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Strain-dependent induction of allergic rhinitis without adjuvant in mice

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Background: To date, no murine models have been reported to show the induction of both antigen-specific IgE and nasal eosinophilia, two of the major hallmarks of allergic rhinitis, after local sensitization in the absence of adjuvants, a phenomenon which reflects natural exposure. In this report, we attempted to establish a murine model representing an initiation of allergic rhinitis.

Methods: BALB/c, CBA/J, and C57BL/6 mice were sensitized intranasally to *Schistosoma mansoni* egg antigen (SEA) solely. After repeated sensitization, serum Ab titers, nasal eosinophilia, and cytokine production by nasal lymphocytes were determined.

Results: BALB/c mice produced SEA-specific IgE after repeated sensitization. High-dose sensitization to SEA induced IgE production in CBA/J mice, while C57BL/6 mice did not show the production throughout the period observed, suggesting that IgE production was regulated genetically. BALB/c mice also exhibited nasal eosinophilia after the nasal challenge. In addition, nasal lymphocytes sensitized with SEA intranasally produced significant amount of IL-5 *in vitro*.

Conclusions: These results suggest that intranasal sensitization with SEA in the absence of adjuvants induces a Th2 immune reaction, reflecting the hallmarks of the initiation of allergic rhinitis both *in vivo* and *in vitro*, which is genetically regulated.

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Production of antigen-specific IgE and nasal eosinophilia are two of the hallmarks of allergic rhinitis. In man, approximately 20–40% of the population react to airborne allergens by producing specific IgE and/or showing nasal eosinophilia (1–3). Age, sex, social class, daily habits, genetic background including specified gene expression, and/or antigenicity

seem to affect the induction of allergy (4–6). However, the precise mechanism by which the environmental “innocuous” airborne antigens induce the production of allergen-specific IgE and concomitant eosinophil accumulation at inflammatory sites in certain populations remains unclear.

In murine experimental models to investigate the regulation of IgE synthesis *in vivo*, administration of antigens via the natural route is desirable because administration of antigen via other routes results in different degrees of IgE production (7–9). Therefore, many trials of intranasal or aerosolized sensitization were performed to investigate the regulation of IgE production in murine airway allergy (7–15). However, in order to generate measurable production of IgE, antigens were usually inoculated with adjuvants (9, 11, 12). It is well known that adjuvants have immunoregulatory effects that may distort the physical antigenicity (16–22). Thus, sensitization of antigens without adjuvants is preferable to reflect the natural exposure. However, attempts with intranasal or aerosolized sensitization resulted in either transient induction of antigen-specific IgE or none at all (7, 8, 14). Moreover, no reports show eosinophil accumulation, demonstrating the induction of antigen-specific IgE in the absence of adjuvants (10, 13, 15).

In this study, we used *Schistosoma mansoni* egg antigen (SEA) as a sensitizing antigen. SEA is known as a strong Th2 inducer, and this antigen induces upregulation of serum IgE and eosinophilia in blood and tissues in both man and experimental animals (23, 24). In addition, airway hyper-reactivity was induced by intratracheal challenge with SEA in presensitized mice (25). To investigate genetic regulation of the production of antigen-specific Abs including IgE and nasal eosinophilia, three different strains of mice were sensitized intranasally with serial doses of SEA in the absence of adjuvants. We found that the production of SEA-specific IgE is under genetic control and correlates with nasal eosinophilia. This model could be useful to investigate the pathophysiology of the initiation of allergic rhinitis.

Material and methods

Animals

Young adult (7–10 weeks old) female mice of the three strains BALB/c (H-2d), CBA/J (H-2k), and C57BL/6 (H-2b) were purchased from Harlan (Indianapolis, IN, USA).

Sensitization of mice

Mice ($n=5$) were given 20 μ l of 0.3, 3.0, or 30 μ g SEA or saline intranasally by microsyringe (Hamilton Co., NV, USA). Two weeks later, this treatment was boosted in this fashion. From day 21, mice were inoculated with the same amount of antigen on 7 consecutive days. This will be referred to as the nasal challenge. Sera were taken from the tail 6 days after the boosting sensitization and at the end of nasal challenge.

