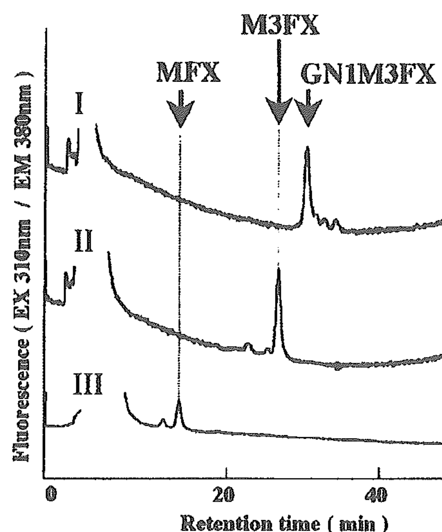

Fig. 7. SF-HPLC of Exoglycosidase Digests of PA-Sugar Chain D.
I, PA-sugar chain D; II, the diplococcal β-*N*-acetylglucosaminidase digest of I; III, the jack bean α-mannosidase digest of II. Arrows (GN1M3FX, M3FX, and MFX) indicate the elution positions of authentic PA-sugar chains, GlcNAc1Man3Xyl1Fuc1GlcNAc2-PA (GN1M3FX), Man3Xyl1Fuc1GlcNAc2-PA (M3FX), and Man1Xyl1Fuc1GlcNAc2-PA (MFX) respectively.

weights of these two Jun a 1 molecules were almost the same, as shown in Fig. 1. Therefore, it seems that the number of glycosylation sites is not be different. From the deduced amino acid sequence of Jun a 1,[7] two potential glycosylation sites have been predicted, but at this moment, it is still obscure whether both asparagine residues are glycosylated.

Plant complex type *N*-glycans containing the Lewis a epitope have been found in various plant glycoproteins, including the sycamore laccase,[7] storage glycoproteins,[16] and a carleticulin from *L. tulipifera*.[17] The structures of *N*-glycans linked to Cry j 1, one of the Japanese cedar pollen allergens, have already been reported.[3,4] In these reports, however, the structures of *N*-glycans of the glycoallergen (Cry j 1) have been reported to have the Galβ1–4(Fucα1–6)GlcNAcβ1– unit instead of the Lewis a epitope. Therefore, it appears that this is the first report to show the occurrence of the Lewis a epitope in the *N*-glycan moiety of pollen allergens. Although the physiological activity of the plant *N*-glycans harboring the Lewis a unit remains to be investigated, it might be worthwhile to assay some immunological activities for the human cellular immune system, since we have already found that the free M3FX can suppress the production of IL 4 from T-cells of pollinosis patients.[5]

Acknowledgments

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Roles of major oligosaccharides on Cry j 1 in human immunoglobulin E and T cell responses

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Summary

Background We have demonstrated that carbohydrates in Cry j 1, the major allergen of *Cryptomeria japonica* pollen, play a major role in promoting Cry j 1-specific Th2 response. However, little is known as to whether the carbohydrates directly participate in allergic responses.
Objective We sought to determine whether Cry j 1-related oligosaccharides function as IgE and/or T cell epitopes. In addition, the regulatory effect of Cry j 1-related oligosaccharide on Cry j 1-specific T cell responses was investigated.
Methods Two monovalent oligosaccharides largely found on Cry j 1, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (M3FX), and GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (GN2M3FX) were prepared. Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (M9A) was used as control. Competitive inhibition ELISA for Cry j 1-specific IgE was performed using these oligosaccharides as inhibitors. In addition, T cell lines specific for Cry j 1 or purified protein derivative of *Mycobacterium tubecurosis* (PPD) were established, and cellular responses against these oligosaccharides were investigated in the presence or absence of the respective antigens.
Results Overall, neither M3FX nor GN2M3FX displayed inhibitory effect on the binding between IgE and Cry j 1. In addition, M3FX did not by itself stimulate Cry j 1 or PPD-specific T cells. However, M3FX significantly inhibited Cry j 1-induced proliferation and IL-4 production in Cry j 1-specific T cells. Such an inhibitory effect was not seen in PPD-specific T cell responses.
Conclusion These results suggest that Cry j 1-related oligosaccharides are not major epitopes for IgE or T cells. However, these oligosaccharides have a novel potential to inhibit Cry j 1-specific T cell responses selectively.

Keywords IgE, oligosaccharide, pollen, T cell, Th2
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Introduction

Carbohydrates expressed on allergens are known to participate in both initiation and exacerbation of allergic responses. Several carbohydrates on allergens/antigens such as *Aspergillus fumigatus* (Af) and *Schistosoma mansoni* egg antigen participate in *in vivo* induction of Th2 response [1–3]. For example, we have recently demonstrated that deglycosylation of Af antigen by periodate treatment significantly abrogates its ability to induce *in vivo* Af-specific IgE production and nasal eosinophilia in a murine model of allergic rhinitis [3]. On the contrary, carbohydrates on phospholipase A2, a major allergen of honeybee venom, are not involved in the induction of Th2 responses *in vivo* [4].

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Several reports demonstrated that carbohydrates on allergens such as phospholipase A2 from bee venom, sea squirt allergen, Lol p 11 from rye grass, Bermuda grass pollen BG60 and Ole e 1 from olive pollen have potential as IgE epitopes [5–10]. In addition, carbohydrate structures such as β[1, 2]-xylose and α[1, 3]-fucose are shared by a number of unrelated glycoallergens, and can act as cross-reactive carbohydrate determinants (CCD) which lead to the multiple sensitization detected *in vitro* [11, 12]. On the other hand, it remains controversial whether the carbohydrates show biological activity [9, 10, 13–15].

Further, carbohydrates on allergens can elicit specific T cell responses. For example, carbohydrates on phospholipase A2 and the pollen extract from *Parietaria judaica* , respectively, were recognized by $CD4^+$ and $CD8^+$ T cells from allergic patients [16, 17]. Also, several T cell clones specific to Bet v 1 from birch pollen and Phl p 5 from *Phleum pratense* do not recognize recombinant isoallergens that do not contain carbohydrate structures unlike the natural isoforms [18,19]. However, little is known whether the carbohydrates on

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allergen directly act as T cell epitopes or whether they contribute to the immunogenicity of glycosylated allergens.

Japanese cedar pollinosis is the most prevalent allergic disease in Japan. A recent epidemiological study demonstrated that approximately 20% of people living in urban areas suffer from the disease [20]. Cry j 1 is one of the major allergens on the pollen, and up to 95% of the patients have Cry j 1-specific IgE in serum [21, 22]. Cry j 1 is known as a glycoprotein with a 6% (W/W) carbohydrate content [21]. Five possible *N*-linked glycosylation sites have been found in the primary amino acid sequence of this allergen although it is not known whether all these sites are glycosylated [23]. Furthermore, *N*-linked oligosaccharide structures of Cry j 1 have been proposed (Fig. 1; [24]). Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (M3FX), and GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (GN2M3FX) but not Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (M9A) were largely found on the allergen. Inhibition assay revealed that carbohydrates on Cry j 1 contribute little or only conformationally to the reactivity of specific IgE in humans [24]. On the other hand, Taniai et al. [25] reported that glycosylation of NGNATPQLTKNAGVLTCSLSKR sequence on Cry j 1 might be required for IgE binding to the peptide moiety. In addition, we have demonstrated that carbohydrates on Cry j 1 play a major role in promoting Cry j 1-specific Th2 response *in vitro* [26]. However, in such investigations, structurally related glycopeptide and/or periodate-treated glycoproteins were used, and it cannot be determined whether the carbohydrates directly function as IgE and/or T cell epitopes or whether they affect the allergenicity of peptide portion.

In the present study, we investigated the direct roles of major oligosaccharides on Cry j 1 in specific IgE and T cell responses by using monovalent oligosaccharides. We believe that findings presented here may provide new insights into not only the pathogenesis of pollinosis, but also the development of novel tools for pollen-specific immunotherapy since M3FX seems to be one of the common oligosaccharides shared by unrelated pollen allergens [11, 12].

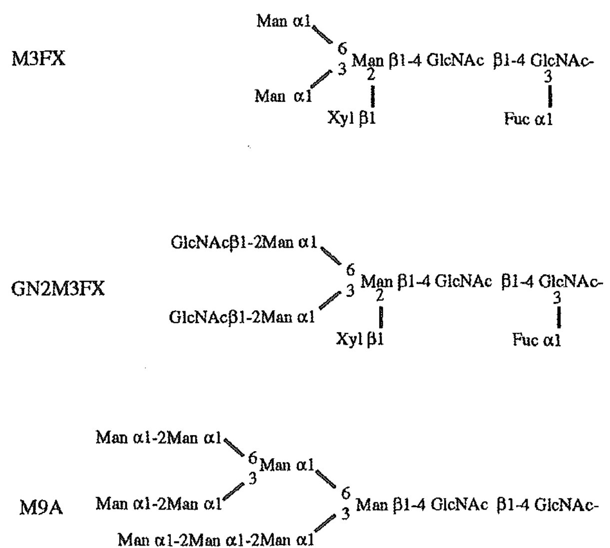

Fig. 1. Structures of oligosaccharides.

Methods

Antigens and momovalent oligosaccharides

Cry j 1 was purified from crude extracts of *Cryptomeria japonica* pollen (JCP) in a well-established procedure [21]. Protein concentration of Cry j 1 was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Purified protein derivative of *Mycobacterium tubecurosis* (PPD) was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). Cry-consensus, a recombinant polypeptide containing major T cell epitopes of p106–120 and p211–225 on Cry j 1, was constructed as described previously [27]. Monovalent GN2M3FX (GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (pyridylaamino)), M3FX (Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3) GlcNAc-PA), and M9A (Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA) were prepared and purified from some plant glycoproteins (*Ginko biloba* pollen glycoprotein [28], *Ricinus communis* agglutinin (RCA 120) [29], and royal jelly glycoproteins [30]) (Fig. 1). In brief, *N*-glycans were released by hydrazinolysis (100 °C, 12 h, in anhydrous hydrazine) from each plant glycoproteins (100–300 mg). After *N*-acetylation of the hydrazinolysate with saturated ammonium bicarbonate (20 mL) and acetic anhydride (0.8 mL), the acetylated hydrazinolysate was desalted by Dowex 50 × 2 resin. Pyridylamination (PA) of the sugar chains was done by the method of Kondo et al. [31]. Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus with a Jasco (Tokyo, Japan) 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-AR column (1.0 × 25-cm, Nacalai Tesque Inc., Kyoto, Japan) or a Shodex Asahipak NH2P-50 column (10 × 25 cm, Showa Denko Co., Tokyo, Japan). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 20% in 60 min at a flow rate 1.5 mL/min. In the case of size-fractionation HPLC using the Asahipak NH2P-50 column, the PA-oligosaccharide was eluted by increasing the water content in the water–acetonitrile mixture from 30% to 50% linearly in 60 min at a flow rate of 1.5 mL/min.

