOL評価法マニュアル．インターメジカ．10–13, 2001.

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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 eosinophila 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 A_2 (PLA_2) from the venom of the honeybee *Apis mellifera* [11,12]. We have demonstrated that carbohydrates on SEA, but not PLA_2, 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].

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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 7days 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-(OD450 nm with inhibitor/OD450nm without inhibitor)) × 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

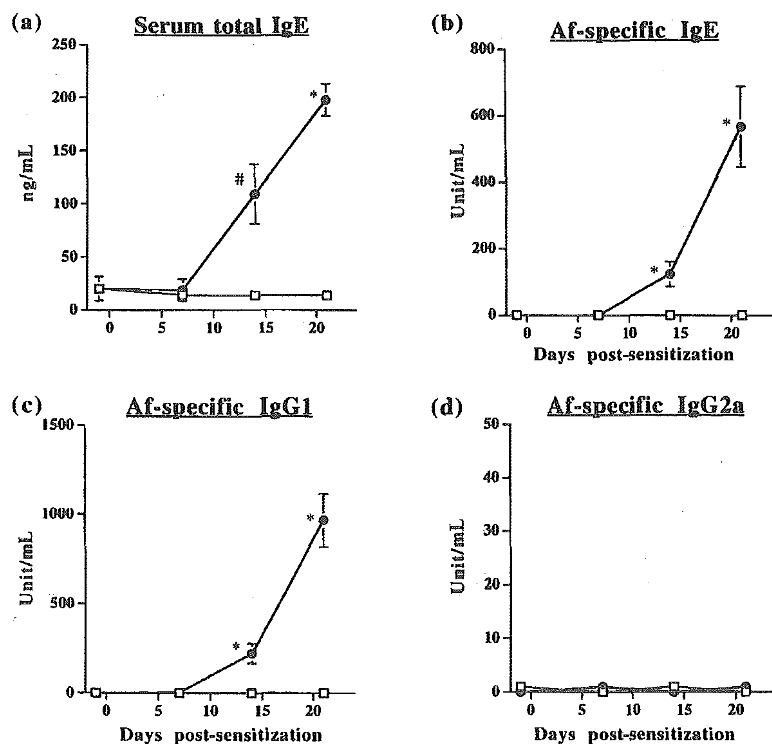

Fig. 1. Levels of serum total IgE (a), Af-specific IgE (b), Af-specific IgG1 (c), and Af-specific IgG2a (d) following intranasal sensitization with native Af. CBA/J female mice (n=6 per group) were sensitized by intranasal administration of 100μg protein of native Af in a total volume of 50μL concentrated 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 2weeks. 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 $\pm$ 44.52, 88.29 $\pm$ 24.67 and 293.16 $\pm$ 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 eosinophila 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 eosinophlia 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 $\pm$ 12.52, 4.25 $\pm$ 2.72, and 52.00 $\pm$ 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 µg/mL biotinylated native Af to plate-bound IgE in a dose-dependent manner. In contrast to all the proceeding data

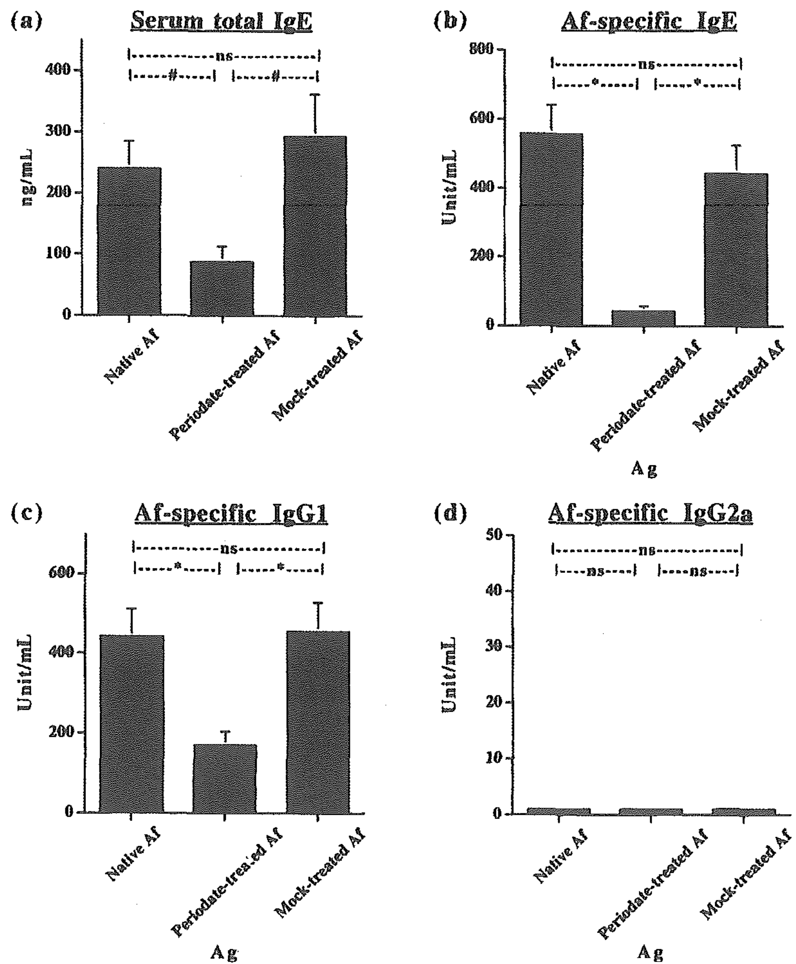

Fig. 2. Antibody production in CBA/J mice after repeated intranasal sensitization with native, periodate-treated, or mock-treated Af. CBA/J mice were sensitized intranasally with native, periodate-treated, or mock-treated Af as described in Materials and Methods. After the ninth sensitization, blood 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. #$P<0.05$; *$P<0.01$.

