

Induction of Th2 Responses and IgE Is Largely Due to Carbohydrates Functioning as Adjuvants on *Schistosoma mansoni* Egg Antigens¹

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Infection with the helminth parasite *Schistosoma mansoni* induces a pronounced Th2-type response that is associated with significant IgE production. To better understand how the parasite drives these responses, we investigated the relative roles of proteins and carbohydrates in driving Th2-type and/or IgE responses using a murine model of intranasal sensitization with soluble egg Ags (SEA) of *Schistosoma mansoni*. We found that repeated intranasal sensitization with soluble egg Ags led to the induction of both total and specific IgE production and nasal eosinophilia. By comparing the responses of mice sensitized with SEA or metaperiodate-treated SEA we were able to demonstrate that carbohydrates on SEA are the major inducers of IgE production and nasal recruitment of eosinophils. Mice sensitized with periodate-treated SEA displayed a significant decrease in both total and specific IgE levels in comparison to mice sensitized with native SEA. Furthermore, sensitization of mice with periodate-treated SEA significantly reduced levels of Ag-specific IgG1, but had no effect on IgG2a production. Nasal lymphocytes from mice sensitized with native SEA, but not with periodate-treated SEA, produced IL-4, IL-5, and IL-10 when restimulated with native SEA in vitro. On the other hand, lymphocytes from mice sensitized with periodate-treated SEA did not produce any of these same cytokines following in vitro restimulation, suggesting that carbohydrates were required for in vivo induction of Th2 response and for that of associated cytokine responses in this model. Lastly, competitive inhibition ELISA showed that although carbohydrates are required for SEA-specific IgE induction, they are not targets of the induced IgE response. *The Journal of Immunology*, 1999, 163: 6712–6717.

Carbohydrates expressed by pathogens are capable of eliciting several types of innate immune activation. Furthermore, glycans on the pathogen surface that are recognized by Abs or lymphocytes also induce various types of immune responses (1–4). Several different sugars have been shown to bind to the serum mannose-binding lectin or to the mannose receptor on cells, facilitating complement binding and/or phagocytosis as part of the innate immune system (5, 6). In addition, carbohydrates activate the alternative pathway of complement (7). However, other than T-independent Ab production, little is known in terms of carbohydrates having any direct roles in induction of cellular responses that lead to the production of Ag-specific or nonspecific Abs in vivo.

Schistosoma mansoni is a helminth parasite of humans that induces a potent Th2-type response and production of nonspecific and specific IgE. Schistosome soluble egg Ags (SEA)³ are strong activator(s) of Th2-type responses and are associated with increases in serum IgE as well as eosinophilia in blood and tissues in

both humans and experimental animals (8–10). Recently, SEA has been shown to contain oligosaccharides such as lacto-*N*-fucopentaose III, which appear to directly activate cells, inducing B and B-1 cells to produce IL-10 and PGE₂ that down-regulate type 1 CD4⁺ T cells (11–13). These observations suggest that carbohydrates on SEA function as immune activators of Th2-associated responses.

We recently developed a murine model of allergic rhinitis, which showed that repeated intranasal sensitization of mice with SEA leads to Ag-specific IgE production and nasal eosinophilia (14). To determine whether carbohydrates on SEA play a role in the induction of Th2 responses, we compared Ab responses, nasal eosinophilia, and nasal lymphocyte cytokine production following intranasal sensitization with native or periodate-treated SEA. Our results demonstrate that carbohydrates on SEA play a critical role in induction of SEA-specific Th2 responses. Furthermore, our study also shows that sugars on SEA function solely as Th2-activating adjuvants and are not themselves epitopes of induced Ab responses in vivo.

Materials and Methods

Animals

Female BALB/c mice (7 wk of age) were purchased from Harlan Breeders (Indianapolis, IN). Female BALB/c xid mice (7 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained at animal facilities at the Harvard School of Public Health according to the guidelines set forth by the Harvard Medical Area Research Committee.