Serum samples

Forty-five patients with Japanese cedar pollinosis (mean age, 33.60 ± 19.61 years; range, 7–81 years; 22 males and 23 females) were enrolled in this study. Sensitization to JCP was confirmed by the presence of specific-IgE antibodies determined by CAP [32] (Pharmacia, Uppsala, Sweden; ranging from 1.27 to 146.34 (mean 28.47 ± 31.36) UA/mL). Of these, 12 patients were sensitized to JCP alone or both JCP and Hinoki cypress (*Chamaecyparis obtuse*) whereas the remaining 33 patients were sensitized not only JCP but also to other unrelated pollens such as orchard grass, short ragweed, mugwort, and Japanese alder as determined by CAP. None of the patients were sensitized to mites, molds, insects, animal

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danders, or foods. In addition, these patients underwent skin scratch test to JCP extract (Torii Co., Tokyo, Japan). A positive result was defined as a skin reaction with a erythema of greater than or equal to 10 mm in combination with a negative response to control fluid (Torii). Based on this evaluation, eight out of 45 patients showed negative results in skin scratch test to Japanese cedar pollen. None of the patients had received immunotherapy or used immunosuppressive drugs, including topical steroids, prior to the enrollment. The present study was approved by the Human Research Committee of Okayama University Medical School.

Cry j 1-specific IgE ELISA and inhibition

The Cry j 1-specific IgE was measured by captured ELISA according to the methods as previously reported [33]. In brief, ELISA plates (Corning Inc., Corning, NY, USA) were coated overnight at 4 °C with 100 μL of 5 μg/mL rat anti-human IgE mAb (clone LO-HE-17; Biosource, Camarillo, CA, USA) in 0.05 M carbonate buffer pH 9.6. Then plates were blocked for 2 h at 37 °C with 200 μL of PBS containing 10% FCS. After blocking, serum samples serially diluted with PBS containing 10% FCS were added to the plates, and the plates were incubated for 2 h at 37 °C. Thereafter, plates were incubated with 6 nM biotinylated Cry j 1 (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) for 2 h at 37 °C, followed by 1/1000 extravidin–peroxidase conjugate (Sigma Chemical Co., St Louis, MO, USA) for 1 h at 37 °C. After the final washing, tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) was added. Finally, 5% phosphoric acid (Wako Pure Chemical Industries Ltd, Osaka, Japan) was added to stop the reaction. The absorbance at 450 nm was measured using an automatic microplate reader (Bio-Rad, Hercules, CA, USA). Between each steps, plates were washed with PBS containing 0.05% Tween 20 four times, except the final wash, which was repeated six times.

Competitive inhibition ELISA for Cry j 1-specific IgE was performed as follows. ELISA plates were coated with rat anti-human IgE mAb as described above. After the blocking, serum samples were added in triplicate wells, then the plates were incubated for 2 h at 37 °C. In a separate plate, biotinylated Cry j 1 (final 6 nM) was mixed with serial concentrations (final 0, 30, 300 and 3000 nM) of M3FX, GN2M3FX or M9A as control. Then it was added to the IgE-bound plate for 2 h at 37 °C, followed by extravidin–peroxidase conjugate for 1 h at 37°C. Finally, plates were developed with TMB substrate and stopped with 5% phosphoric acid. Plates were read in a microplate reader at 450 nm. Results were presented as percentage binding calculated as follows: (OD 450 nm with M3FX or GN2M3FX/OD 450 nm with M9A) × 100.

Antigen-specific T cell lines

Short-term cultured Cry j 1-specific T cell lines (TCLs) were generated from peripheral blood mononuclear cells (PBMCs) of 7 patients with Japanese cedar pollinosis (four males and three females: age 21–43, mean 31.29 ± 8.48 years old) as described previously [26]. PPD-specific TCLs were also generated from these patients in the same manner.

Cellular responses to Cry j 1-related oligosaccharide

M3FX-driven proliferation and cytokine production of Cry j 1-specific TCL was tested *in vitro* as described previously [34]. Briefly, in flat-bottomed microtitre plates (Corning), 2×10^4 T cell blasts mixed with 1×10^5 autologous PBMCx in the presence or absence of either 0.28 μM of Cry j 1 or 10 μg/mL of Cry-consensus were cultured in 0.2 mL CM with serial concentrations (final 0, 0.5, 5.0 and 50 μM) of M3FX or M9A as negative control. In addition, these cells were cultured with 0.28 μM of Cry j 1 alone. Culture supernatants were collected after 65 h incubation, and stored at − 80 °C until the assay for cytokine production. For the last 7 h of the 72 h culture, 1 μCi ^{3}H-thytmidine (ICN Radiochemicals, Irvine, CA, USA) was added to each well. Uptake of ^{3}H-thytmidine by responding cells was counted by liquid scintillation spectrometry (Aloka Co., Tokyo, Japan). The data recorded was the mean count per minutes (c.p.m.) of triplicate cultures.

In separate experiments, 2×10^4 T cell blasts specific for PPD co-cultured with 1×10^5 autologous PBMCx were cultured in 0.2 mL CM with 2 μg/mL PPD in the presence or absence of serial concentrations (final 0, 0.5, 5.0 and 50 μM) of M3FX or M9A. Proliferative responses and cytokine production by PPD-specific TCLs were determined as described above.

Cytokine determination

Levels of IL-4 and IFN-γ in culture supernatants were measured by means of Opt EIA sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions [26]. The detection limit in these assays was 3 pg/mL for IL-4 and 20 pg/mL for IFN-γ.

Statistical analysis

Data were analysed using the Bartlett test followed by Wilcoxon's signed-rank test and Fisher's exact probability test. $P < 0.05$ was considered statistically significant. Values were given as mean ± standard deviation (SD).

Results

Inhibition of binding between IgE and Cry j 1 by pollen-related oligosaccharides

In captured ELISA, pooled serum from patients with pollinosis reacts with biotinylated Cry j 1 when the serum was diluted at 1 : 32, and the signal was lost when it was heated at 56°C for 1 h (data not shown). On the other hand, that from healthy subjects did not react with biotinylated Cry j 1, so that individual sera at 1 : 32 dilution were examined for further experiments. Cry j 1-specific IgE titre is significantly and positively correlated with JCP-specific IgE titre determined by CAP (Fig. 2a, $r = 0.615$, $P < 0.0001$), and 40 out of 45 samples showed a positive IgE signal against Cry j 1 twice

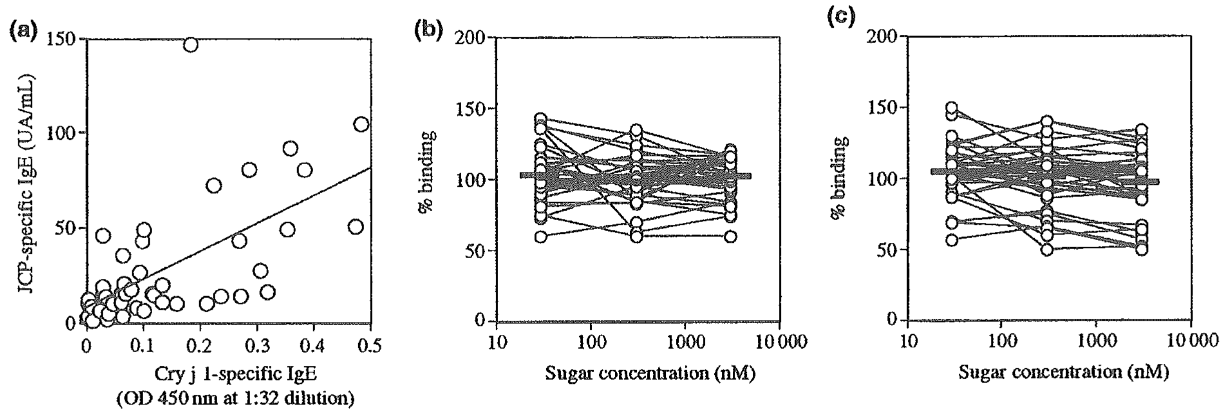

Fig. 2. (a) Correlation of IgE-binding to crude extract of Japanese cedar pollen (JCP) against IgE-binding to Cry j 1. Titers for JP-specific IgE were expressed as UA/mL determined by CAP [31]. Titres for Cry j 1-specific IgE were presented as the absorbance at 450 nm from triplicate wells of 1 : 32 serum dilution. (b, c) Competitive inhibition of the binding between Cry j 1 and IgE by monovalent oligosaccharides. Biotinylated Cry j 1 (6 nM) was mixed with M3FX (b), GN2M3FX (c), or M9A at 30, 300 and 3000 nM, then they were added to the IgE-bound plate. Results are presented as percentage binding. *P*-value was obtained through use of the Wilcoxon signed-rank test.

that from the background. Thus we examined inhibition ELISA with these 40 samples.

We used M3FX, GN2M3FX and M9A as an inhibitor at the concentration of 30, 300 and 3000 nM which yields the ratio between carbohydrates on biotinylated Cry j 1 and free carbohydrates of about 1 : 1, 1 : 10 and 1 : 100, respectively, since five possible *N*-linked glycosylation sites are found in the primary sequence of Cry j 1 molecule [23]. Overall, no significant inhibition was observed in the presence of M3FX and GN2M3FX, the Cry j 1-related oligosaccharides, as compared with the response in the presence of M9A, the Cry j 1-unrelated oligosaccharide (Figs 2b and c). The mean absorbances at 450 nm in the presence of 30, 300 and 3000 nM of M9A were 0.191 ± 0.290, 0.194 ± 0.286 and 0.189 ± 0.294, respectively. These results suggest that neither M3FX nor GN2M3FX were the major IgE epitopes in these patients with Japanese cedar pollinosis.