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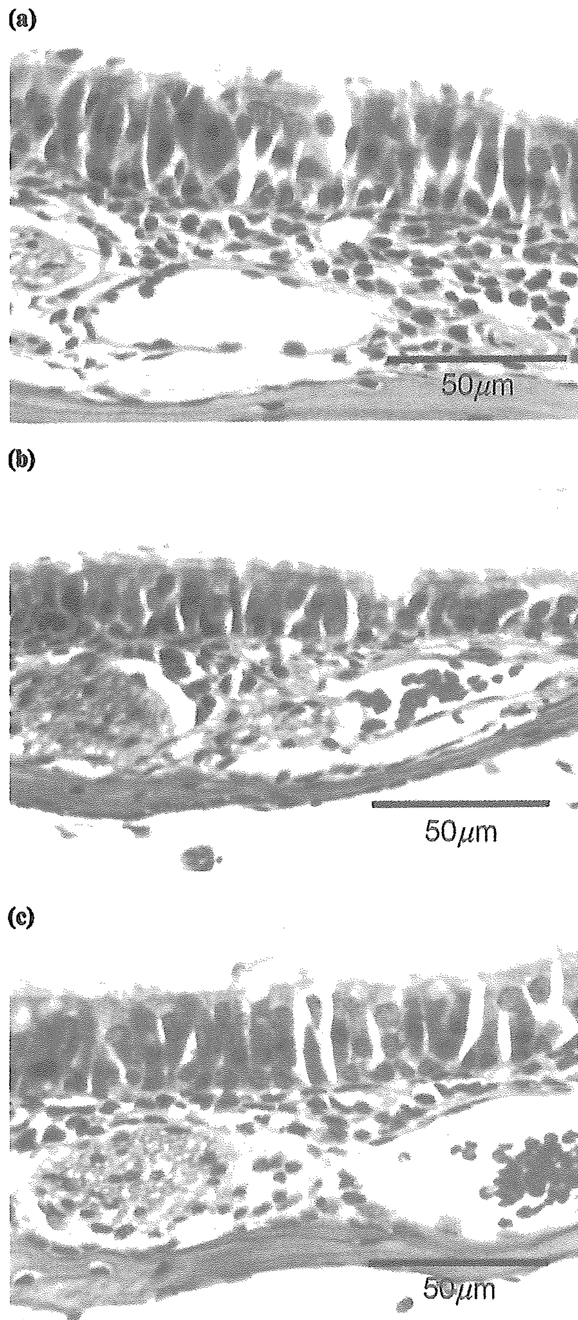

Fig. 3. Nasal eosinophilia in CBA/J mice intranasally sensitized with native Af (a), periodate-treated Af (b), or mock-treated Af (c). 12h after the final nasal challenge, as described in Materials and Methods, the mice were killed. Nasal sections were fixed followed by decalcification, and Luna staining 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

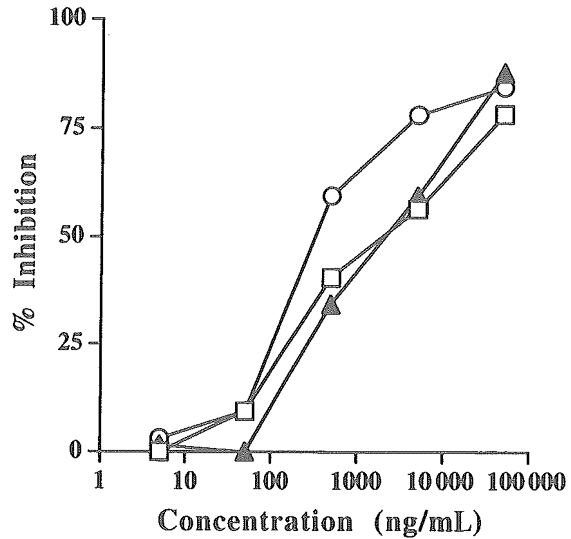

Fig. 4. Inhibition of binding between Af and serum IgE. CBA/J mice were bled following the ninth intranasal sensitization with native Af. The binding between biotinylated Af and serum IgE was inhibited by adding serial concentrations of inhibitors; native Af (open square), periodate-treated Af (closed triangle), or mock-treated Af (open circle). Results show the mean percentage inhibition ± 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 50μg/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 PLA_2 from honeybee venom. Carbohydrates on PLA_2 had no inductive effect on the Th2 response since CBA/J mice sensitized intranasally with deglycosylated PLA_2 produced large amounts of PLA_2-specific IgE and IgG1 as did those treated with native PLA_2 [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 α1–3 and α1–6 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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Intranasal application of purified protein derivative suppresses the initiation but not the exacerbation of allergic rhinitis in mice

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Summary

Background Several epidemiological and experimental studies have demonstrated that exposure to pathogens such as those from the genus *Mycobacterium* leads to the suppression of allergic sensitization and inflammation. However, little is known as to whether pathogen-derived soluble antigens have the potential to modulate the pathogenesis of allergic rhinitis.

Objective We sought to determine whether application of purified protein derivative (PPD) from *Mycobacterium tuberculosis* can suppress the initiation and/or exacerbation of allergic rhinitis using a recently developed murine model.

Methods First, we investigated whether a single intranasal application of PPD could elicit cytokine production in the nose by RT-PCR. BALB/c mice were repeatedly sensitized with *Schistosoma mansoni* egg antigen (SEA) intranasally without an adjuvant. PPD was applied through different routes either before or after sensitization. The production of SEA-specific antibodies, nasal eosinophilia and cytokines by nasal lymphocytes was compared among mice that had or had not received PPD treatment.

Results IFN-γ, but not IL-4, was detected in the nasal tissue 12 to 48 h after a single intranasal application of 10 μg PPD. Repeated intranasal application of PPD prior to and during sensitization with SEA significantly inhibited the production of both SEA-specific IgE/IgG1 and nasal eosinophilia. Moreover, it partially inhibited the production of IL-4 by nasal lymphocytes in response to SEA. Conversely, this treatment led to a significant increase in IFN-γ production. On the other hand, PPD applied through the footpad had no effect over the same period. Repeated intranasal application of PPD after sensitization with SEA had no exacerbative effect on allergic inflammation.

Conclusion These results indicate that the local application of PPD, and the subsequent induction of IFN-γ, inhibits the initiation, but not the exacerbation, of allergic rhinitis in mice. This suggests that pathogen-derived antigens have potential for use in the prevention and prophylaxis of allergic rhinitis.