Antigens

SEA was prepared as described previously (15). Periodate oxidation was performed using 10 mM sodium metaperiodate as described previously (13), which was then dialyzed against PBS overnight at 4°C and stored at –20°C until further use. Using Abs specific for carbohydrates on SEA, we

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³ Abbreviations used in this paper: SEA, soluble egg Ags; PLA₂, phospholipase A₂.

have previously demonstrated that treatment of SEA with 10 mM sodium metaperiodate leads to structural alteration in carbohydrates without any significant effect on proteins in SEA (13). Mock-treated SEA was produced by following the identical periodate oxidation protocol as previously described (13) but with the omission of sodium metaperiodate. Mock-treated SEA was used as a buffer control for the periodate-oxidized SEA. Biotinylated SEA was prepared using 1.6 mg/ml SEA in sodium bicarbonate buffer (pH 8.5), and then incubated with biotin (long arm) *N*-hydroxy succinimide ester (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. The reaction was stopped by the addition of 5 μ l of ethanolamine, then dialyzed overnight against PBS containing 0.05% sodium azide. Protein concentrations were determined by bicinchoninic acid (BCA) assay according to the manufacturer's instruction (Pierce, Rockford, IL).

Sensitization of mice

Intranasal sensitization of mice with SEA was performed as described with slight modifications (14). Mice ($n = 6$) were sensitized weekly for 3 wk by intranasal administration of 5 μ g protein of native, periodate-treated, mock-treated SEA, or saline in a total volume of 20 μ l. One week following the third sensitization, mice were challenged daily with intranasal administration of 1 μ g protein in a 20 μ l volume for 7 consecutive days.

Serum Ab levels

Peripheral blood was sampled from tail snips after the final nasal challenge. Blood was centrifuged at $200 \times g$ and stored at -20°C until use. Serum total IgE and Ag-specific IgE was measured by sandwich ELISA as described previously (14). Ag-specific IgG was detected by indirect ELISA as described previously (14). Optimal concentrations of peroxidase-conjugated anti-mouse IgG isotypes were 1/4000. Results are expressed as OD at 450 nm.

Competitive inhibition ELISA for Ag-specific IgE

The competitive inhibition ELISA protocol that we previously described was followed (16). In brief, ELISA plates were coated with rat anti-mouse IgE mAb (5 μ g/ml) in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C . Plates were blocked with a solution of 10% FCS containing 0.3% Tween 20 for 2 h at 37°C and washed four times with PBS and 0.05% Tween 20 (PBS-5T). Then 100 μ l of 1:6 diluted pooled sera from SEA-challenged mice was added to triplicate wells and incubated for 2 h at 37°C , after which the plates were washed in PBS-5T. In a separate plate, biotinylated SEA (1 μ g/ml) was mixed with serial concentrations (0, 0.08, 0.31, 1.25, 5.0, 20.0, and 80.0 μ g/ml) of native SEA, periodate-treated SEA, mock-treated SEA, or human serum albumin as control, then it was added to the ELISA plates for 1 h at 37°C . Next it was washed with PBS-5T, then by addition of a 1:1000 dilution of extravidin-peroxidase conjugate to the wells for 1 h at 37°C . The plates were then washed eight times with PBS-5T, and color developed by addition of tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and was stopped by addition of phosphoric acid (5.0%). Absorbance at 450 nm was measured with a Molecular Devices (Menlo Park, CA) automated plate reader. Percent inhibition was calculated as follows: $[100 - (\text{OD}_{450} \text{ with inhibitor} / \text{OD}_{450} \text{ without inhibitor})] \times 100$.

In vitro culture of nasal lymphocytes

Three hours after the final nasal challenge, nasal lymphocytes were isolated by enzyme extraction with collagenase as described by Asanuma et al. (17). Cell suspensions were pooled from two mice and contained 2 million lymphocytes/ml. Cells were cultured with or without SEA (5 μ g/ml) for 72 h at 37°C in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FCS, 5×10^{-5} M 2-ME (Sigma, St. Louis, MO), and 100 U/ml and 100 μ g/ml penicillin/streptomycin (Sigma) in flat-bottom 48-well plates (Corning Glass, Corning, NY). Cell supernatants were collected and stored at -80°C until assayed.