Tentatively defining a positive inhibition as that in which the binding between IgE and Cry j 1 was inhibited more than 20% in the presence of M3FX or GN2M3FX as compared with M9A, most sera (31/40 : 77.5%) showed no inhibition. However, 2 (5.0%), 4 (10.0%) and 3 (7.5%) samples showed a positive inhibition in the presence of M3FX alone, GN2M3FX alone, and both M3FX and GN2M3FX, respectively. These results suggest that pollen-related oligosaccharides may function as a minor IgE epitope in nine out of 40 subjects (22.5%).

Clinical characterization of patients sensitized to Cry j 1-related oligosaccharides

In 40 patients showing sensitization to Cry j 1, 12 patients were sensitized to Japanese cedar alone or both Japanese cedar and Hinoki cypress, whereas the remaining 28 patients were sensitized to other unrelated pollens such as orchard grass, short ragweed, mugwort, and Japanese alder. Eight out of nine patients (88.9%) who were sensitized to M3FX and/or GN2M3FX showed multiple sensitization to unrelated pollens whereas 20 out of 31 patients (64.5%) who were not sensitized to the oligosaccharides showed the multiple sensitization. However, there was no significant difference regarding multiple sensitization to unrelated pollens between patients with or without sensitization to Cry j 1-related oligosaccharides ($P = 0.1617$). In addition, seven out of 40 patients showed the negative results in skin scratch test to Japanese cedar pollen. There was also no significant difference regarding reactivity in skin scratch test against Japanese cedar pollen between patients with or without sensitization to Cry j 1-related oligosaccharides ($P = 0.8246$).

T cell responses against Cry j 1-related oligosaccharide

In order to determine whether Cry j 1-specific T cells recognize Cry j 1-related oligosaccharide as a T cell epitope, we established a panel of Cry j 1-specific T cell lines from seven patients with Japanese cedar pollinosis. All of the Cry j 1-specific T cell lines responded to native Cry j 1 at the concentration of 0.28 μM (mean: 32 592 ± 32 527 c.p.m., $P = 0.0180$), and produced a higher amount of IL-4 (mean: 563 ± 466 pg/mL, $P = 0.0180$) and a lesser amount of IFN-γ (mean: 93 ± 161 pg/mL, $P = 0.0431$). However, these T cells responded to neither M3FX nor M9A at the concentration of 0.5, 5.0 or 50 mM (Fig. 3a). Two out of seven patients were sensitized to GN2M3FX and/or M3FX, and we found no significant differences in the cellular responses to M3FX whether the patients were sensitized or not to the oligosaccharides. In addition, production of neither IL-4 or IFN-γ was induced in response to these oligosaccharides (Figs 3b and c). PPD-specific TCLs also did not respond either to M3FX or to M9A (data not shown).

Selective inhibition of Cry j 1-specific T cell responses by Cry j 1-related oligosaccharide

Finally, we decided to determine whether Cry j 1-related oligosaccharide affect specific T cell responses. Thus serial concentrations of M3FX or M9A were added in the interaction between specific T cells and Cry j 1. Presence of M9A did not affect the Cry j 1-specific cellular responses (Figs 4a–c).

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However, presence of M3FX significantly inhibited specific T cell responses in a dose-dependent manner (Fig. 4d). In addition, M3FX at 50 μM inhibited IL-4 production in Cry j 1-specific T cell (Fig. 4e). On the other hand, the presence of M3FX did not play any role in IFN-γ production (Fig. 4f). On the contrary, addition of either M3FX or M9A did not affect Cry-consensus-specific T cell proliferation or cytokine production (Fig. 5). Addition of M3FX had no effect on either PPD-specific T cell proliferation or cytokine production (data not shown).

Discussion

Several reports demonstrated that carbohydrates on allergens contribute to the binding capacity of IgE to the allergen [5–12, 14, 15]. For example, 14 out of 17 Cup a 1-positive sera abolished the IgE binding to the periodate-treated allergen [10]. On the contrary, other reports showed that the carbohydrates on allergens such as Pla l 1 from *Plantago lanceolata* pollen do not constitute a relevant allergenic epitope [4, 13, 35]. However, it remains unclear whether the carbohydrates expressed on allergen directly act as IgE epitopes or indirectly contribute the epitope activity of a peptide moiety by affecting the conformational structure. Allergens expressed in prokaryotic or eukaryotic vectors may be potential tools for investigating the role of carbohydrates on allergen in the immune responses. In addition, the effect of glycosylation of the individual glycosylation sites can be studied using engineered mutants expressed in prokaryotic and eukaryotic systems. However, these investigations may reflect indirect roles of carbohydrates, and cannot exclude the possibility that alteration of conformational structure of protein moieties such as refolding may affect the responses. For example, Akdis et al. [36] demonstrated that non-refolded recombinant allergen alters both IgE binding and proliferation/cytokine production by T cells. And Taniai et al. [25] reported that some conformational structure might be involved in the effective binding between IgE and Cry j 1. In this manuscript, we focused on the direct roles of carbohydrates on Cry j 1 in specific IgE and T cell responses using monovalent oligosaccharides. The result presented here that GN2M3FX and M3FX, the major and core oligosaccharide expressed on Cry j 1, respectively, did not display the significant inhibition of the binding between Cry j 1 and human IgE (Fig. 3) is consistent with the report by Ogawa et al. [24], and gives direct evidence that these two oligosaccharides are not the major IgE epitopes in patients

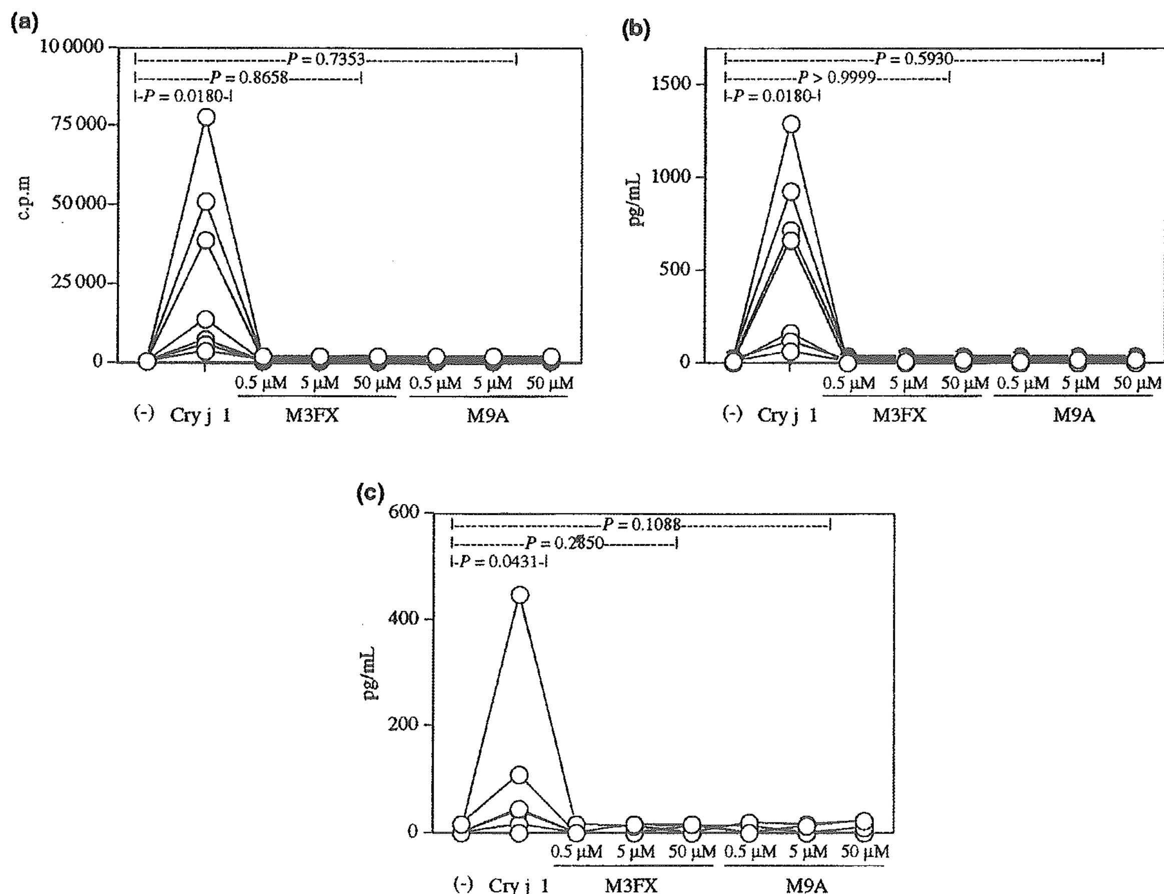

Fig. 3. Proliferation (a) IL-4 production (b) and IFN-γ production (c) of Cry j 1-specific TCLs against stimulation with monovalent oligosaccharides. Seven individual TCLs were cultured with M3FX or M9A at serial concentrations for 72 h. As a positive control, these cells were cultured with 0.28 μM of Cry j 1. *P*-value was obtained through use of Wilcoxon signed-rank test.