Keywords allergic rhinitis, eosinophil, IFN-γ, IgE, IL-4, mice, mycobacterium, nose, PPD

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Introduction

Allergic rhinitis is the most common allergic disease and is characterized by the production of allergen-specific IgE and nasal eosinophilia [1]. In developed countries, more than 10% of the population suffer from allergic rhinitis and the disease creates burdens such as medical expenses and loss of productivity [2]. Previous studies have indicated that Th2-type cytokines, such as IL-4 and IL-5, play a pivotal role in the pathogenesis of the disease [3]. It is widely accepted that IL-4 induces the differentiation and expansion of Th2 lymphocytes, which in turn produce IL-4, IL-5, IL-6, IL-10 and IL-13 [4,5]. IL-4 also plays a critical role in the production of IgE, which mediates immediate hypersensitivity reactions [6,7]. Furthermore, IL-4 up-regulates the expression of VCAM-1 on vascular endothelial cells. VCAM-1 is a ligand for VLA-4, which is expressed on eosinophils and basophils but is absent on neutrophils [8]. IL-5 is particularly important in allergic inflammation as it selectively promotes chemotaxis, activation and the survival of eosinophils [9–11].

One strategy to prevent the initiation and/or exacerbation of allergic disease involves skewing local inflammatory sites (i.e. the nasal mucosa of subjects with allergic rhinitis) towards a Th1-dominated micro-environment, as Th1-associated cytokines down-regulate Th2 function [6]. In fact, several epidemiological studies support this hypothesis [12–14]. For example, the prevalence of allergic rhinitis in subjects with a history of viral hepatitis, which causes Th1 immune responses, is significantly lower than that of other segments of

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the population [13]. In addition, an inverse relationship has been observed between the total amount of serum IgE measured and the degree of delayed-type hypersensitivity (DHT) following intradermal injection with purified protein derivative of *Mycobacterium tuberculosis* (PPD), in Japan [14]. Furthermore, studies using experimental animals have shown that when a Th1-dominated immune environment is established by prior infection or vaccination with live, attenuated or killed pathogen, Th2 allergic inflammation is suppressed [15–17]. Of the pathogens studied, those from the genus *Mycobacterium* appear to be the most potent inducers of Th1-type responses [18].

We recently developed a murine model of allergic rhinitis [19]. Using this model, the repeated intranasal administration of *Shistosoma mansoni* egg antigen (SEA), in the absence of an adjuvant, induced a Th2 response and led to the production of SEA-specific IgE as well as eosinophil infiltration into the nasal mucosa [19]. We used this model to determine whether pathogen-derived antigen can solely suppress the initiation and/or exacerbation of allergic rhinitis, in the present study. We chose PPD, rather than Bacillus Calmette-Guerin (BCG), as the Th1-inducer as the *in vivo* application of BCG is known to induce granuloma formation in the lung and liver [16, 17] and thus, may be harmful for clinical use. Our results are the first to demonstrate that the local application of PPD induces IFN-γ in the nasal mucosa and suppresses the initiation of allergic rhinitis. These results provide evidence with regard to the role of pathogens and pathogen-derived antigens in altering inflammation in allergic rhinitis. Moreover, they suggest the potential merits of a new approach for regulating allergic rhinitis via the intranasal application of Th1-inducible antigens during the critical period of sensitization.

Materials and methods

Animals

Female BALB/c mice aged 7 to 10 weeks old were purchased from Charles River Japan (Yokohama, Japan). The mice were maintained in SPF conditions at Okayama University Medical School in accordance with the guidelines set forth by the Okayama University Medical Area Research Committee.

Antigens

A Puerto Rican strain of *Schistosoma mansoni* was maintained using Biomphalaria glabrata snails and CD-1 mice (Taconic Farms, Germantown, PA, USA). SEA was prepared from eggs harvested from the livers of CD-1 mice 7 to 8 weeks after intraperitoneal infection with 200 cercaria, as previously described [20]. PPD was purchased from Nihon BCG Seizo Co. (Tokyo, Japan).

Determination of cytokine signals after intranasal application of antigens

To determine this, 0.1, 1 or 10 μg of PPD, or 5 μg of SEA dissolved in 20 μL PBS ($n = 4$/group), were intranasally applied to BALB/c mice. Before and 12, 24, 48 and 72 h after application, mRNA was extracted from the nasal tissue, then IL-4, IFN-γ and β-actin were detected by RT-PCR, as described elsewhere [21].

Sensitization of mice

We designed two procedures for intranasal sensitization in order to investigate the effects of PPD application on either the initiation or exacerbation of allergic rhinitis in our recently developed murine model [19]. In order to determine whether PPD application can alter the initiation of allergic rhinitis, 10 μg of PPD in 20 μL PBS was applied intranasally once a week for 3 weeks, then 5 μg of SEA mixed with 10 μg of PPD was inoculated into the nostril once a week for 3 weeks. One week later, 1 μg of SEA mixed with 2 μg of PPD was challenged intranasally for 7 consecutive days (Fig. 1a). Blood was sampled from the tail 1 day prior to sensitization with SEA, 5 days after the third sensitization with SEA, and 12 h after the final nasal challenge. In addition, 12 h after the final challenge, the mice were sacrificed and nasal sections were obtained (Fig. 1a). In another set of experiments, an equal amount of PPD to that used for intranasal application was administered through the footpad in order to determine whether the route of PPD application makes a difference in altering SEA sensitization.

Next, we sought to determine whether intranasal application of PPD can alter the exacerbation of allergic inflammation in intranasally pre-sensitized mice. First, mice were sensitized intranasally with 5 μg of SEA once a week for 3 weeks. Sensitization to SEA was confirmed by the presence of SEA-specific IgE 5 days after the third sensitization. Then we applied 10 μg of PPD intranasally one, three or five times, over 2 weeks. Following the 2-week PPD treatment, mice were challenged

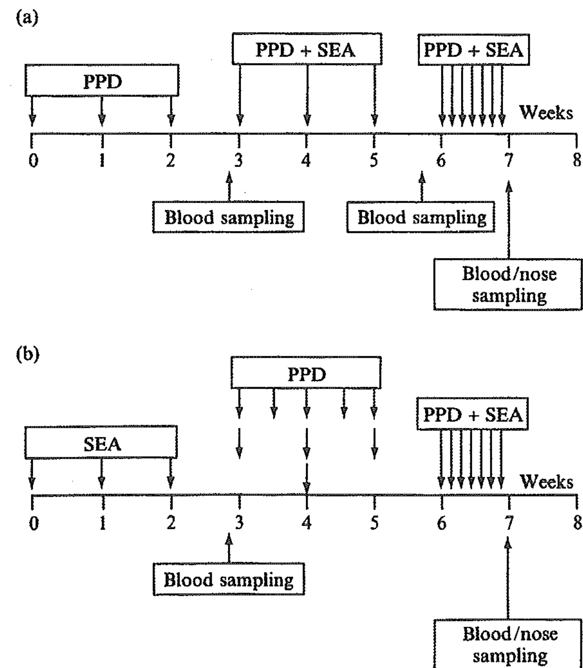

Fig. 1. Experimental design used to investigate the effect of PPD application on the initiation (a) or exacerbation (b) of allergic rhinitis in mice. BALB/c mice were sensitized and subsequently challenged with SEA in the absence of an adjuvant. PPD was given intranasally or injected into the footpad prior to and during sensitization with SEA (a), or after sensitization (b). Sensitization was confirmed by the presence of SEA-specific IgE.