Serum total IgE

Serum total IgE was measured by sandwich ELISA. ELISA plates (Corning, Inc., NY, USA) were coated overnight at 4°C with 100 μ l of 5 μ g/ml rat anti-mouse IgE mAb (Biosource, CA, USA) in 0.05 M carbonate buffer, pH 9.6. Then plates were blocked for 2 h at 37°C with 200 μ l of PBS containing 10% FCS and 0.3% Tween 20. After blocking, serum samples or standard mouse IgE mAb (Pharmingen, CA, USA), which was diluted serially with PBS containing 10% FCS, was added to the plates, and the plates were incubated for 2 h at 37°C. Thereafter, plates were incubated with 0.5 μ g/ml biotinylated rat anti-mouse IgE mAb (Pharmingen) for 2 h at 37°C, followed by 1/1000 extravidin-peroxidase conjugate (Sigma Chemical Co., MO, USA) for 1 h at 37°C. After the final washing, tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Inc., MD, USA) was added. Finally, 5% phosphoric acid (Fisher Scientific, PA, USA) was added to stop the reaction. The absorbance at 450 nm was measured with an automatic microplate reader (Molecular Devices, CA, USA). The amount of total IgE in ng/ml was analyzed with the Softmax software program. Between each step, the plates were washed with PBS containing 0.05% Tween 20 four times except that the final wash was repeated eight times.

Antigen-specific IgE

The SEA-specific IgE was also measured by sandwich ELISA using biotinylated SEA as a second reagent. ELISA plates were coated and blocked in the same way as the measurement of serum total IgE. After the incubation of samples for 2 h at 37°C, biotinylated SEA (1 μ g/ml) was added to plates for 2 h at 37°C, followed by extravidin-peroxidase conjugate as described above. Finally, the plates were developed with substrate and stopped with 5% phosphoric acid. Plates were read in a microplate reader at 450 nm. For biotinylation, SEA (1.6 mg/ml) in sodium bicarbonate buffer, pH 8.5, was

incubated with biotin (long-arm) *N*-hydroxy succinimide ester (Vector Laboratory, CA, USA) for 2 h at room temperature. The reaction was stopped by adding 5 μ l of ethanolamine, and dialyzed overnight with PBS containing 0.05% sodium azide.

SEA-specific IgG, IgM, IgA, and IgG isotypes

Antigen-specific Abs except IgE were detected by indirect ELISA. ELISA plates were coated with SEA (2 μ g/ml) overnight at 4°C, and blocked as described above. Then plates were incubated with samples in serial dilutions for 2 h at 37°C, followed by goat anti-mouse Ig mAb-peroxidase conjugate (Boehringer-Mannheim, IL, USA) for 1 h at 37°C. Thereafter, plates were developed with substrate, and a stop solution was added. Finally, the OD value was determined at 450 nm with a microplate reader. Optimal concentrations of peroxidase-conjugated anti-mouse IgM and IgG mAb, and anti-mouse IgA and IgG isotypes were 1/10 000 and 1/2000, respectively.

In vitro culture of nasal lymphocytes

After the final nasal challenge, nasal lymphocytes were isolated by enzyme extraction with collagenase, as described by Asanuma et al. (26). Cell suspensions were pooled from two mice and contained 2 million lymphocytes per ml. Cells were cultured with or without SEA (5 μ g/ml) for 72 h at 37°C in RPMI 1640 medium (Gibco) containing 10% FCS, 5×10^{-5} M 2-mercaptoethanol (Sigma), and 100 units/ml and 100 μ g/ml penicillin/streptomycin (Sigma) in flat-bottomed 48-well

plates (Corning). Cell supernatants were collected and stored at -80°C until assayed.