Cytokine determination

Levels of IL-4, IL-5, IL-10, and IFN- γ in culture supernatants were measured by capture ELISA as previously described (18). Recombinant IL-4, IL-5, IL-10, and IFN- γ standards were plated at 0–5,000 pg/ml, 0–20,000 pg/ml, 0–20,000 pg/ml, and 0–10,000 pg/ml, respectively. The detection limits in these assays were 3 pg/ml for IL-4, 10 pg/ml for IL-5, 20 pg/ml for IL-10, and 20 pg/ml for IFN- γ .

Histological analysis

Histological examination was performed as described previously (14). In brief, 3 h after the final nasal challenge, the mice were killed with carbon dioxide. Then heads were removed, fixed with 10% formalin, and treated with a decalcifying solution (VWR Scientific Products, Bridgeport, NJ) for 7 days. Then, coronal sections were stained with hematoxylin and eosin, and local eosinophilia in nasal septum was observed.

Statistical analysis

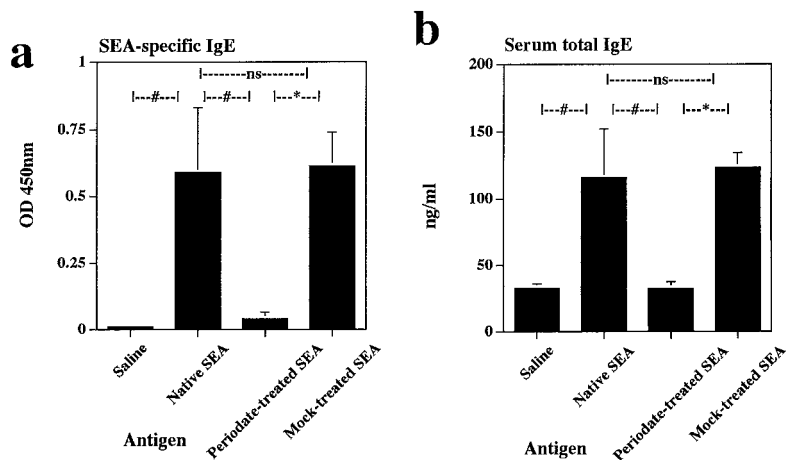
Statistical significance was determined by Student's unpaired *t* test. A *p* value < 0.05 was considered statistically significant. Values were given as mean $\pm$ SEM.

Results and Discussion

Carbohydrates on SEA induce Th2 Ab production

As we described previously, sensitization and subsequent challenge with SEA in the absence of adjuvants led to the production of SEA-specific and total IgE in BALB/c mice (Fig. 1, *a* and *b*). Using SEA carbohydrate-specific Abs, we have previously demonstrated that low concentrations (10–20 mM) of sodium metaperiodate treatment selectively alters glycan structure without affecting protein (13). Therefore, we used native, mock-treated, or periodate-treated SEA to determine the role of carbohydrates on SEA in induction of Th2-type Ab response and cytokine production after intranasal sensitization. Mice sensitized with periodate-treated SEA displayed marked decreases (94.31 $\pm$ 1.76%) in SEA-specific IgE production and displayed levels of SEA-specific IgE similar to those observed in mice sensitized with saline. Similarly, significant decreases in total serum IgE were also observed in sera from mice sensitized with periodate-treated SEA compared with

FIGURE 1. Serum SEA-specific IgE (*a*) and total IgE (*b*) after intranasal sensitization with SEA. BALB/c mice were sensitized and challenged intranasally with native, periodate-treated, or mock-treated SEA as well as with saline in the absence of adjuvants as described in *Materials and Methods*. Following the final nasal challenge, mice were bled and serum SEA-specific IgE (*a*) and total IgE (*b*) were determined by ELISA. Levels of SEA-specific IgE were presented as the absorbance at 450 nm from duplicate wells of 1:4 serum dilution. Results show the mean $\pm$ SEM from six individual sera per group. Data are representative of three separate experiments. #, $p < 0.05$; *, $p < 0.01$; ns, not significant.