with 1 μg of SEA mixed with 2 μg of PPD for 7 consecutive days, and blood samples and nasal sections were obtained 12 h after the final challenge (Fig. 1b).

Serum Ab titres

Antigen-specific IgE was determined by sandwich ELISA using biotinylated antigen as the detection reagent, as previously described [7, 19]. Titres are expressed as the mean OD at 450 nm of six individual serum samples diluted to 1:4. Ag-specific IgG1 and IgG2a Ab were detected by indirect ELISA, as previously described [7, 19]. Titres are expressed as the mean OD at 450 nm of six individual serum samples diluted to 1:100.

Histological examination

Histological examination was performed as previously described with a slight modification [7, 19]. In brief, 12 h after the final nasal challenge, mice were killed by cervical dislocation. Their heads were removed, fixed in 10% formalin and decalcified with 2.5% EDTA-2Na solution. Coronal nasal sections were stained with Luna solution and the number of eosinophils in the nasal mucosa was counted microscopically under a high power field (10 × 40).

In vitro culture of nasal lymphocytes

Twelve hours following the final nasal challenge, mice were sacrificed and their nasal lymphocytes were isolated by enzyme extraction using collagenase, as described by Asanuma *et al.* [22]. Viable lymphocytes were counted by trypan blue exclusion with a Neuebauer haemocytometer. Cell suspensions were adjusted to 2×10^6 cells/mL in RPMI 1640 medium (Gibco, NY, USA) containing 10% FCS, and 100 units/mL : 100 μg/mL of penicillin : streptomycin (Sigma). Cells were cultured in flat bottomed 48-well plates (Corning) with SEA (5 μg/mL) and either ConA (2 μg/mL), which served as a positive control, or supplemented medium, which served as a negative control. Following incubation at 37 °C for 72 h in 5% CO_2, supernatant was collected and stored at − 80 °C until assayed for cytokine production.

Cytokine determination

IL-4 and IFN-γ production by stimulated (SEA and ConA) and unstimulated nasal lymphocytes was measured by sandwich ELISA, as previously described [23]. Detection limits for IL-4 and IFN-γ were 0.1 U/mL and 1.0 U/mL, respectively

Statistical analysis

Student's unpaired *t*-test was used to determine the statistical significance of the values obtained. $P < 0.05$ was considered statistically significant. Data are expressed as the mean ± standard error of mean (SEM) for each subject group.

Results

Effect of intranasal application of PPD on the induction of cytokines

A total of 10 μg of PPD was intranasally applied to BALB/c mice and mRNA was subsequently extracted from the nasal tissues. Then IL-4, IFN-γ and β-actin were detected by RT-PCR. IL-4 was not detected during the period of study; however, INF-γ could be detected 12 to 48 h following a single application of 10 μg PPD (Fig. 2a). On the other hand, IL-4 was detected within the nasal tissues 12 to 28 h following the intranasal application of 5 μg SEA (Fig. 1b). In addition, 1 μg or less of PPD did not induce nasal production of IFN-γ (data not shown). These results indicate that 10 μg of PPD appears to induce a Th1-dominated micro-environment in BALB/c noses; thus we have chosen this amount for *in vivo* application in our further investigations.

Effect of intranasal application of PPD on the initiation of allergic rhinitis

We sought to determine the effect of PPD on the initiation of allergic rhinitis in a murine model we have recently developed [7]. As described in Materials and methods, the intranasal application of PPD was performed prior to and during sensitization with SEA (Fig. 1a). The production of SEA-specific IgE was significantly lower in mice treated with PPD, compared with mice that did not receive PPD treatment (Fig. 3a). In addition, the production of specific IgG1 was also lower in mice treated with PPD (Fig. 3b). However, the production of specific IgG2a was inversely increased in these mice, although the difference observed was not significant (Fig. 3c).

Following nasal challenge with SEA, eosinophil infiltration into the nasal septum was significantly lower ($P < 0.01$) in mice treated with PPD, compared with those that did not receive PPD treatment (Fig. 4). On the other hand, no histological changes were seen in the lungs of either PPD-treated or untreated SEA-sensitized mice (data not shown). The average number ± SEM of eosinophils infiltrating into the nasal septum per field (10 × 40) was 79.83 ± 9.06 in mice sensitized with SEA alone and 43.17 ± 9.13 in mice treated with PPD.

After the final nasal challenge, nasal lymphocytes were isolated and stimulated with ConA or SEA for 72 h, and the culture supernatant was assayed for cytokine production. SEA-stimulated nasal lymphocytes from PPD untreated mice produced Th2-associated IL-4 but not Th1-associated IFN-γ

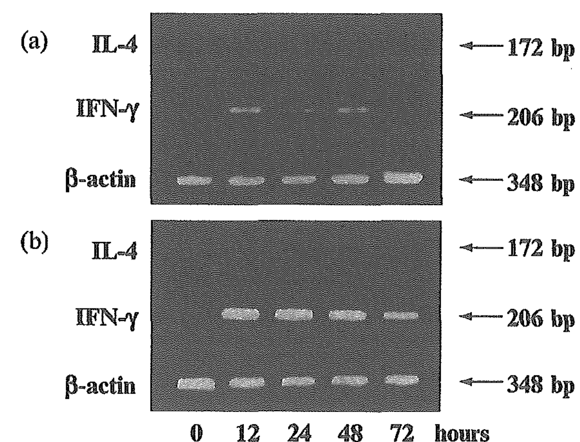

Fig. 2. Expression of IL-4, IFN-γ and β-actin in the nasal tissues after a single intranasal application of PPD (a) or SEA (b). 10 μg PPD (a) or 5 μg SEA (b) in 20 μL PBS was applied intranasally through the nostril in BALB/c mice ($n = 4$). Before and 12, 24, 48 and 72 h after application, mRNA was extracted from the nose, then the levels of IL-4, IFN-γ and β-actin were detected by RT-PCR, as described in Materials and methods.