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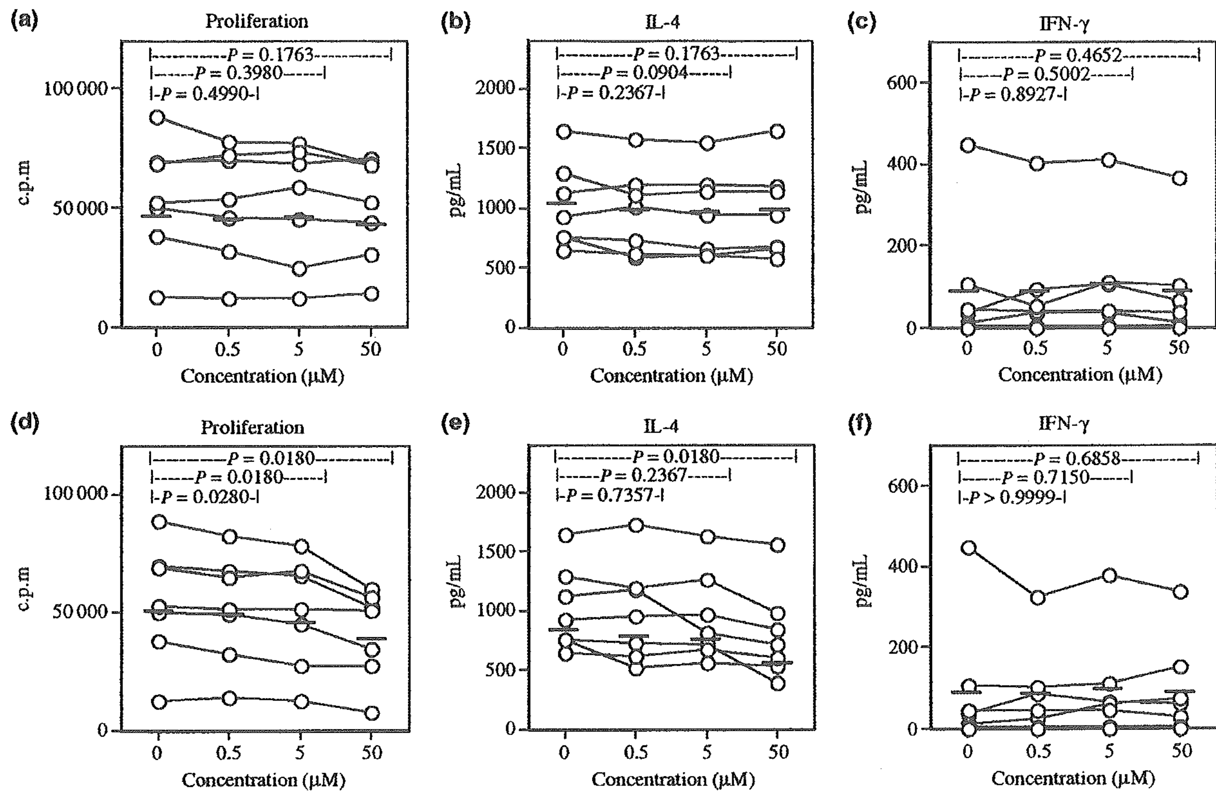

Fig. 4. Inhibition of Cry j 1-specific T cell responses by monovalent oligosaccharide. Seven individual TCLs were cultured with 0.28 μM of Cry j 1 in the presence of M9A (a–c) or M3FX (d–f) at serial concentrations for 72 h. Proliferation (a, d), IL-4 production (b, e) and IFN-γ production (c, f) of Cry j 1-specific T cell responses were determined as described in Methods. *P*-value was obtained through use of the Wilcoxon signed-rank test.

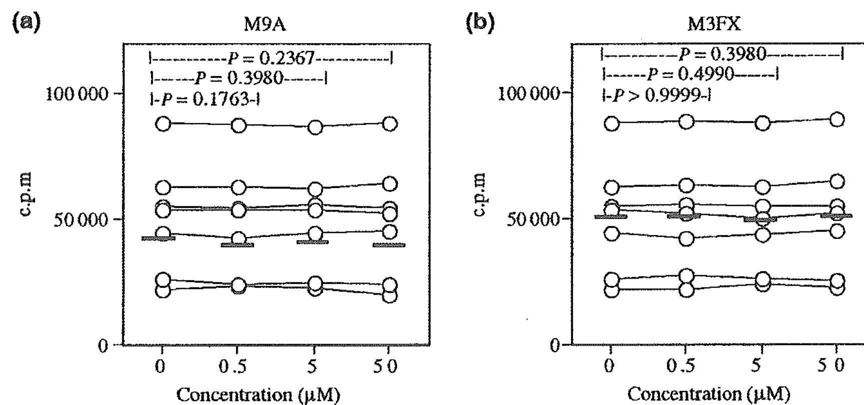

Fig. 5. Effect of momovalent oligosaccharide on peptide-driven T cell responses. Seven individual TCLs were cultured with 10 μg/mL of Cry-consensus in the presence of M9A (a) or M3FX (b) at serial concentrations for 72 h. Proliferation of peptide-specific T cell responses was determined as described in Methods. *P*-value was obtained through use of Wilcoxon signed-rank test.

with Japanese cedar pollinosis. In these experiments, we used M9A as a negative control of inhibition since high concentration of oligosaccharides may affect the inhibition via changing the culture condition such as osmolarity, and M9A is suitable as a negative control since Cry j 1 does not contain this oligosaccharide [24].

However, these oligosaccharides may function as minor IgE epitopes since a partial inhibition of the binding between IgE and Cry j 1 was observed in the presence of GN2M3FX and/or M3FX in 22.5% of the patients sensitized to Cry j 1 (Fig. 4). van Ree et al. [7] demonstrated that sera of 38.8% of patients allergic to Lol p 11 contained anti-carbohydrate IgE. And more recently, Calabozo et al. [34] reported that sugar residues of Pla l 1 participate in the binding of IgE epitopes that are minor allergic determinants, contributing in a low proportion to the total IgE-binding capacity of the allergen.

On the other hand, Batarero et al. [9] reported that 65% and 100% of patients sensitized to olive pollen and Ole e 1, respectively, recognized carbohydrate determinant on Ole e 1. In terms of the prevalence of anti-carbohydrate IgE, our results were similar to the first two reports in this paragraph. This may be due to the composition, valency and/or structural homology of carbohydrates among the allergens. Both Cry j 1 and Lol p 11 contain M3FX, which has fucose α1-3 linked to the innermost GlucNAc, but Ole e 1 is not known to contain such oligosaccharide or fucose [13, 24].

Carbohydrates on allergens are known to act as CCD, which lead to the broad cross-reactivity of IgE in food pollen and invertebrate animal allergens [11, 12, 14, 15]. Iacovacci et al. [37] established mAb 5E6, which recognized M3FX on Cup a 1 from *Cupressus arizonica* pollen. This mAb also reacted with not only pollens from Cupressaceae family, but also taxonomically unrelated pollens such as olive and grass pollen. We divided 40 patients sensitized to Cry j 1 into two groups. The first group enrolled patients who were sensitized to *C. japonica* alone or to both *C. japonica* and *Chamaecyparis obtusa*. *C. japonica* and *C. obtusa* are taxonomically related tree pollens, and we have previously reported that 76.4% of patients with spring pollinosis were sensitized to both pollens [38]. The second group enrolled patients who were sensitized not only to *C. japonica*, but also to other unrelated pollens such as orchard grass, short ragweed, mugwort, and Japanese alder. There was no significant difference regarding multiple sensitization to unrelated pollens between patients with or without sensitization to pollen-related carbohydrates. This result suggests that M3FX and/or GN2M3FX is not acting significantly as CCD in Japanese cedar pollinosis.

The role of carbohydrates on allergens in the biological implications of IgE-mediated reactions remains controversial [9, 10, 13–15]. Natural and recombinant PLA2 show similar histamine-releasing capability [13]. And recently, Mari et al. [15] reported that positive ELISA results of antigens eliciting negative skin test responses could be ascribed to IgE for carbohydrate epitopes. On the other hand, blood cells from patients with olive pollinosis gave specific histamine release after stimulation of isolated carbohydrate from Ole e 1. And there was no observed histamine release by basophils from Cupressaceae-allergic subjects whose reactivity was essentially a reactivity against carbohydrate moiety after stimulation with recombinant Cup a 1 [10]. Further, more recently, Foetisch et al. [39] reported that some patients with tomato allergy have biologically relevant CCD-specific IgE. In order to determine whether the recognition of oligosaccharides on Cry j 1 are involved in the biologic activity, we divided the patients according to their reactivity in skin scratch test to *C. japonica* pollen extract. No significant correlation was observed between the reactivity in skin scratch test against Japanese cedar pollen and sensitization to M3FX and/or GN2M3FX, suggesting that these oligosaccharides are not important in these biological activities. Further analysis such as histamine release assay using these oligosaccharides and selection of subjects with positive IgE in CAP and negative symptoms against *C. japonica* may be required for precise investigation.

In the present study, we stimulated Cry j 1-specific T cells with M3FX, the core oligosaccharide on Cry j 1 containing α1-3 fucose and β1-2 xylose (Fig. 3). We have previously shown that 18 out of 19 T cell clones specific for Cry j 1 showed significant proliferative responses as indicated by values of stimulation index greater than 2, suggesting that carbohydrates on Cry j 1 are not the major T cell epitopes [26]. The result presented here that Cry j 1-specific T cell lines did not display proliferation or cytokine production in response to M3FX is consistent with our former report as well as the report by Dudler et al. [16] that carbohydrate-dependent T cell clones acting against PLA2 did not respond to asparagine-linked oligosaccharide alone. Although the carbohydrate structures of Cry j 1 and PLA2 are not similar, e.g. with respect to the presence or absence of β1-2 xylose, these results suggest that the oligosaccharides on allergens appear not to be the major T cell epitopes [23, 24, 40]. In fact, we have previously found six major T cell eitopes (p16–30, p81–95, p106–120, p111–125, p211–225, and p301–315) in Cry j 1, and no glycosylation sites exist adjacent to these T cell epitopes [27].

On the other hand, addition of M3FX inhibited the Cry j 1-specific T cell responses of proliferation and IL-4 production (Fig. 5). Our study has for the first time demonstrated that monovalent oligosaccharide can regulate allergen-specific T cell responses. It is known that monovalent oligosaccharides can inhibit cellular responses against glycoproteins. For example, Velupillai and Harn [41] reported that, in spleen cells from mice infected with *Schistosoma mansoni*, proliferation in response to human serum albumin conjugated with lacto-*N*-fucopentaose III (LNFIII) and lacto-*N*-neotetraose (LNnT) could be inhibited by the addition of monovalent LNFIII and LNnT, respectively, in a sugar-specific manner. In addition, M3FX did not inhibit proliferation and cytokine production in PPD-specific TCLs. PPD may contain several immunogenic glycoproteins such as Apa [42]. Although the composition and structure of carbohydrates of Cry j 1 and PPD may differ, these results suggest that M3FX selectively regulates cellular responses against glycoproteins containing this oligosaccharide [23, 24, 43]. Result that addition of M3FX did not affect Cry-consensus-specific T cell responses (Fig. 5) may support this since Cry-consensus is a prokaryotically expressed polypeptide and did not contain carbohydrates. This in turn suggests the explanation that carbohydrate-specific ligand on antigen presenting cells and/or T cells can affect Cry j 1-specific T cell responses [44]. However, we have previously reported that a blockade of mannose receptor (MR), one of the most characteristic carbohydrate-specific receptors, induced few changes in Cry j 1-specific T cell responses, suggesting that the involvement of MR was negligible [26]. Identification of the receptor specific for M3FX must be made in further investigation. It then may be found that this orphan receptor is not simply involved in antigen uptake but also controls Th differentiation, since the addition of M3FX affects the IL-4 but not IFN-γ production by Cry j 1-stimulated TCLs.