IL-5 Determination

Levels of IL-5 in culture supernatants were measured by capture ELISA. Maxisorp 96-well plates (Nunc Laboratories, Denmark) were coated with 50 μ l of rat anti-mouse IL-5 mAb (Pharmingen) at 2.0 μ g/ml, in 0.05 M carbonate buffer, pH 9.6, overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 and then blocked with PBS-10% FCS for 2 h at 37°C. Culture supernatant or recombinant IL-5 standards (0–20 000 pg/ml) were then plated in duplicate and incubated overnight at 4°C. The wells were then washed and plated with biotinylated rat anti-mouse IL-5 mAb (Pharmingen, 1 μ g/ml), and incubated for 1 h at 37°C. Plates were washed, and then streptavidin-alkaline phosphatase (Pharmingen) diluted 1:2000, was added to each well for 45 min at 37°C. Finally, the plates were washed, and *p*-nitrophenol phosphate substrate (Sigma) was added; absorbance was read at 405 nm.

Histologic examination

Three hours after the final nasal challenge, the mice were killed with carbon dioxide. Then the heads were removed, treated with a decalcifying solution (VWR Scientific Products, NJ, USA) for 7 days, and fixed with 10% formalin. Then, coronal nasal sections were stained with hematoxylin and eosin, and the number of eosinophils was counted microscopically in a high-power field (40 $\times$ 10).

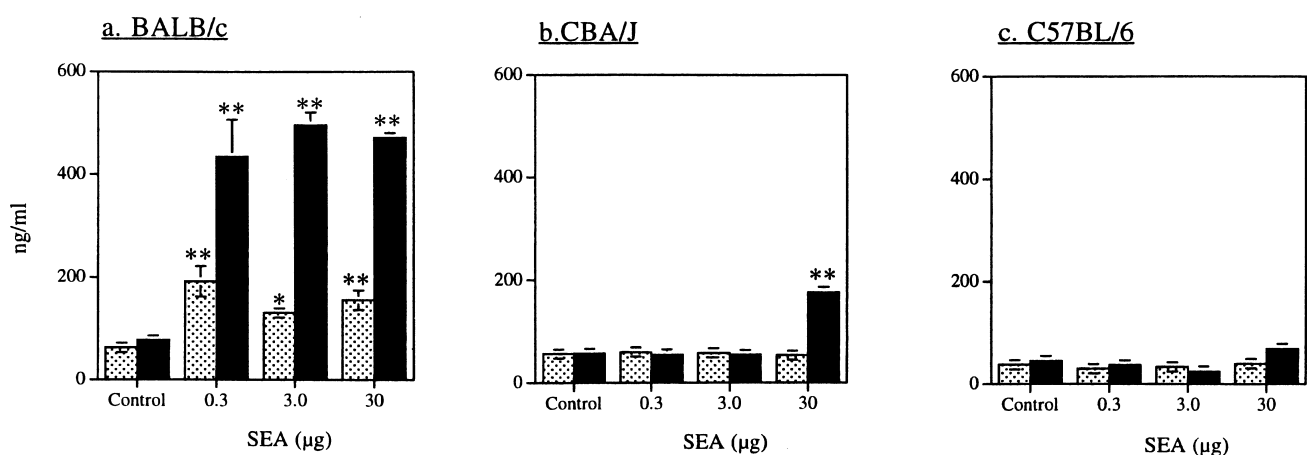


Figure 1. Levels of serum total IgE after intranasal sensitization with SEA in three different strains of mice. BALB/c (a), CBA/J (b), and C57BL/6 (c) mice ($n=5$) were intranasally sensitized with 0.3, 3.0, and 30 μ g SEA or saline, and sera were taken 6 days after boosting sensitization (dotted bar) and at end of nasal challenge (closed bar). Pooled sera were assayed for serum total IgE in ELISA assays. Results show mean $\pm$ SEM from triplicate wells. Data represent two separate experiments. * $P<0.05$, ** $P<0.01$ compared with control group.