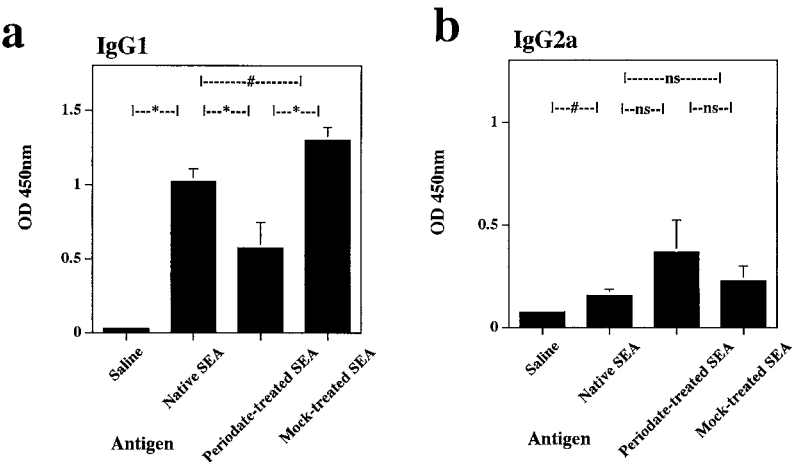


FIGURE 2. Serum SEA-specific IgG1 (a) and IgG2a (b) isotypes after intranasal sensitization with SEA. BALB/c mice were sensitized and bled as described in Fig. 1. Serum SEA-specific IgG1 (a) and IgG2a (b) were determined by ELISA. Levels of SEA-specific IgG1 and IgG2a were presented as the absorbance at 450 nm from duplicate wells of 1:100 serum dilution. Results show the mean $\pm$ SEM from six individual sera per group. Data are representative of three separate experiments. #, $p < 0.05$; *, $p < 0.01$; ns, not significant.