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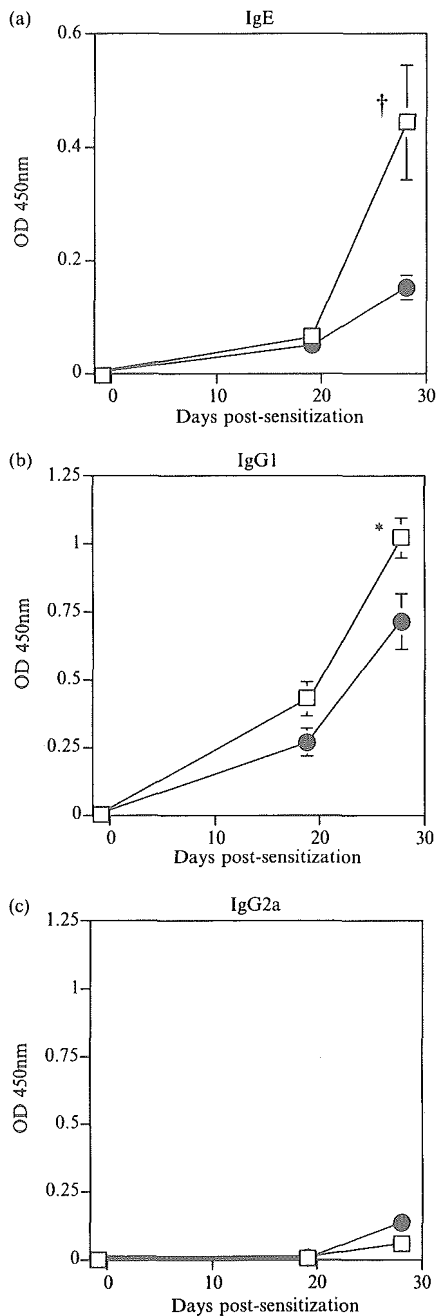

Fig. 3. Effect of intranasal application of PPD on the induction of antibody production. BALB/c mice ($n = 6$ per group) were intranasally sensitized with SEA. PPD (●) or PBS (□), which served as a control, was applied *intranasally prior to and during sensitization and subsequent challenge*, as shown in Fig. 1(a). Blood was sampled from the tail 1 day prior to sensitization with SEA, 5 days after the third sensitization with SEA, and 12 h after the final nasal challenge. Titres of SEA-specific IgE (a), IgG1 (b) and IgG2a (c) were determined by ELISA. Results show (a) the mean OD at 450 nm ± SEM of six serum samples from each group at a 1:4 dilution, and (b, c) the mean OD at 450 nm ± SEM of six serum samples from each group at a 1 : 100 dilution. Data are representative of three separate experiments. $^*P < 0.05$; $^{\dagger}P < 0.01$.

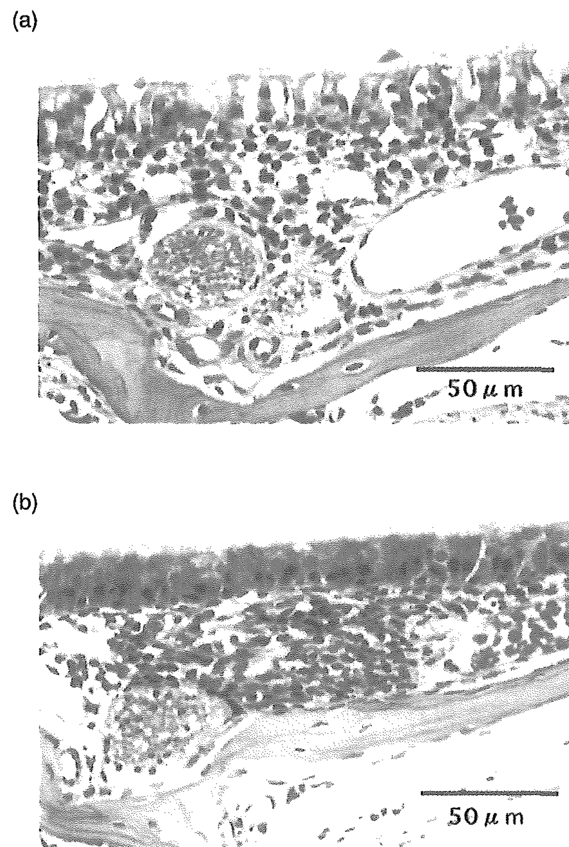

Fig. 4. Effect of intranasal application of PPD on the induction of nasal eosinophilia. BALB/c mice ($n = 6$ per group) were intranasally sensitized with SEA. PBS (a) or PPD (b) was applied intranasally prior to and during sensitization and subsequent challenge. 12 h after the final nasal challenge with SEA, mice were sacrificed. Nasal sections were fixed, decalcified, and Luna staining was performed to detect eosinophils in the nasal mucosa.

(Fig. 5). Nasal lymphocytes from mice intranasally treated with PPD showed an increased production of IFN-γ in response to SEA. In addition, the production of IL-4 in response to SEA was reduced in mice treated with PPD, albeit the difference observed was not significant ($P < 0.1$). Similar results were also seen when cells were stimulated with ConA. These results indicate that the intranasal application of PPD prior to and during sensitization with SEA can suppress the initiation of allergic rhinitis in BALB/c mice.