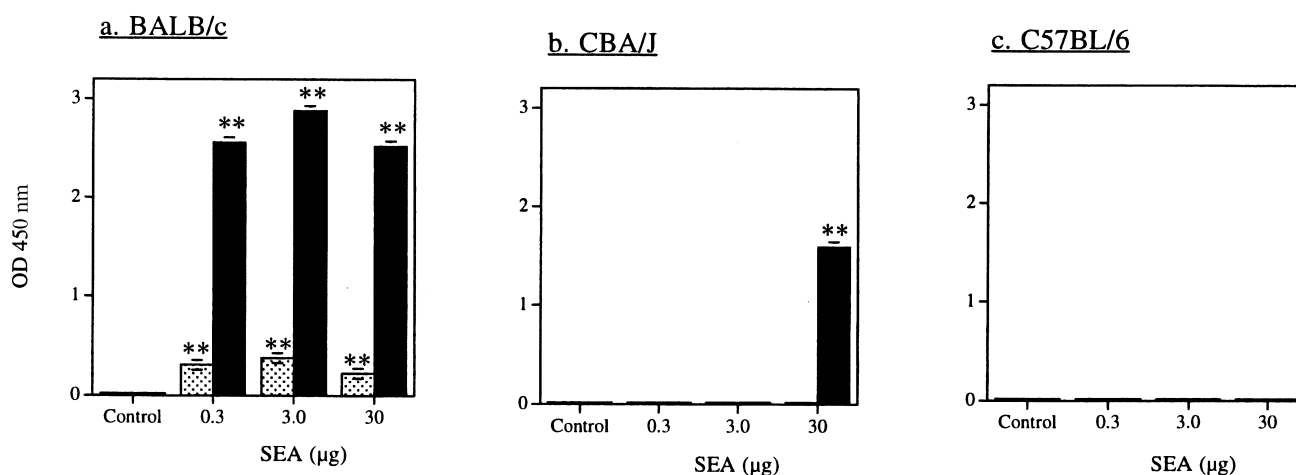


Figure 2. Production of SEA-specific IgE after intranasal sensitization with SEA. Pooled sera from BALB/c (a), CBA/J (b), and C57BL/6 (c) mice sensitized intranasally with SEA were collected as described in Fig. 1. SEA-specific IgE were assayed in sandwich ELISA using biotinylated SEA as detection reagent. Results show mean OD at 450 nm $\pm$ SEM from triplicate wells of 1:4 serum dilution. Data represent two separate experiments. * $P < 0.05$, ** $P < 0.01$ compared with control group.

Statistical analysis

Data were analyzed with two-way ANOVA by Tukey's multiple comparison. $P < 0.05$ was considered statistically significant. Values were given as mean $\pm$ standard error of the mean (SEM).

Results

Elevation of serum total IgE after repeated intranasal sensitization with SEA

Among the three different strains of mice sensitized, only BALB/c mice showed elevation of serum total IgE after the boosting sensitization (Fig. 1a). This elevation was seen in all the doses tested (0.3, 3.0, and 30 μ g). Two-way ANOVA revealed the interaction on the levels of serum total IgE between the number of the intranasal sensitizations and the doses of SEA, suggesting that increase in serum total IgE was synergistically enhanced after the nasal challenge compared with after the boosting sensitization ($P < 0.01$). CBA/J mice showed a modest but significant elevation of serum total IgE after the nasal challenge with 30 μ g of SEA (Fig. 1b). On the other hand, an increase of serum total IgE was never found in C57BL/6 mice in the study (Fig. 1c).

Induction of SEA-specific IgE after intranasal sensitization with SEA

Next, we investigated the detection of SEA-specific IgE. In indirect ELISA (the same method for detection of SEA-

specific IgG; see Material and methods), signals for IgE were not detected (data not shown). However, the method using biotinylated antigen as a detection reagent in sandwich ELISA could detect signals (Fig. 2). This signal diminished when we used sera heated at 56°C for 2 h (99.5%, data not shown), suggesting that this signal indeed reflects the reagenic IgE activity. As with the increase of serum total IgE, BALB/c mice produced SEA-specific IgE after the boosting sensitization (Fig. 2a). SEA-specific IgE was also enhanced after the nasal challenge according to two-way ANOVA. Interestingly, the amount of SEA-specific IgE was not statistically different in terms of doses intranasally inoculated in BALB/c mice. CBA/J mice also showed the production of SEA-specific IgE after the nasal challenge with 30 μ g of SEA (Fig. 2b). However, SEA-specific IgE was never detected in C57BL/6 mice during the period observed (Fig. 2c).