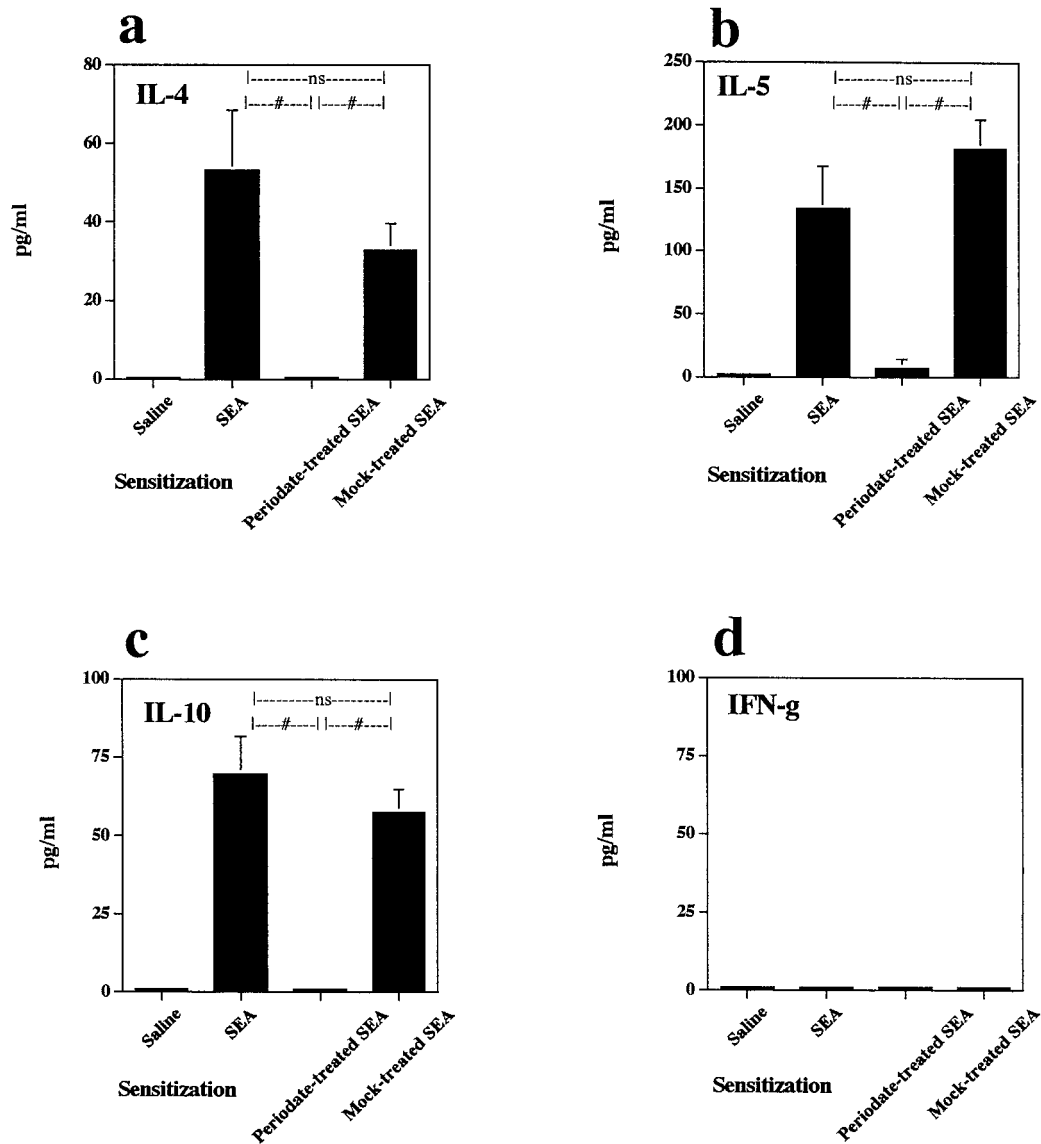


FIGURE 3. In vitro cytokine production by nasal lymphocytes. Nasal lymphocytes from mice intranasally sensitized with saline, native SEA, periodate-treated SEA, or mock-treated SEA were isolated by enzymatic extraction following the final nasal challenge as described in *Materials and Methods* (15). Lymphocytes were pooled from two noses of mice and cultured with 5 μ g/ml of native SEA for 72 h, then supernatants were collected. Amount of IL-4 (a), IL-5 (b), IL-10 (c), and IFN- γ (d) were determined by ELISA. Results show the mean $\pm$ SEM from three individual pools of lymphocytes. Data are representative of two separate experiments. #, $p < 0.01$; ns, not significant.

mice sensitized with native or mock-treated SEA (Fig. 1b). Levels of total IgE and SEA-specific IgE were nearly identical between native and mock-treated SEA sensitized animals.

We then examined the role of carbohydrates on induction of Th1- or Th2-associated Igs by examining differences in induction of IgG2a or IgG1, respectively, following intranasal sensitization in BALB/c mice. We found that mice sensitized with periodate-treated SEA produced significantly less SEA-specific IgG1 compared with those sensitized with native SEA (Fig. 2a). In contrast, there was no significant difference in levels of SEA-specific IgG2a among the groups (Fig. 2b).