Effect of footpad application of PPD on the initiation of allergic rhinitis

In order to determine whether the suppressive effect of PPD on the initiation of allergic rhinitis is dependent on the route of application, we injected mice with 10 μg of PPD via the footpad, following the same time line used to study the effects of intranasal application. Unlike that observed for mice that were intranasally inoculated with PPD, the footpad application of PPD had no effect on the initiation of allergic rhinitis. Mice that received repeated PPD applications into their footpad produced SEA-specific IgE and IgG1, the amounts of which were

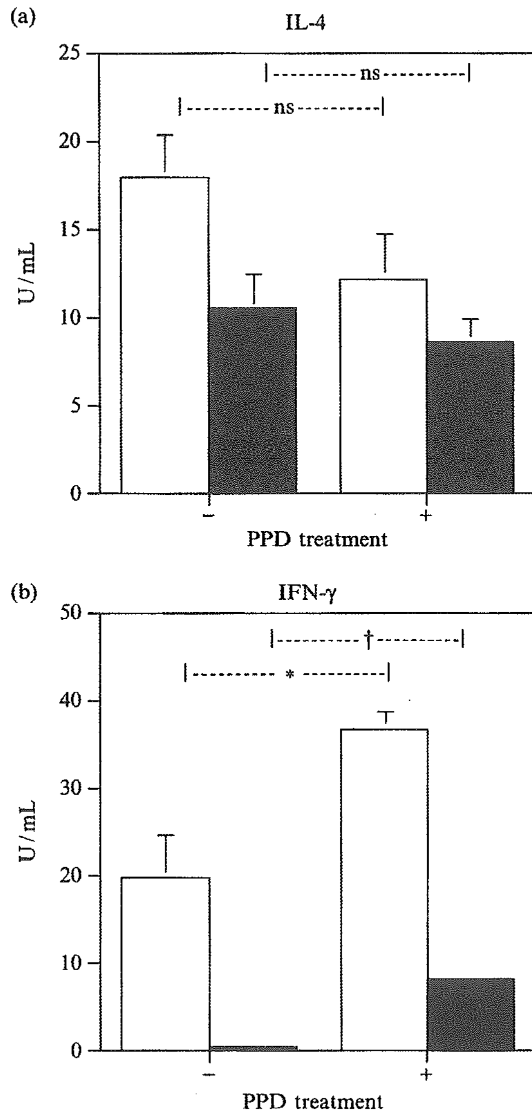

Fig. 5. Production of IL-4 (a) and IFN-γ (b) by nasal lymphocytes from BALB/c mice treated with PPD or PBS intranasally prior to and during sensitization and subsequent challenge with SEA. Nasal lymphocytes were isolated and cultured *in vitro* for 72 h with ConA (□) or SEA (■). Cytokines were measured by ELISA. Results show the mean ± SEM of three separate experiments. *$P < 0.05$; †$P < 0.01$.

comparable with that produced by mice that had not received PPD treatment (Fig. 6). In addition, following nasal challenge with SEA, eosinophil infiltration into the nasal septum was similar among mice that had and had not received PPD treatment via the footpad (data not shown).

Effect of intranasal application of PPD on the ongoing exacerbation of allergic rhinitis in intranasally pre-sensitized mice

Next, we investigated whether intranasal application of PPD is also effective at suppressing ongoing allergic inflammation in

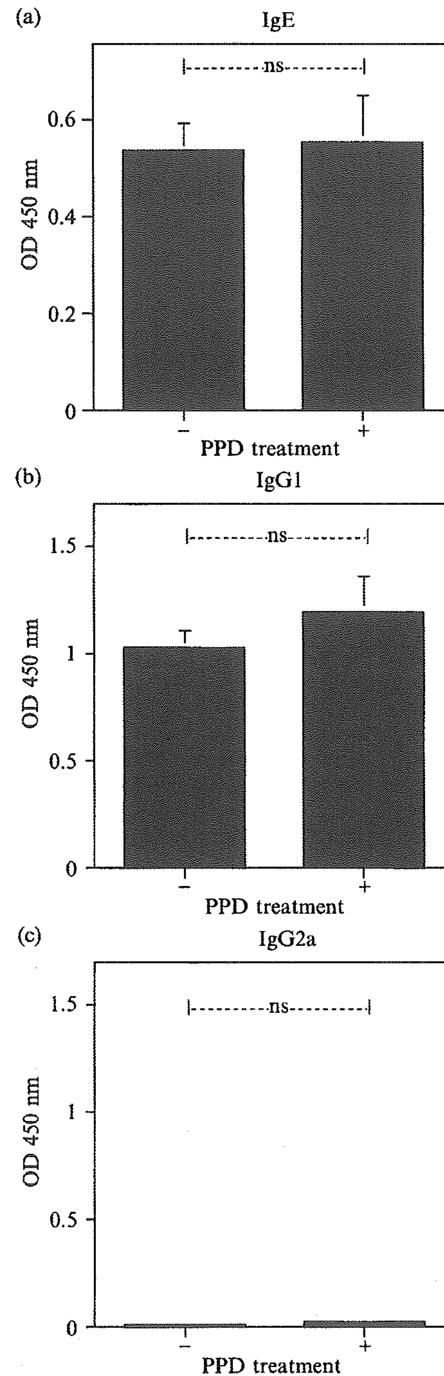

Fig. 6. Effect of footpad application of PPD on the induction of antibody production. BALB/c mice ($n = 6$ per group) were intranasally sensitized with SEA. PPD, or PBS as a control, was delivered into the footpad prior to and during sensitization and subsequent challenge. Blood was sampled from the tail 12 h after the final nasal challenge with SEA. Titres of SEA-specific IgE (a), IgG1 (b) and IgG2a (c) were determined by ELISA. Results show (a) the mean OD at 450 nm ± SEM of six serum samples from each group at a 1:4 dilution, and (b, c) the mean OD at 450 nm ± SEM of six serum samples from each group at a 1:100 dilution. Data are representative of two separate experiments.

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intranasally pre-sensitized mice (Fig. 1b). In this experiment, mice treated with PPD produced increased amounts of specific IgE and IgG1, similar to that produced by PPD untreated mice (Fig. 7). In addition, similar numbers of nasal eosinophila were seen in both PPD-treated and untreated mice (Fig. 8). The average number ± SEM of eosinophils infiltrating the nasal septum per field (10 × 40) after intranasal sensitization with SEA was 124.67 ± 17.17, 106.50 ± 17.17 and 108.67 ± 10.30 in mice treated with PPD intranasally one, two and three times, respectively; 131.67 ± 16.20 eosinophils per field were detected in the nasal septum of PPD untreated mice. These results suggest that the intranasal application of PPD does not have a suppressive effect on the exacerbation of allergic inflammation in intranasally pre-sensitized mice.