Strain-dependent induction of SEA-specific IgG production

BALB/c mice produced SEA-specific IgG after the boosting sensitization with 30 μ g of SEA (Fig. 3a). In this strain of mice, inoculation with 3 μ g of SEA also induced significant IgG production after the nasal challenge. On the other hand, CBA/J mice produced SEA-specific IgG only after the nasal challenges with 30 μ g of SEA (Fig. 3b). C57BL/6 mice did not show the induction of SEA-specific IgG throughout the study (Fig. 3c). None of the strains of mice produced specific IgG after the priming of SEA (data not shown).

In BALB/c mice, IgG1 was the predominant IgG isotype specific for SEA, followed by IgG2b and IgG2a. IgG3 was not

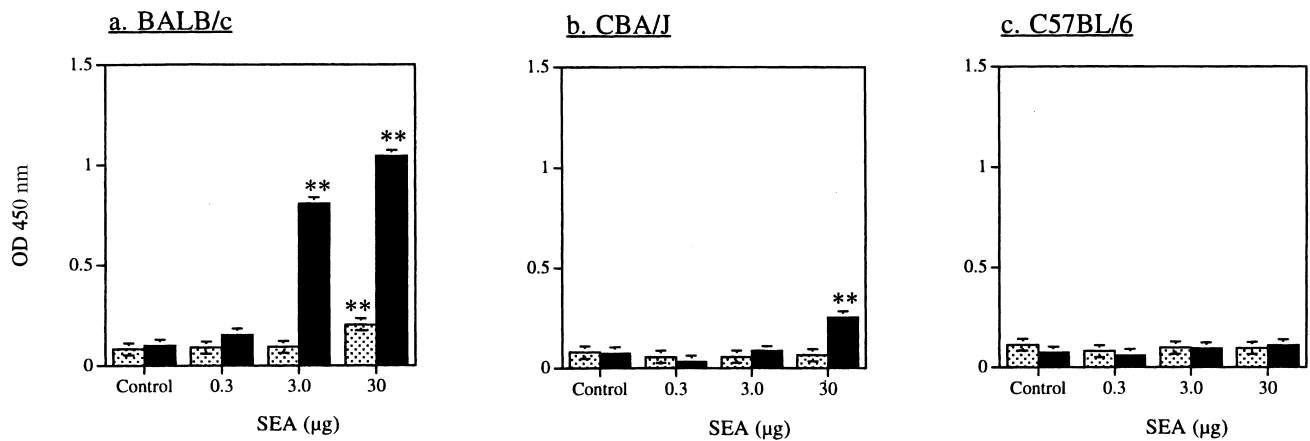


Figure 3. Production of SEA-specific IgG after intranasal sensitization with SEA. Pooled sera from BALB/c (a), CBA/J (b), and C57BL/6 (c) mice sensitized intranasally with SEA, as described in Fig. 1, were assayed for SEA-specific IgG in indirect ELISA. Results show mean OD at 450 nm $\pm$ SEM from triplicate wells of 1:100 serum dilution. Data represent two separate experiments. * $P < 0.05$, ** $P < 0.01$ compared with control group.

detected after the nasal challenge with SEA (Fig. 4). The predominance of this isotype did not change due to the dose of SEA inoculated. We confirmed that peroxidase-labeled anti-IgG isotype Abs do not cross-react with other IgG isotypes. In addition, the absorbance values for the binding of Abs over a given concentration range of matched purified IgG isotypes (Pharminogen) were remarkably similar.