These results indicate that although carbohydrates on SEA induce the production of the Th2-associated immunoglobulins IgE and IgG1, they have no effect on the Th1-associated isotype IgG2a (19). There are several explanations of how SEA-associated carbohydrates might function as adjuvants. For example, because carbohydrates on SEA minimally contain fucose and galactose residues (11), glycoprotein and/or glycolipid molecules in SEA that contain these sugars might be taken up preferentially by APC via lectin-like receptors such as mannose receptor or mannose-binding lectin receptor (5, 10, 20). Furthermore, dendritic cells are one of the most efficient APC, and they express carbohydrate-binding receptors, which mediate endocytosis of glycosylated Ags (21, 22). Considering the intranasal sensitization model that we used for this study, dendritic cells would likely accumulate in the respiratory tract, including in nasal turbinates and nasal-associated lymphoid tissue (23, 24). Additionally, the intranasal route for Ag delivery is likely to have advantages over other routes of Ag delivery, e.g., avoidance of acid and proteolytic enzymes in saliva and gastric fluid (25). Thus, it may be hypothesized that the immune environment where SEA was introduced in this study induced Th2-associated Ab responses and allowed a rapid interaction of SEA with APC via glycans. We are currently investigating this hypothesis in the ongoing studies in our laboratory.

Inhibition of in vitro Th2 cytokine production in mice sensitized with periodate-treated SEA

The ability of carbohydrates on SEA to drive Th2 responses was examined by recall stimulation of nasal lymphocytes. We measured levels of Th2-associated IL-4, IL-5, and IL-10, and Th1-associated IFN- γ . We found that nasal lymphocytes from mice sensitized and challenged intranasally with native SEA produced significantly higher levels of IL-4, IL-5, and IL-10 after stimulation than those from control mice sensitized with saline (Fig. 3, a–c). In contrast to native or mock-treated SEA, nasal lymphocytes from mice sensitized with periodate-treated SEA produce significantly less of these cytokines. In addition, we found that nasal lymphocytes from mice sensitized and challenged with native SEA produced IL-5 if they were restimulated with periodate-treated SEA. The amount of IL-5 produced from these cells, however, was not statistically different from those restimulated with native or mock-treated SEA in vitro (Fig. 4). Furthermore, a recent study from our laboratory demonstrates that removal of N- and O-linked glycans from the SEA using trifluoro methane sulfonic acid completely abrogates its adjuvant activity to induce Th2 response.⁴ Together, these results suggest that although carbohydrates on SEA are required for the initiation of the Th2 responses in vivo, they are not required for recall production of IL-5 by sensitized nasal lymphocytes.

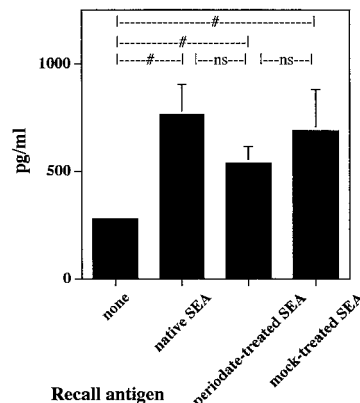


FIGURE 4. IL-5 production by nasal lymphocytes primed with native SEA. Nasal lymphocytes from mice intranasally sensitized with native SEA were cultured with 5 μ g/ml of native SEA, periodate-treated SEA, or mock-treated SEA without re-stimulation for 72 h, then supernatants were collected and amounts of IL-5 were determined by ELISA. Results show the mean $\pm$ SEM from three individual pools of lymphocytes. Data are representative of two separate experiments. #, $p < 0.05$; ns, not significant.

Next we measured levels of the Th1 cytokine IFN- γ . Surprisingly, we were unable to detect IFN- γ production regardless of the type of SEA used to sensitize the mice (Fig. 3d). However, the same lymphocytes did produce significant amounts of IFN- γ when stimulated with Con A (data not shown). These results suggest that interaction between carbohydrates on SEA and nasal lymphocytes leads to induction of Th2 but not of Th1 type responses. Furthermore, these results support other studies that show that nasal lymphocytes, including those in nasal-associated lymphoid tissue, contribute to the induction of Ab production after intranasal sensitization (26). In fact, we recently found that carbohydrates purified from SEA enhance *in vivo* Ab production and induce naive CD4⁺ T cells to produce IL-4 *in vitro*.⁴ These results indicate that CD4⁺ T cells in nasal lymphocytes are likely to be one of the targets for carbohydrates on SEA.⁴