In summary, we have provided *in vitro* evidence that pollen-related ologosaccharides from Cry j 1 are not the major IgE or T cell epitopes. However, monovalent core oligosaccharide containing α1-3 fucose and β1-2 xylose selectively inhibits Cry j 1-specifc T cell responses. These observations may provide a basis for future therapeutic approaches in the management of pollinosis by using monovalent oligosaccharides since these oligosaccharides do not induce IgE reaction in the majority of patients.

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Roles of FcγRIIB in Nasal Eosinophilia and IgE Production in Murine Allergic Rhinitis

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The low-affinity IgG Fc receptor, FcγRIIB, displays inhibitory potential in experimental models such as autoimmune diseases. However, whether this receptor is involved in the onset of allergic diseases remains unknown. This study examines the role of FcγRIIB in the initiation of allergic rhinitis in mice. Repeated intranasal sensitization with *Schistosoma mansoni* egg antigen (SEA) induced SEA-specific IgE and marked nasal eosinophilia in high-responder BALB/c mice. FcγRIIB gene-deficient (−/−) BALB/c mice displayed severe eosinophilia compared with that of wild-type counterparts. However, FcγRIIB −/− mice conversely produced less SEA-specific IgE. The production of interleukin (IL)-4 but not of IL-5 or IFN-γ by nasal mononuclear cells was also decreased in FcγRIIB −/− mice, suggesting that the exacerbation of nasal eosinophila in FcγRIIB −/− mice is independent of the local IL-5 levels. The findings in low responder C57BL/6 mice were similar. In addition, nasal eosinophilia in FcγRIIB −/− mice passively sensitized with SEA was exacerbated, and conversely, specific IgE production was inhibited after a nasal challenge. These results suggest that FcγRIIB plays a regulatory role in the initiation of allergic rhinitis that is independent of either mouse strain or type of sensitization.

Keywords: Fc receptor; rhinitis; mouse; IgE; eosinophil

Allergic rhinitis is the most prevalent allergic condition initiated by immediate hypersensitivity. Over 10% of the population in developed countries suffers from allergic rhinitis, which creates societal burdens such as medical expenses and loss of productivity (1, 2). Antigen-specific IgE production and nasal eosinophilia are characteristic hallmarks of this condition (1, 3). In addition, atopic humans and mice often produce antigen-specific IgGs, especially IgG4 and IgG1, respectively (4–8). Atopic humans usually express higher IgG4 levels than healthy individuals (4, 5), and antigen-specific IgG1 is often produced before specific IgE in mice (7, 8). Thus, the role of antigen-specific IgG and the interaction between IgG and Fc receptors in the pathogenesis of allergic rhinitis should be understood.

Among Fc receptors in mice, FcγRI, FcγRIII, and FcεRI share a common γ chain that contains an immunoreceptor tyrosine-based activation motif in the intracytoplasmic domain (9, 10). These receptors appear to elicit cellular responses such as phagocytosis, antibody-dependent cell-mediated cytotoxicity, anaphylaxis, neutrophil chemotaxis, or autoimmune diseases via binding antibody–antigen complexes (11–15). On the other hand, FcγRIIB is the major Fc receptor expressed on B cells, mast cells, macrophages, neutrophils, and eosinophils in humans and mice (9, 16, 17). This receptor consists solely of an α chain containing an immunoreceptor tyrosine-based inhibition motif in the intracytoplasmic domain, and it suppresses cell activation triggered by cross-linking B-cell receptors or Fc receptors (18–20). In several models of autoimmune diseases, mice deficient in the FcγRIIB gene (FcγRIIB −/−) develop enhanced immune-complex–mediated alveolitis, collagen-induced arthritis, systemic lupus erythematosus, and Goodpasture's syndrome (21–24). In addition, FcγRIIB −/− mice produce more IgM, IgA, and IgG in response to both thymus-dependent and -independent antigens, and both IgG- and IgE-mediated anaphylactic responses are enhanced (25, 14). However, little is known about whether FcγRIIB is involved in the initiation of atopic diseases such as allergic rhinitis (26). Moreover, the role of FcγRIIB in antigen-specific IgE production has not been demonstrated.

We recently developed a strain-dependent murine model of allergic rhinitis based on repeated intranasal sensitization with *Schistosoma mansoni* egg antigen (SEA) in the absence of adjuvants (6). This study examines the involvement of FcγRIIB in the initiation of allergic rhinitis in both high- and low-responder mouse strains. To our knowledge, we are the first to describe the role of FcγRIIB in the initiation of allergic diseases through not only systemic but also local sensitization by antigen without adjuvants in models that mimic natural exposure. In addition, we either passively or intraperitoneally sensitized the mice with SEA to determine whether the findings from FcγRIIB −/− mice after intranasal sensitization with SEA depended on a particular route and/or type of sensitization.

METHODS

Animals and Antigens

All experiments were performed using young adult (6–10 weeks old) female BALB/c and C57BL/6 (FcγRIIB +/+) mice purchased from Charles River Japan (Yokohama, Japan). Homozygous FcγRIIB gene-deficient (FcγRIIB −/−) BALB/c and C57BL/6 founder mice were gifts from Dr. Jeffery V. Ravetch (Rockefeller University, New York, NY) (24). FcγRIIB −/− mice were bred and maintained under specific pathogen-free conditions at Okayama University Medical School according to the guidelines established by the Okayama University Medical Area Research Committee. All experimental protocols and procedures in this study were approved by institutional animal care and use committee. SEA was prepared as described (6). Concanavalin (Con) A was purchased from Sigma (St. Louis, MO).

Sensitization of Mice

Mice were either actively or passively sensitized with SEA. We designed both local and systemic sensitization (active sensitization) before nasal challenge with SEA. In local sensitization, mice were intranasally sensitized with SEA in the absence of an adjuvant as described (6, 27). In systemic sensitization, the mice were intraperitoneally sensitized with 5 μg of SEA adsorbed to 1 mg of alum (Kyowa Kagaku, Kagawa, Japan)

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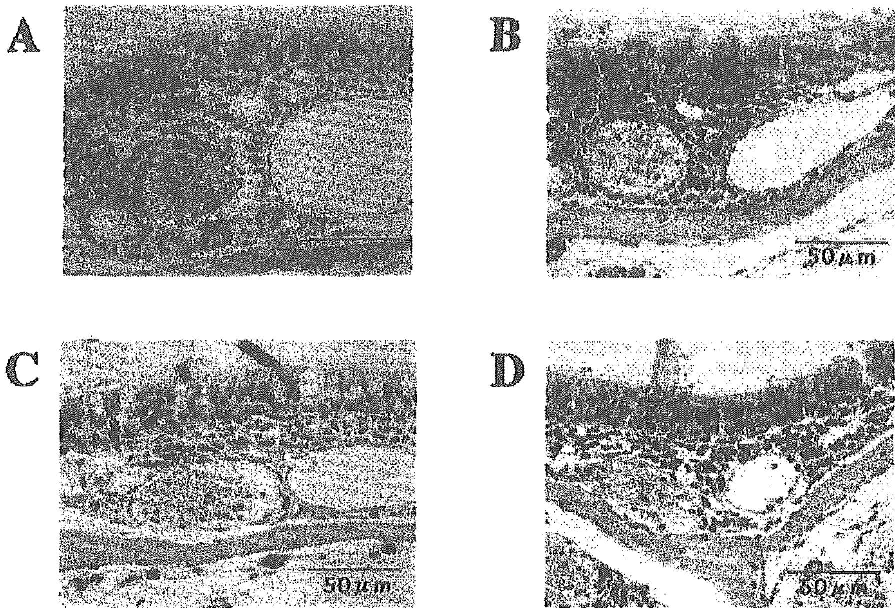

Figure 1. Nasal eosinophilia after nasal challenge in BALB/c (*A* and *B*) and C57BL/6 (*C* and *D*) mice. Wild-type BALB/c (*A*) and C57BL/6 (*C*) mice and counterpart FcγRIIB −/− mice (*B* and *D*, respectively) were intranasally sensitized with *Schistosoma mansoni* egg antigen (SEA). At 12 hours after the final nasal challenge with SEA, mice were killed. Nasal sections were fixed, decalcified, and eosinophils in nasal mucosa were detected by Luna stain. Luna stain is specific for eosinophils and renders their cytoplasm red–brown on a blue background. (C) Peeled mucin and erythrocyte cluster were stained as bright red.

in a total volume of 200 μl. Two weeks later, the mice were boosted in the same manner. Alternatively, mice were passively sensitized by an intravenous injection of 200 μl of pooled sera from mice presensitized by a repeated intranasal application of SEA or from nonsensitized mice. One hour after passive sensitization, the mice were challenged intranasally with 20 μl of 1-μg SEA for 7 consecutive days.

SEA-specific Antibody Determination

Peripheral blood collected from the tail vein 12 hours after the final challenge was centrifuged at 200 × *g*, and the levels of SEA-specific antibody, including IgE, IgG1, and IgG2a, were measured by ELISA as described (6, 27). Titers are expressed as endpoint titers where the endpoint equals the final serum dilution yielding an absorbance equal to twice that of the background.

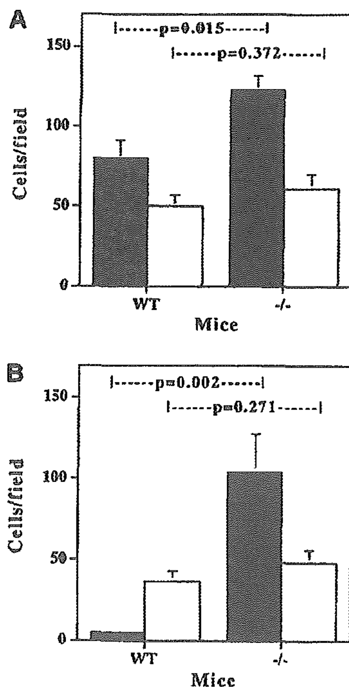

Figure 2. Quantitation of cellular infiltration into nasal mucosa after nasal challenge in BALB/c (*A*) and C57BL/6 (*B*) mice. Wild-type (WT) and FcγRIIB −/− mice were intranasally sensitized and challenged with SEA, as described in Figure 1. The number of eosinophils (*closed bar*) and mononuclear cells (*open bar*) in the posterior portion of nasal septum was determined microscopically in high-power fields (10 × 40). Results show mean numbers of infiltrating cells per field ± SEM of six nasal sections from each group. Data are representative of two separate experiments.