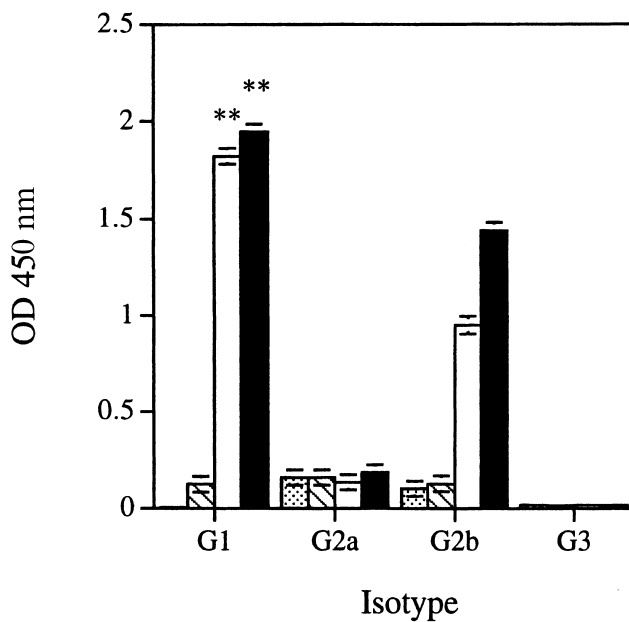


Figure 4. IgG isotypes specific for SEA after intranasal sensitization with SEA in BALB/c mice. Mice ($n=5$) were sensitized with 0.3 µg (hatched bar), 3.0 µg (open bar), 30 µg (closed bar) SEA, or saline (dotted bar), as described in Fig. 1. After nasal challenge with SEA/saline, sera were taken and pooled. IgG1, IgG2a, IgG2b, and IgG3 specific for SEA were assayed in indirect ELISA. Results show mean OD at 450 nm $\pm$ SEM from triplicate wells of 1:100 serum dilution. Data represent two separate experiments. * $P < 0.05$, ** $P < 0.01$ compared with other isotypes.

Nasal eosinophilia in SEA-sensitized mice

In mice inoculated intranasally with saline, eosinophils were rarely seen in the nasal mucosa (Fig. 5a). However, BALB/c mice showed the apparent accumulation of eosinophils in nasal mucosa after the nasal challenge with SEA (Fig. 5b). Eosinophilia was mainly detected in the meatus nasi medius and maxillary sinus, and also in the nasal turbinate and nasal septum. Eosinophils accumulated not only in the lamina propria, but also in the epithelial layer. Numbers of accumulated eosinophils in nasal septum did not differ among the groups sensitized with different doses of SEA in BALB/c mice (Fig. 5c). CBA/J mice also showed nasal eosinophilia after the nasal challenge with 30 µg, but not 0.3 or 3.0 µg of SEA (Fig. 5d). However, nasal eosinophilia was not seen in C57BL/6 mice (Fig. 5e).

IL-5 production by nasal lymphocytes

Next, we investigated IL-5 production by nasal lymphocytes after the nasal challenge with 3 µg of SEA for 7 consecutive days. Nasal lymphocytes cultured with SEA for 72 h produced a significant amount of IL-5 (Fig. 6). On the other hand, nasal lymphocytes from control BALB/c mice sensitized with saline did not produce IL-5 either with or without *in vitro* restimulation of SEA.

Discussion

In this study, we demonstrated that IgE production by intranasal sensitization with SEA was genetically influenced. BALB/c (H-2d), CBA/J (H-2k), and C57BL/6 (H-2b)