Carbohydrates on SEA enhance induction of nasal eosinophilia

As we described previously, BALB/c mice showed remarkable eosinophilia in the nasal septum after challenge with native SEA, compared with saline (Fig. 5, a and b). In contrast, intranasal sensitization with periodate-treated SEA did not induce nasal eosinophilia, whereas nasal eosinophilia was seen in mice challenged with mock-treated SEA, and the degree of eosinophilia was similar to that observed in mice which received native SEA (Fig. 5, c and d). Eosinophils are known as primary effector cells which induce airway damage by the release of detrimental mediators such as major basic protein and eosinophil cationic protein (25). IL-5 plays a central role not only in eosinophil differentiation and proliferation but also in eosinophil accumulation in inflammatory sites (26, 27, 30, 31). Thus, it appears that carbohydrates on SEA enhance nasal eosinophilia by inducing local IL-5 production by nasal lymphocytes.

Th2-stimulating carbohydrates on SEA are not epitopes for IgE

To determine whether carbohydrate molecules on SEA are epitopes for anti-SEA Abs, we analyzed whether SEA-specific Abs specifically bind carbohydrates on SEA. An inhibition ELISA that measured the binding of biotinylated SEA to plate-bound IgE was used to test serum from sensitized mice for IgE Abs against carbohydrates. In contrast to all the preceding data presented in this study, we found that all types of SEA, including periodate-treated

⁴ M. S. Ryan, S. Disanayake, and D. A. Harn, Jr. 1999. *Schistosoma mansoni* egg Ag carbohydrates display potent adjuvant properties selectively enhancing Th2 type immune responses to co-injected Ags. Submitted for publication.