Histologic Examination

Twelve hours after the final nasal challenge, mice were killed with carbon dioxide. The heads were removed, fixed, and decalcified (6, 27). Coronal nasal sections were visualized by either hematoxylin/eosin or Luna stain. *Luna stain is specific for eosinophils and renders their* cytoplasm red–brown on a blue background (28). The number of infiltrating cells such as eosinophils and mononuclear cells in the posterior portion of nasal septum was determined microscopically in a blinded manner and was expressed as numbers per high-power field (10 × 40).

Detection of *In Vivo* Apoptosis in Nasal Mucosa

Detection of *in vivo* apoptosis in nasal mucosa was performed using the TdT-mediated dUTP-biotin nick labeling (TUNEL) method. In brief, the head of each mouse was cut, and skin, muscles, and brain tissues were removed. Each head was fixed in 10% phosphate-buffered formaldehyde, embedded in paraffin, and cut into 6-μm thick sections in the coronal plane. Each selected section was deparaffinized and rehydrated. After incubation with 20 μg/ml of proteinase K (Boheringer Mannheim, Mannheim, Germany), endogenous peroxidase was blocked by using 2% H_2O_2 in methanol for 30 minutes. TdT enzyme (Takara In Situ Apoptosis Detection Kit; Takara Bio Inc., Shiga, Japan) was dropped on the sections and incubated at 37°C for 60 minutes. Then antifluorescein isothiocyanate horseradish peroxidase conjugate (Takara In Situ Apoptosis Detection Kit) was dropped on the sections and incubated at 37°C for 30 minutes. The sections were stained with diaminobenzidene tetrahydrochloride (Sigma) for 10–15 minutes.

In Vitro Culture of Nasal Mononuclear Cells and Cytokine Determination

Mice were killed 12 hours after the final nasal challenge, and nasal mononuclear cells were isolated by enzymatic extraction using collagenase as described (6, 27). Cells were cultured in flat-bottomed 48-well plates (Corning, Corning, NY) with SEA (1 μg/ml), Con A (2 μg/ml) as a positive control, or supplemented medium as a negative control. After an incubation at 37°C for 72 hours in 5% CO_2, supernatants were collected and stored at −80°C. Levels of interleukin (IL)-4, IL-5, and IFN-γ production induced by stimulated (SEA and Con A), and unstimulated nasal mononuclear cells were measured by capture ELISA as described (6, 27). The detection limits for IL-4, IL-5, and IFN-γ in this system were 0.1 IU/ml, 10 pg/ml, and 0.1 IU/ml, respectively.

Statistical Analysis

Data are expressed as the mean ± SEM for each subject group. Statistical analysis was performed using Student's unpaired *t* test. Differences in antibody endpoint titers were determined using the Mann-Whitney *U* prime test.

RESULTS

Role of FcγRIIB in the Induction of Allergic Th2 Responses After Intranasal Sensitization with SEA in High-responder BALB/c Mice

After a nasal challenge with SEA, the nasal mucosa from wild-type BALB/c mice contained diffuse inflammatory infiltrate primarily comprised of eosinophils (6). Figures 1A and 1B show severe eosinophilia in the nasal mucosa in FcγRIIB −/− BALB/c mice as compared with the wild type. The numbers of eosinophils infiltrating the nasal septum per field (10 × 40) were 80.0 ± 11.3 and 122.7 ± 9.2 in FcγRIIB +/+ and −/− BALB/c, respectively (mean ± SEM, n = 6, p = 0.015). On the other hand, the magnitude of infiltration of mononuclear cells into nasal mucosa was similar between FcγRIIB −/− mice and wild-type counterparts after the intranasal sensitization with SEA (50.3 ± 6.7 vs. 61.2 ± 9.2, p = 0.372) (Figure 2). In addition, neutrophils and apoptotic cells were rarely seen in both mice (Figure 3).

Serum SEA–specific IgE, IgG1, and IgG2a levels were determined in FcγRIIB +/+ and FcγRIIB −/− BALB/c mice after nasal challenge with SEA (Figure 4). Wild-type BALB/c mice predominantly expressed SEA-specific IgE and IgG1 after repeated intranasal sensitization with SEA (6). FcγRIIB −/− BALB/c mice produced significantly less SEA-specific IgE (p = 0.008) but more IgG1 (p = 0.115) than the wild type. All groups produced similar amounts of IgG2a (p = 0.515; Figure 4).

We examined cytokine production from nasal mononuclear cells after challenge. Nasal mononuclear cells isolated by enzyme extraction were stimulated with SEA or Con A for 72 hours, and then we assessed IL-4, IL-5, and IFN-γ production in the culture supernatants. SEA-stimulated nasal mononuclear cells from wild-type BALB/c mice produced detectable levels of both IL-4 and IL-5, whereas the cells with medium alone did not produce these cytokines. IL-4 production in those from FcγRIIB −/− BALB/c mice was significantly decreased, whereas the levels of IL-5 were similar between the groups. In addition, IFN-γ was not detectable in either FcγRIIB +/+ or −/− BALB/c mice in response to SEA. The findings were similar when the T cells were stimulated with Con A (Figures 5A–5C).

The Role of FcγRIIB in the Induction of Allergic Th2 Responses after Intranasal Sensitization with SEA in Low-responder C57BL/6 Mice

We examined whether the effect of FcγRIIB on the initiation of allergic rhinitis is dependent on strain using FcγRIIB −/− C57BL/6 and the wild type, which has low responses to intranasal sensitization with SEA (6). Wild-type C57BL/6 mice under specific pathogen-free conditions produced detectable amounts of SEA-specific IgE and IgG1, whereas FcγRIIB −/− C57BL/6 mice produced significantly more IgG1 (p = 0.019) and conversely less IgE (p = 0.019; Figure 6). In addition, eosinophil infiltration of the nasal mucosa was more apparent in FcγRIIB −/− C57BL/6 mice than in the wild type after nasal challenge with SEA (Figures 1C and 1D). The numbers of eosinophils infiltrating the nasal septum per field (10 × 40) were 5.0 ± 1.6 and 103.8 ± 23.5 in FcγRIIB +/+ and −/− C57BL/6, respectively (mean ± SEM, n = 6, p = 0.002) (Figure 2).

Detectable amounts of IL-4 and IL-5 were seen in nasal mononuclear cells from wild-type C57BL/6 mice intranasally sensitized with SEA in response to *in vitro* Con A stimulation (Figure 7h medium alone did not produce these cytokines. In nasal mononuclear cells from FcγRIIB −/− C57BL/6 mice, IL-4 but not IL-5 production was significantly decreased as compared with that of the wild type after Con A stimulation (Figure 7).

Selective Suppression of IgE Production after Intraperitoneal Sensitization with SEA

We examined whether the role of FcγRIIB in specific antibody synthesis is restricted in intranasal sensitization. We intraperitoneally sensitized FcγRIIB −/− and wild-type BALB/c mice with SEA adsorbed to alum. Like the results obtained after intranasal sensitization, SEA-specific IgE production was significantly decreased in FcγRIIB −/− mice after an intraperitoneal boost with SEA compared with the wild type (p = 0.005; Figure 8A). Conversely, FcγRIIB −/− mice produced more SEA-specific IgG1, although the difference between the groups was not significant (p = 0.117; Figure 8B). Neither mouse produced detectable amounts of IgG2a under these conditions (Figure 8C).

Role of FcγRIIB in the Exacerbation of Allergic Th2 Responses in Mice Passively Sensitized with SEA

We investigated the role of FcγRIIB in the exacerbation of allergic Th2 responses in passively sensitized mice. As described in Methods, FcγRIIB −/− and wild-type BALB/c mice passively sensitized with SEA by an intravenous injection of presensitized or nonsensitized sera were challenged intranasally with SEA daily for 7 consecutive days. Figure 9A shows that specific IgE synthesis was significantly decreased in FcγRIIB −/− mice compared with the wild type (p = 0.019). Conversely, significantly more specific IgG1 was produced in FcγRIIB −/− mice (p = 0.031; Figure 9B). On the other hand, IgG2a was undetectable in either FcγRIIB −/− or in wild-type mice (Figure 9C). In addition, the degree of nasal eosinophilia was significantly higher in FcγRIIB −/− mice than in the wild type, although the degree of eosinophilia was

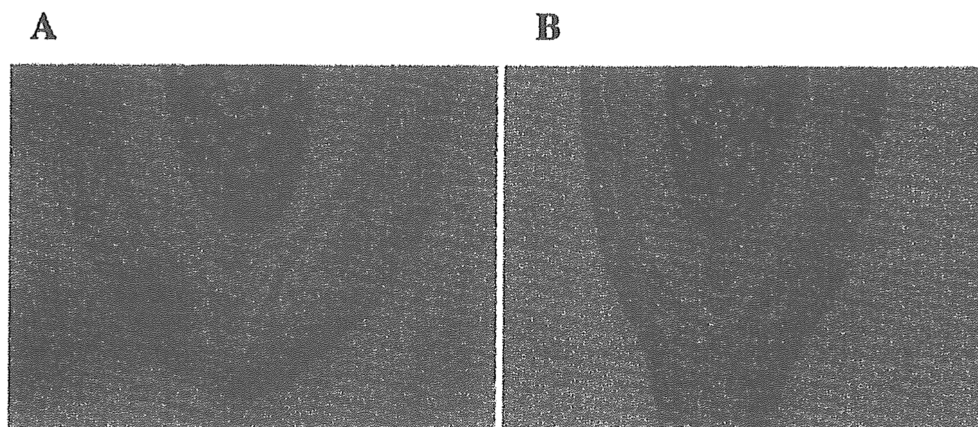

Figure 3. Detection of *in vivo* apoptosis in nasal mucosa. WT (*A*) and FcγRIIB −/− (*B*) BALB/c mice were intranasally sensitized with SEA. At 12 hours after the final nasal challenge, mice were killed. Detection of *in vivo* apoptosis in nasal mucosa was performed using TdT-mediated dUPT-biotin nick labeling (TUNEL) method.