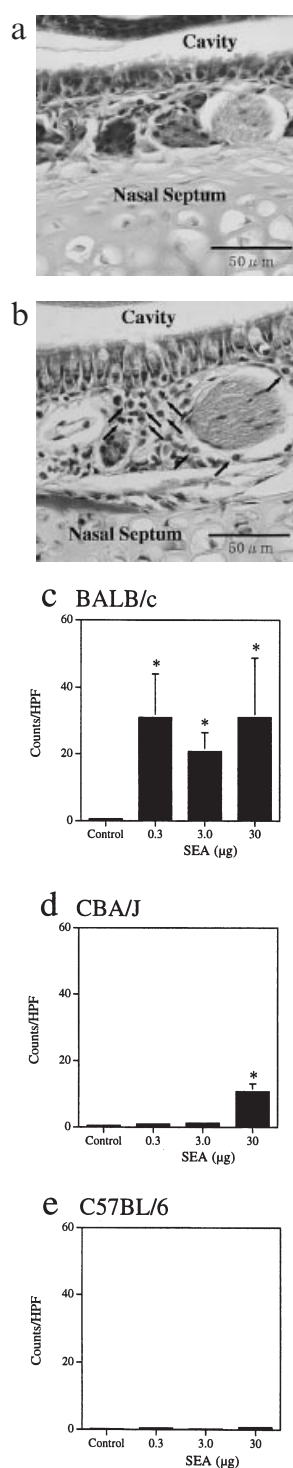


Figure 5. Nasal eosinophilia of mice intranasally sensitized with SEA. BALB/c mice were sensitized with SEA or saline as described in Fig. 1. After nasal challenge, mice were killed and their heads were removed. Nasal sections were decalcified, fixed, and stained with hematoxylin and eosin. Typical nasal sections from mice intranasally sensitized with saline (a) or SEA (b) are shown. Arrows indicate eosinophils. Summary of nasal eosinophilia after intranasal sensitization with 0.3, 3.0, 30 μg SEA or saline in BALB/c (c), CBA/J (d), and C57BL/6 (e) mice. Results show mean numbers of eosinophils per high-power field (10×40) $\pm$ SEM in nasal septum from four nasal sections in each group. * $P < 0.05$ compared with control group.

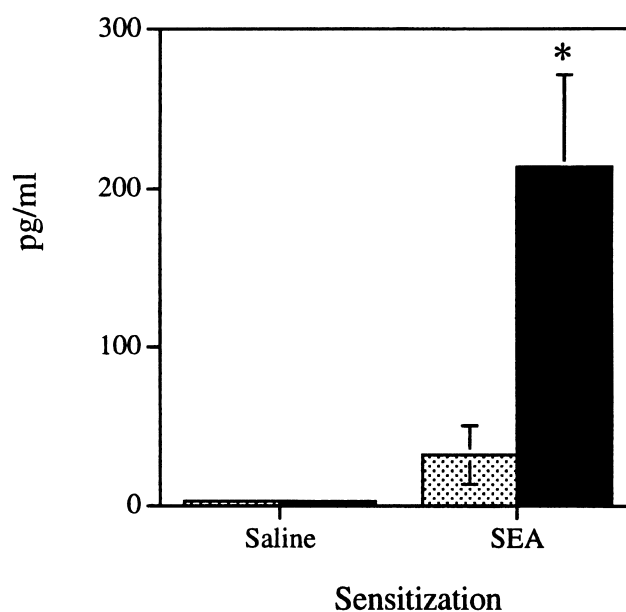


Figure 6. Production of IL-5 by nasal lymphocytes from BALB/c mice sensitized intranasally with 3.0 μg SEA or saline. Nasal lymphocytes were isolated and cultured *in vitro* for 72 h with (closed bar) or without (dotted bar) 5 μg/ml SEA. Cytokines were measured by ELISA. Results show mean $\pm$ SEM of three different experiments. Detection limit for IL-5 was 20 pg/ml. * $P < 0.05$ compared with control group.

were high, moderate, and low responders, respectively, in terms of IgE responsiveness to SEA. It is known that Ab production is genetically restricted, especially with MHC class II antigen, in experimental models with intranasal administration of antigens as well as systemic immunization (9, 16, 27, 28). Tamura et al. showed that BDF₁ (H-2b/d), BALB/c, and C₃H (H-2k) were high, intermediate, and low responders with respect to ovalbumin (OVA) by intranasal immunization of OVA together with the cholera toxin B subunit (9). On the other hand, Robinson et al. demonstrated that the H-2b haplotype is positively associated with IgG₁ production in intranasal immunization with alcalase together with the detergent matrix (27). We also demonstrated that CBA/J mice (H-2k) responded well with IgE production after intranasal sensitization with phospholipase A₂ from bee venom, whereas C57BL/6 mice (H-2b) responded poorly (in press). Thus, genetic restriction by intranasal sensitization seems to depend on the nature of the antigen, although BALB/c mice have been high responders in intratracheal immunization (29). In addition, the susceptibility to *S. mansoni* infection in mice is known to be genetically regulated (30–32). CBA/J, C₃H/HeJ, and BALB/c mice developed bigger hepatic granulomas and higher portal hypertension, whereas C57BL/6 mice developed relatively smaller granulomas and lower portal hypertension (30, 31). We previously demonstrated that peritoneal B-1 cell out-

growth due to *S. mansoni* infection was strain-dependent, occurring in CBA/J, C3H/HeJ, and BALB/c mice, but not in C57BL/6 mice (32). Our current results are consistent with these reports. Amiri et al. demonstrated that complete suppression of the total IgE response resulted in decreases in worm burden and egg production in *S. mansoni*-infected normal and IFN- γ knockout mice (33). Therefore, our results suggest that genetic susceptibility to IgE production against SEA would be involved in the course of *S. mansoni* infection.