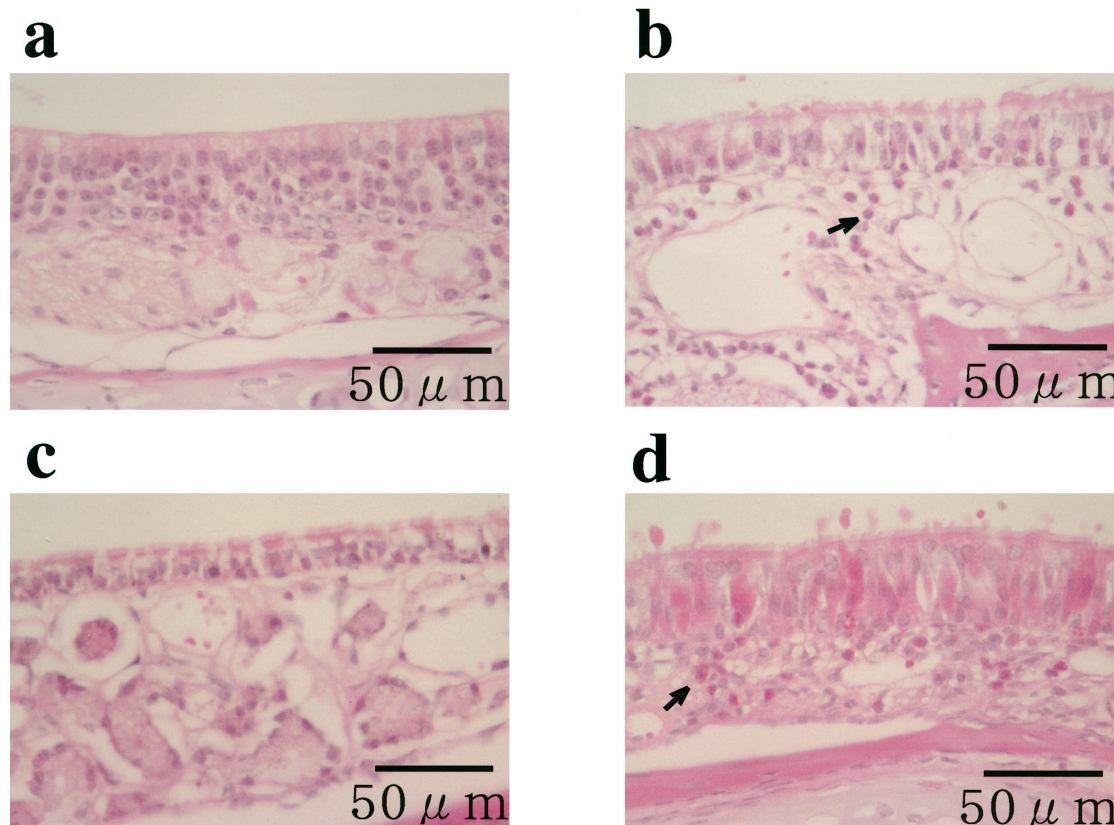


FIGURE 5. Hematoxylin-eosin stained nasal septum. BALB/c mice were sensitized and challenged intranasally with saline (a), native SEA (b), periodate-treated SEA (c), or mock-treated SEA (d). After the final nasal challenge, mice were killed and their heads were removed. Nasal tissues were fixed and decalcified, and sections were stained with hematoxylin and eosin.

SEA, were able to inhibit (>90.0% at 80 $\mu\text{g/ml}$ inhibitor) the binding between biotinylated SEA and serum IgE in a dose-dependent manner (Fig. 6). Taken together, these findings demonstrate that carbohydrates on SEA are not major epitopes for induced SEA-specific IgE.

Previous findings in several studies indicate that the ability of glycans on Ags to induce specific IgE generally depends on the allergen studied (28, 29). For example, Su et al. (28) reported that carbohydrate residues on Bermuda grass pollen Ag BG60 are essential for binding with specific IgE because IgE binding was re-

duced after periodate treatment. On the other hand, Muller et al. (29) demonstrated that native and deglycosylated phospholipase A_2 (PLA $_2$) gave rise to similar skin reactions in patients, indicating that sugar residues were of little relevance for IgE binding to PLA $_2$. We also demonstrated that IgE from mice intranasally sensitized with PLA $_2$ binds to deglycosylated PLA $_2$ in a dose-dependent manner, providing further evidence that carbohydrates on PLA $_2$ are not required, nor are they targets of anti-PLA $_2$ IgE (16). These two examples, taken together with the results of our study indicate that the Th2-inducing adjuvant-like property of carbohydrates on glycosylated Ags largely depend on the composition and structure of sugar residues.

In summary, we have provided *in vivo* evidence that carbohydrate residues on SEA play a major role in immune responses by functioning as adjuvants in priming and induction of Th2-type Ab and cytokine responses. Moreover, such sugar-primed cells maintain their Th2 bias when recalled with protein component in the absence of sugar. These results have implications for future investigations on the induction, maintenance, and pathophysiology of allergy and allergic asthma, and also on allergy caused by infectious diseases. Our results also point to the development of novel carbohydrate-based adjuvants that could be used to enhance Ab-dependent vaccines.

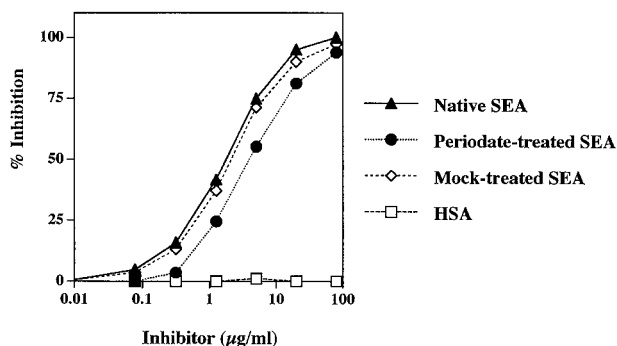


FIGURE 6. Inhibition of binding between SEA and serum IgE. BALB/c mice were bled following the nasal challenge with SEA. The binding between biotinylated SEA and serum IgE was inhibited by adding serial concentrations of inhibitors; native SEA (▲), periodate-treated SEA (●), mock-treated SEA (◇), or human serum albumin as a control (□). Results show the mean percentage of inhibition $\pm$ SEM of triplicate wells. Data are representative of two separate experiments.

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