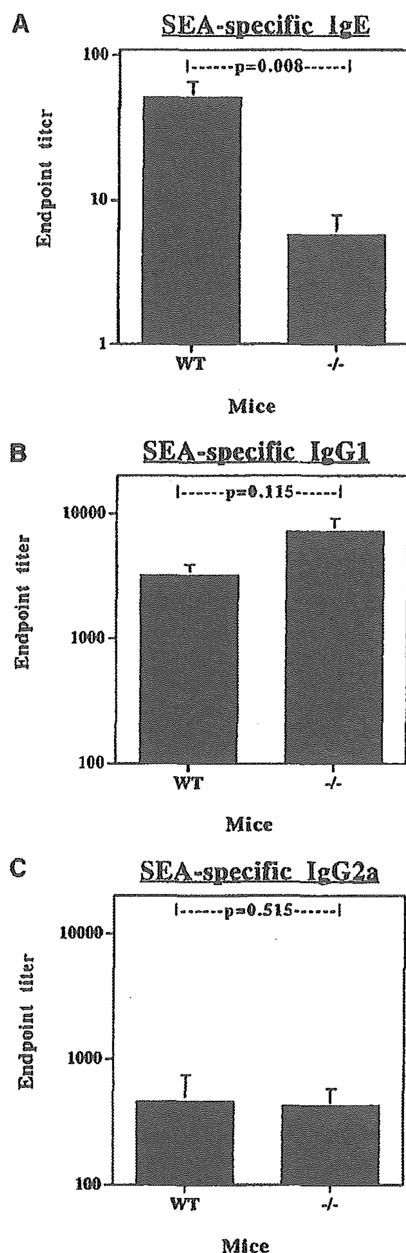

Figure 4. Antibody production in high-responder BALB/c mice after intranasal sensitization with SEA. After final nasal challenge, blood was sampled from WT and FcγRIIB −/− (−/−) mice, and levels of serum SEA–specific IgE (*A*), IgG1 (*B*), and IgG2a (*C*) were determined by ELISA. Results show mean endpoint titer ± SEM of six serum samples from each group. Data are representative of three separate experiments.

lower than that observed in mice actively sensitized with SEA (Figure 1). The numbers of eosinophils infiltrating into nasal septum per field (10 × 40) were 11.8 ± 2.8 and 25.2 ± 3.6 in FcγRIIB +/+ and −/− BALB/c mice, respectively (mean ± SEM, n = 6, p = 0.016). Furthermore, either SEA-specific IgE, IgG1, or IgG2a was not detected in serum from mice passively sensitized with sera from SEA nonsensitized mice and subsequently challenged intranasally with SEA.

DISCUSSION

FcγRIIB plays an inhibitory role in both IgE- and IgG-mediated anaphylactic responses during the effector phase of allergy (14). This study examined the role of FcγRIIB during the induction phase of allergy using a murine model of allergic rhinitis (6, 27). To date, the involvement of FcγRIIB in the production of IgE has not been investigated. In terms of pre–B-cell receptor–positive pre-B cells, recent study demonstrated that FcγRIIB negatively regulates pre–B-cell receptor–mediated signaling for apoptosis (29). We found that SEA-specific IgE production in FcγRIIB −/− mice was significantly decreased after intranasal sensitization with SEA as compared with that of wild-type BALB/c mice (Figure 4). This result suggests that signals through FcγRIIB play an important role in increasing antigen-specific IgE synthesis in this model. The decreased production of IL-4 by nasal mononuclear cells from FcγRIIB −/− mice in response to SEA restimulation (Figure 5) supports this result as IL-4 plays a critical role in *in vivo* IgE synthesis (30). Although IL-4 displays the inhibitory effect on the expression and function of FcγRII (31), the mechanisms governing why a deficiency of FcγRIIB leads to the inhibition of IL-4 production remain to be elucidated.

After intranasal sensitization with SEA, FcγRIIB −/− BALB/c mice developed a severe eosinophilia in the nasal mucosa as compared with wild-type BALB/c (Figure 1). Several investigators have demonstrated that FcγRIIB plays an inhibitory role in the induction of tissue inflammation (14, 21–23). For example, FcγRIIB −/− mice with immune complex–mediated alveolitis developed enhanced alveolar hemorrhage, increased interstitial neutrophil infiltration, and perivascular edema as compared with wild-type mice in (21). FcγRIIB −/− mice immunized with collagen type IV develop massive pulmonary hemorrhage with neutrophil and macrophage infiltration and crescent glomerulonephritis (23). In addition, hemorrhage in ileum villi is increased in FcγRIIB −/− mice during IgE-mediated systemic anaphylaxis (15). Our results are consistent with these findings and suggest that FcγRIIB inhibits eosinophilic inflammation.

Although it is unknown whether the functions of human eosinophils are mediated by FcγRIIB, FcγRIIA/C, or both, human and mouse eosinophils express FcγRII, and murine FcγRIIB is involved in the survival, degranulation, or apoptosis of eosinophils (17, 32, 33). In addition, ligation of FcγRIIB on not only developing but also mature eosinophils isolated from hepatic granulomas from *S. mansoni*–infected mice induces their destruction through Fas-mediated apoptosis (17). More recently, Kim and colleagues demonstrated that FcγRII ligation pivotally regulates both the survival and death of eosinophils (33). This study found that nasal mononuclear cells from both FcγRIIB +/+ and −/− mice produced similar amounts of IL-5 after intranasal sensitization with SEA (Figure 5). One explanation of why nasal eosinophilia was exacerbated in FcγRIIB −/− mice irrespective of local IL-5 production is a lack of direct ligation of *S. mansoni*–specific IgG on FcγRIIB on eosinophils. In addition, TUNEL-positive cells in the nasal mucosa were rarely seen in both wild type and FcγRIIB −/− mice after the intranasal challenge with SEA (Figure 3), suggesting that *in vivo* apoptosis of eosinophils triggered by FcγRIIB less contributes to the suppression of nasal eosinophilia in this model.

The production of both antigen-specific IgE and IgG1 is positively regulated by Th2 responses in mice, and antigen-specific IgE and nasal eosinophilia are two of the major indicators of the initiation of allergic rhinitis. However, recent investigations have demonstrated that the production of antigen-specific IgE and of specific IgG1 and/or local eosinophilia is not definitely correlated (30, 34). IL-4 appears not to be essential for IgG1 class switching or nasal eosinophilia but plays a crucial role in

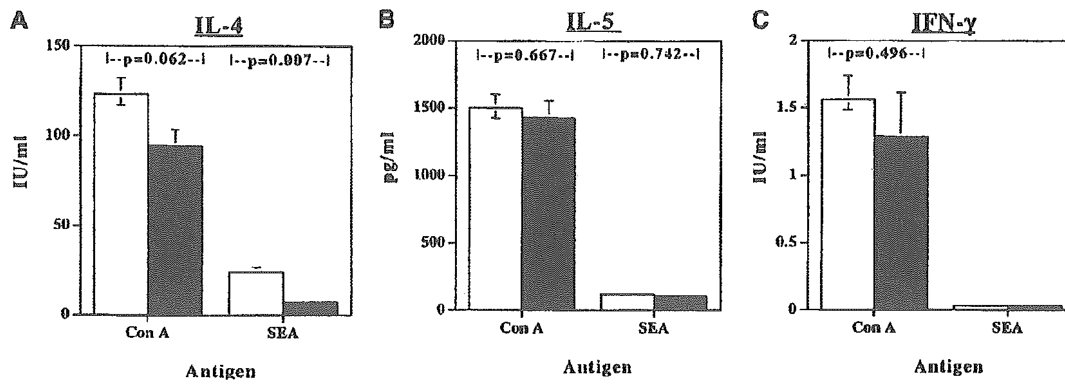

Figure 5. Production of interleukin (IL)-4 (*A*), IL-5 (*B*), and IFN-γ (*C*) by nasal mononuclear cells from BALB/c wild type (*open bars*) and FcγRIIB −/− (*closed bars*) mice. Mice were intranasally sensitized and subsequently challenged with SEA. At 12 hours after final nasal challenge with SEA, nasal mononuclear cells were isolated and cultured *in vitro* for 72 hours with Concanavalin (Con) A, SEA, or medium alone. Cytokines were measured by ELISA. Results show means ± SEM of three individual pools. Data are representative of two separate experiments.

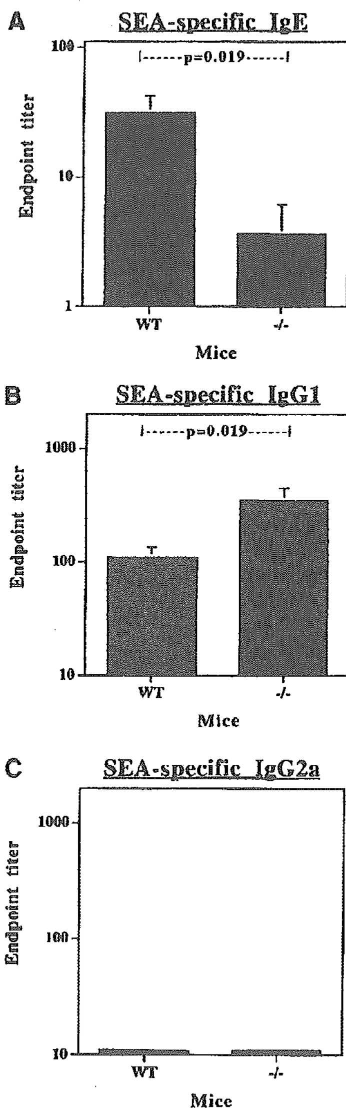

Figure 6. Antibody production in low-responder C57BL/6 mice after intranasal sensitization with SEA. WT and FcγRIIB −/− C57BL/6 mice were intranasally sensitized with SEA under a specific pathogen-free (SPF) condition. After final nasal challenge, blood was sampled, and levels of serum SEA-specific IgE (*A*), IgG1 (*B*), and IgG2a (*C*) were determined by ELISA. Results show mean endpoint titer ± SEM of six serum samples from each group. Data are representative of two separate experiments.

IgE production (30). For example, we recently reported that titers of antigen-specific IgE in IL-4 gene-deficient (IL-4 −/−) BALB/c mice sensitized with SEA are negative, whereas those of specific IgG1 are significant lower but not abolished compared with wild-type control mice (30). We also identified sex-related differences in the production of IgE but not of IgG1 in CBA/J mice after intranasal sensitization (34).