We found that a significant amount of specific IgE as well as IgG could be produced in mice by intranasal sensitization without adjuvants, using SEA as an antigen. Adjuvants such as toxins are commonly used in intranasal immunization to induce definitive Ab production since most soluble antigens are less immunogenic by themselves (9, 11, 34, 35). However, adjuvants are known to direct the process of isotype switching via induction of certain cytokines in the differentiation of Th cell subsets in both man and experimental animals (16–22). In addition, some adjuvants could inhibit IgE production against antigens. For example, intraperitoneal administration of OVA coupled with murine red blood cells induced anti-OVA IgG production without IgE synthesis (36). Pullulan, a polymer of α -glucose, also suppressed antigen-specific IgE while enhancing antigen-specific IgG when this carbohydrate was conjugated with protein antigen (37, 38). Therefore, it is desirable to use antigen in the absence of adjuvants to investigate IgE production *in vivo*.

One of the reasons that we could induce specific IgE without adjuvants may be the use of SEA as an antigen. Allergens that are normally associated with allergic rhinitis in man appear to induce minimal changes in experimental animals when they are inoculated intranasally without adjuvants (15, 39). For example, extract of house-dust mite, which is one of the most common allergens in allergic rhinitis, does not induce local eosinophilia after intranasal inhalation (39). SEA is known to induce a strong Th2 response in hosts (23, 24). SEA contains a lactosamine carbohydrate, lacto-*N*-fucopentaose III, which can induce B and B-1 cells to produce IL-10 and prostaglandin E₂, two molecules that downregulate Th1 CD4⁺ T cells (40–42). And we recently found that this carbohydrate acts as a Th2 adjuvant to induce IgE production against protein when the carbohydrate is physically linked to a protein antigen (paper submitted). Therefore, carbohydrate motives on SEA might function as an internal adjuvant to induce Th2 immune reaction, and studies are now underway to clarify the

adjuvant activity of SEA. Another reason may be the route of sensitization. Intranasal administration of antigen effectively induces Th2 immune reaction (43). This may be due to the existence of dendritic cells which possess a strong property of antigen presentation (44). In addition, intranasal sensitization offers several advantages over the oral route, such as avoidance of an acidic and proteolytic stomach, a dose basis, and less colonization by commensal microorganisms (34, 45). For all these reasons, SEA is likely to induce antigen-specific IgE production without adjuvants by intranasal sensitization.

BALB/c mice intranasally sensitized with SEA showed eosinophil recruitment in nasal mucosa. Eosinophils have been known as primary effector cells inducing airway damage by the release of detrimental mediators such as major basic protein and eosinophil cationic protein (46, 47). Recently, Lukacs et al. demonstrated the recruitment of neutrophils and eosinophils into the lower airway after intraperitoneal inoculation with *S. mansoni* eggs and subsequent intranasal and intratracheal challenge with SEA (25, 48). They did not mention nasal eosinophilia. However, our results are consistent with these reports in terms of eosinophil migration by local administration of SEA. Furthermore, we demonstrated that nasal lymphocytes from mice intranasally sensitized with SEA produced a significant amount of IL-5, whereas those from control mice did not. IL-5 played a central role not only in eosinophil differentiation and proliferation but also in eosinophil accumulation in inflammatory sites (49–51). Moreover, abrogation of IL-5 resulted in ablation of eosinophil accumulation in airway membranes in experimental models of airway hyperactivity (52, 53). Thus, IL-5 production by local nasal lymphocytes may account for nasal eosinophilia in SEA-sensitized mice.

In conclusion, we demonstrated that the induction of both antigen-specific IgE and nasal eosinophilia by intranasal sensitization with SEA without the use of adjuvants in mice is strain-dependent. In addition, mice representing two hallmarks of the initiation of allergic rhinitis showed sneezing and nasal rubbing after nasal challenge with SEA (data not shown). This could be a useful model to investigate not only the induction of pathophysiology and the heterogeneity of allergic rhinitis but also the effect of environmental agents on their immunomodulatory activities.

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