Discussion

In the present study, we used PPD as a potential moderator of the initiation and exacerbation of allergic rhinitis as this antigen is known to induce strong IFN-γ production in both humans and mice [24, 25]. However, little is known regarding the effect of PPD on the nasal mucosa, the actual inflammatory site of allergic rhinitis. We first demonstrated that a single application of 10 μg PPD into the nostril induces the signals for IFN-γ in the nose. IFN-γ was detected 12 to 48 h after application. However, IL-4 was not detected throughout the period of observation. Our results are consistent with a report in which the intravenous embolization of polymer beads bound with PPD into the lungs of BALB/c mice induced INF-γ but not IL-4 production by lung granulomas on day 2 [25]. Moreover, no significant histological changes, such as granuloma formation, were observed in the lungs of mice involved in our procedure. These results encouraged us to argue the potential merits of intranasal application of PPD for the suppression of Th2 allergic inflammation of the nose because the application of PPD appears to be harmless, in comparison with BCG, in terms of granuloma formation [16, 17].

Mice that received PPD intranasally prior to and during intranasal sensitization with SEA, produced significantly less SEA-specific IgE and IgG1 Abs, which are positively regulated by Th2 cytokines, especially IL-4 [6]. In addition, this treatment increased the production of Th1-associated IgG2a, although the difference observed was not significant. Herz et al. demonstrated that intravenous BCG infection, prior to systemic OVA sensitization, suppresses the production of OVA-specific IgE and IgG1 Abs and doubles the production of anti-OVA IgG2a Ab [17]. Scott et al. reported that the concomitant application of heat-killed *Brucella abortus* (BA) coupled with sensitization with OVA adsorbed to alum, suppresses the high IgE response [15]. On the other hand, Erb et al. reported that intranasal BCG infection before systemic OVA sensitization does not affect the production of OVA-specific Abs [16]. Our results are consistent with the former reports and suggest that the intranasal application of a stimulus capable of inducing IFN-γ production can inhibit the synthesis of Th2-type Abs after local sensitization without an adjuvant. In fact, local isotytpe switching to IgE following nasal challenge was observed in the nasal mucosa by the detection of clones of deleted switch circular DNA (Sepsilon/Smu), representing a mu to epsilon switch in nasal lavage cells [26].

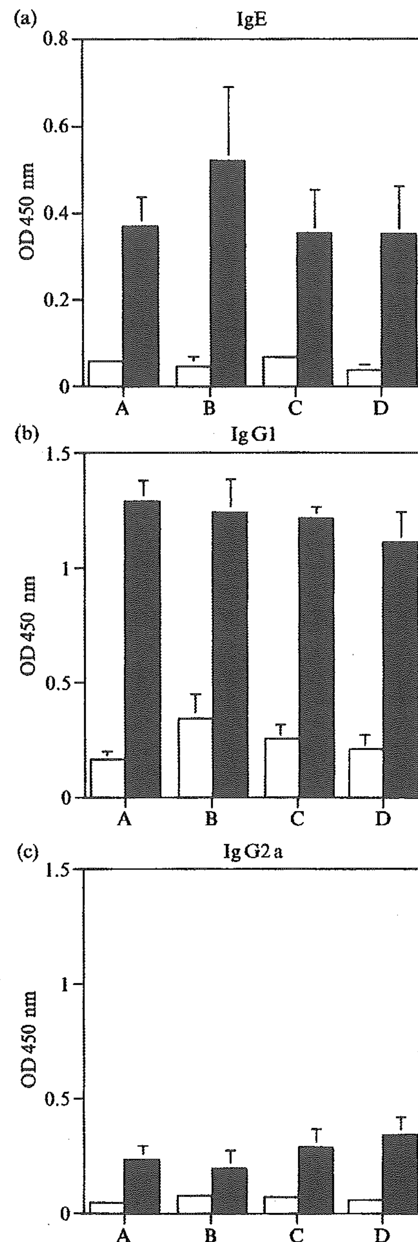

Fig. 7. Effect of intranasal application of PPD on the exacerbation of antibody production. BALB/c mice ($n = 6$ per group) were intranasally sensitized with SEA. Serum was withdrawn 5 days after the third sensitization, at which point we could detect similar amounts of SEA-specific IgE, IgG1 and IgG2a among the groups (□). Then 10 μg of PPD was applied intranasally one (B), three (C) or five times (D) over 2 weeks, as shown in Fig. 1(b). After the 2-week PPD treatment, mice were challenged with 1 μg of SEA mixed with 2 μg of PPD for 7 consecutive days, and blood samples were obtained 12 h after the final challenge (■). Control mice were given SEA alone throughout the study (A). Titres of SEA-specific IgE (a), IgG1 (b) and IgG2a (c) were determined by ELISA. Results show (a) the mean OD at 450 nm ± SEM of six serum samples from each group at a 1:4 dilution, and (b, c) the mean OD at 450 nm ± SEM of six serum samples from each group at a 1:100 dilution. Data are representative of two separate experiments.

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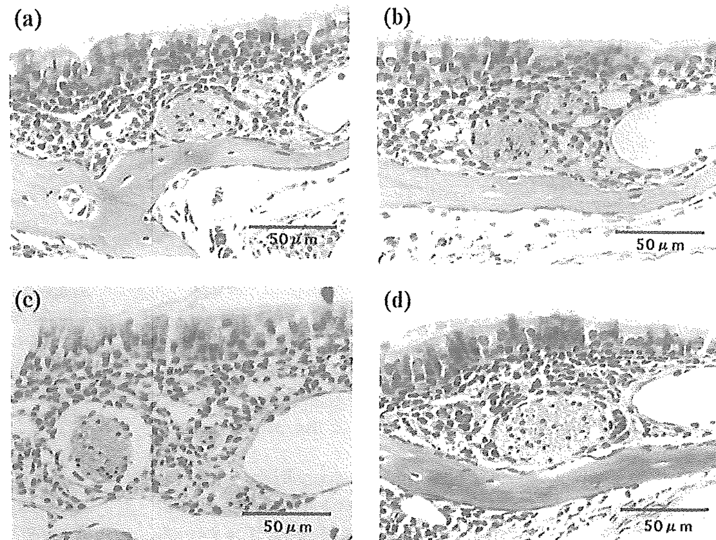

Fig. 8. Effect of intranasal application of PPD on the exacerbation of nasal eosinophilia. BALB/c mice ($n = 6$ per group) were intranasally sensitized with SEA. Then 10 mg of PPD was applied intranasally one (b), three (c) or five times (d) over 2 weeks. After the 2-week PPD treatment, mice were challenged with 1 μg of SEA mixed with 2 μg of PPD for 7 consecutive days, and their heads were removed 12 h after the final challenge. Control mice were given SEA alone throughout the study (a). Nasal sections were fixed, decalcified, and Luna staining was performed to detect eosinophils in the nasal mucosa.