In addition, antibody production and the development of nasal eosinophilia were seen in C57BL/6 mice after intranasal sensitization with SEA under specific pathogen-free conditions. SEA-specific IgG1 levels were elevated. Nasal eosinophilia was exacerbated, and conversely, levels of specific IgE were decreased in FcγRIIB −/− C57BL/6 mice compared with wild-type C57BL/6 mice (Figures 1 and 6). The genetic background of these mice has potential for modulating the initiation of Th2 responses (6, 35). In fact, SEA-specific IL-4 production by nasal mononuclear cells was not detected in either wild-type or FcγRIIB −/− C57BL/6 mice despite that both of the mice could produce the detectable amounts of SEA-specific IgE after the intranasal sensitization. As described by Bix and colleagues, IL-4 production was less in C57BL/6 mice as compared with BALB/c mice (36). Thus, this discrepancy may arise from a possibility that SEA-specific IL-4 production by C57BL/6 mice was too small to detect above the detection limit (0.1 IU/ml). This difference in IL-4 production may lead to the ability in SEA-specific IgE production between the two strains (Figures 4 and 6). These results suggest that the regulatory effect of FcγRIIB on the initiation of allergic rhinitis after intranasal sensitization with SEA is not restricted to specific mouse strains as it appears in both high- and low-responder strains.

In contrast, specific IgG1 production was significantly increased in FcγRIIB −/− C57BL/6 mice. IgM and IgG production was augmented in FcγRIIB −/− mice after immunization with both thymus-dependent and thymus-independent antigens (25). In a murine model of collagen-induced arthritis, more IgG1, IgG2a, IgG2b, and IgM antibodies specific for bovine type II collagen were produced in FcγRIIB −/− mice after immunization with bovine type II collagen (22). In addition, FcγRIIB −/− mice produced more IgG1, IgG2a, IgG2b, IgG3, and IgM but not IgA specific for bovine type IV collagen after immunization with bovine type IV collagen (23). Our results appear to be consistent with the findings of the reports described previously here and suggest that FcγRIIB plays an inhibitory role in the production of antigen-specific IgG1 (the dominant IgG isotype

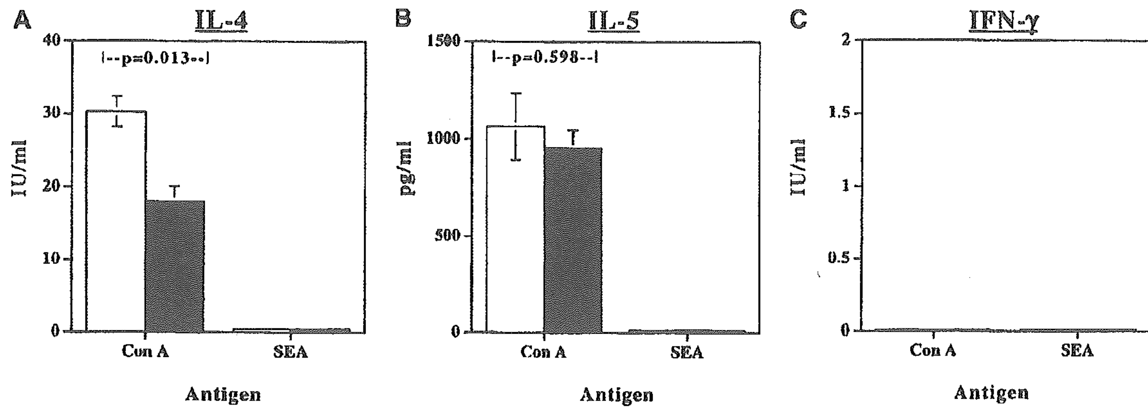

Figure 7. Production of IL-4 (*A*), IL-5 (*B*), and IFN-γ (*C*) by nasal mononuclear cells from C57BL/6 wild-type (*open bar*) and FcγRIIB −/− (*closed bar*) mice under SPF conditions. Mice were intranasally sensitized and subsequently challenged with SEA. Twelve hours after final nasal challenge with SEA, nasal mononuclear cells were isolated and cultured *in vitro* for 72 hours with Con A, SEA, or medium alone. Cytokines were measured by ELISA. Results show mean ± SEM of three individual pools. Data are representative of two separate experiments.

in this model) after intranasal sensitization with SEA (6). On the other hand, similar amounts of SEA-specific IgG2a were seen in FcγRIIB −/− and wild-type mice, suggesting that FcγRIIB does not play an inhibitory role in the production of minor IgG isotypes in this model.

The route of sensitization and/or the use of adjuvants can influence the initiation of Th2 responses (35, 37). We therefore sensitized FcγRIIB +/+ and −/− mice intraperitoneally with SEA adsorbed to alum, an adjuvant that is frequently used to induce experimental allergy in mice (38). Compared with wild-type BALB/c mice, specific IgE production in FcγRIIB −/− mice was significantly decreased (Figure 8A). These results suggest that the role of FcγRIIB in the regulation of specific IgE production was the same regardless of the route of SEA sensitization.

Furthermore, when FcγRIIB −/− mice were passively sensitized with SEA by intravenous injection of presensitized sera, nasal eosinophilia increased, and conversely, IgE production decreased after a nasal challenge with SEA (Figure 9). Passive sensitization together with local antigen challenge results in allergic inflammation characterized by local eosinophilia (39). These results suggested that the role of FcγRIIB in local eosinophilia and IgE synthesis is the same between active and passive sensitization with SEA.

In conclusion, we found that nasal eosinophila is exacerbated, whereas the production of specific IgE is decreased in FcγRIIB −/− mice after intranasal sensitization with SEA. Moreover, the involvement of FcγRIIB in the initiation of allergic rhinitis seems not to be restricted to a specific mouse strain. In addition, the route and type of sensitization did not alter the effect of FcγRIIB. These results suggest that the regulation of expression and/or function of FcγRIIB will be a useful tool with which to control allergic inflammation characterized by nasal eosinophilia.

Conflict of Interest Statement: T.W. has no declared conflict of interest; M.O. has no declared conflict of interest; H.H. has no declared conflict of interest; T.Y. has no declared conflict of interest; N.O. has no declared conflict of interest; N.O. has no declared conflict of interest; Y.S. has no declared conflict of interest; Y.O. has no declared conflict of interest; T.T. has no declared conflict of interest; K.N. has no declared conflict of interest.

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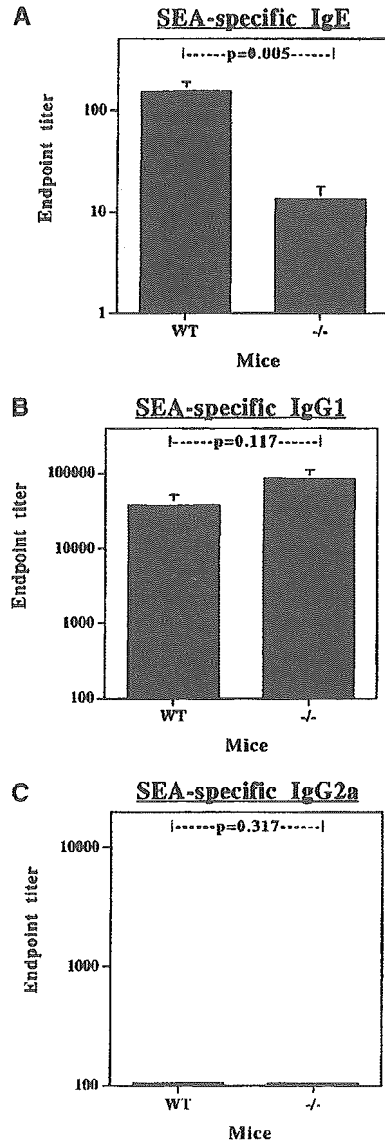

Figure 8. Antibody production after intraperitoneal sensitization with SEA. WT and FcγRIIB −/− BALB/c mice were primed and intraperitoneally boosted with SEA adsorbed to alum. At 1 week after boosting, blood was sampled, and levels of serum SEA–specific IgE (*A*), IgG1 (*B*), and IgG2a (*C*) were determined by ELISA. Results show mean endpoint titer ± SEM of six serum samples from each group. Data are representative of two separate experiments.

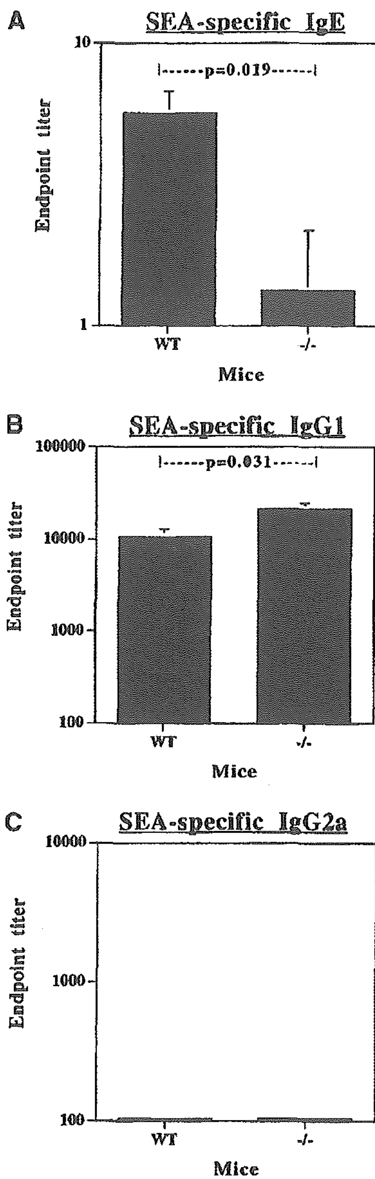

Figure 9. Antibody production after passive sensitization with SEA. WT and FcγRIIB −/− BALB/c mice were passively sensitized with SEA by intravenous injection of pooled sera from presensitized or nonsensitized mice. One hour after passive sensitization, the mice were challenged intranasally with 20 μl of 1 μg SEA for 7 consecutive days. Blood was sampled, and levels of serum SEA–specific IgE (*A*), IgG1 (*B*), and IgG2a (*C*) were determined by ELISA. Results show mean endpoint titer ± SEM of six serum samples from each group. Data are representative of two separate experiments.

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