Mice that received PPD intranasally prior to and during intranasal sensitization with SEA, further showed a significant reduction in eosinophil infiltration into the nasal mucosa after intranasal challenge with SEA. Previous reports have demonstrated that BCG infection prior to systemic sensitization with OVA suppresses the accumulation of eosinophils in BAL fluid after local challenge with OVA [16, 17]. Our results confirm this and suggest that, not only can the mycobacterium itself, but also a soluble protein antigen from the pathogen, elicit a suppressive effect on local eosinophilia when inoculated locally and prior to sensitization with allergens.

In addition, the nasal lymphocytes from mice that received PPD treatment prior to and during sensitization with SEA produced lesser amounts of IL-4 and significantly greater amounts of IFN-γ in response to SEA or ConA, compared with those that did not receive PPD treatment. Hertz et al. reported that splenic mononuclear cells from mice that were injected intravenously with BCG prior to intraperitoneal sensitization with OVA produced significantly lower amounts of IL-4 and greater amounts of IFN-γ in response to ConA, compared with those that did not receive BCG treatment [17]. Yang et al. also showed that OVA-driven IFN-γ production is significantly greater in BCG-infected mice compared with those not infected [27]. Summarizing these results, local PPD application prior to and during intranasal sensitization with SEA inhibits initiation of the allergic Th2 response by skewing the microenvironment towards Th1.

Importantly, the suppressive effect of PPD on the initiation of allergic rhinitis depends on the route of application. Intranasal application of PPD was observed to suppress the initiation of allergic rhinitis, whereas application of antigen into the footpad had no such effect. There is a debate with regard to whether BCC vaccination, or infection with mycobacterium, can inhibit the prevalence of allergic disease [14, 28]. Shirakawa et al. demonstrated the existence of an inverse relationship between the total amount of serum IgE measured and the degree of delayed-type hypersensitivity (DHT) experienced, due to infection with a purified protein derivative of *Mycobacterium tuberculosis* (PPD) in Japan [14]. In Japan, natural exposure to mycobacterium is common, which suggests that infection with *Mycobacterium* may inhibit the onset of atopy [14]. On the other hand, receiving a BCG vaccination before 6 months of age had no observed effect on the prevalence of atopy in Sweden, where the risk of tuberculosis is low [28]. Our finding that the footpad application of PPD had no effect may explain why percutaneous, but not local, BCG vaccination is ineffective in preventing airway allergy in humans living in non-endemic areas for tuberculosis. Erb et al. also demonstrated that intranasal infection with BCG is a more potent stimulus for eosinophil recruitment into airways, compared with intraperitoneal or subcutaneous infection with the pathogen [16]. Thus, direct application of PPD into the nose appears to be useful in preventing allergic rhinitis.

On the other hand, intranasal application of PPD did not regulate either the production of SEA-specific Abs or nasal eosinophilia in mice intranasally pre-sensitized with SEA. In addition, we confirmed that intranasal PPD treatment during the effector phase had no effect on allergic inflammation in mice systemically pre-sensitized with OVA (data not shown). Several reports showed that reversal of an established Th2 response is difficult and has not been effectively achieved by using recombinant cytokines [29,30]. Results presented here are consistent with the reports and suggest that the use of PPD might have limitations with regard to suppressing ongoing Th2 allergic inflammation. In contrast, Scott et al. demonstrated that the application of heat-killed BA down-regulated the secondary OVA-specific IgE response and enhanced the IgG2a response in OVA-pre-sensitized mice [15]. Yeung et al. also reported that heat-killed *Listeria monocytogenes* can act as an adjuvant and convert established Th2-dominated responses against keyhole limpet haemocyanin (KLH) into Th1-dominated responses [31]. In addition, Wang et al. showed that a single injection of heat-killed *Mycobacterium vaccae* into OVA-pre-immunized BALB/c mice suppressed an increase of serum IgE [32]. Therefore the suppressive effect of Th1-inducible intracellular pathogen itself and soluble protein

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stimuli on the pre-existing and/or ongoing Th2 responses appears to be different.

There are several explanations of how intracellular pathogen but not soluble protein stimuli could promote the suppression of the pre-existing and/or ongoing Th2 responses. The most likely explanation is that the pathogen can exert the powerful and long-term suppression against allergic Th2 responses. For example, signals for INF-γ could be detected for up to 48 h in the nose following a single intranasal application of PPD (Fig. 2a), whereas a single intranasal infection with BCG led to a significant increase of IFN-γ production for up to 4 weeks [16].

A second possible explanation is that the intracellular pathogen can activate $CD8^+$ T cells, while exogenous soluble antigen usually can not lead to the activation. In fact, immunization with heat-killed *M. vaccae* results in the generation of $CD8^+$ T cells, which secrete IFN-γ and enhance the production of IL-12 [33].

Another possible explanation is that the deposition, retention and clearance in murine tissues might differ between the particlated pathogen and soluble protein. For example, it is known that aerosolized pollutant was cleared from the respiratory tract by direct absorption into blood, while particlated pollutant was cleared by both blood absorption and mucociliary clearance followed by ingestion and faecal excretion [34]. In addition, protein stimuli appear to be more susceptible to degradation by mucous fluids containing enzymes such as lysozyme and peroxidase as compared with the pathogen itself [35].

In summary, our data demonstrate that intranasal application of PPD before and during sensitization suppresses the initiation of allergic rhinitis by skewing the nasal microenvironment towards Th1, although it has no exacerbative effect on ongoing allergic inflammation in pre-sensitized mice. As the use of local Th1-inducible stimuli has minimal side-effects, including a lack of granuloma formation, which has been observed to occur when the pathogen itself is used, clinical trials using local Th1-inducible stimuli should yield promising insight into the prevention of allergic rhinitis. In addition, further investigations should focus on the local application of other pathogen-derived stimuli, such as CpG oligodeoxynucleotide, as parental application of Th1-inducing stimuli may induce autoimmune diseases, most of which are controlled by Th1 immune responses [36, 37].

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