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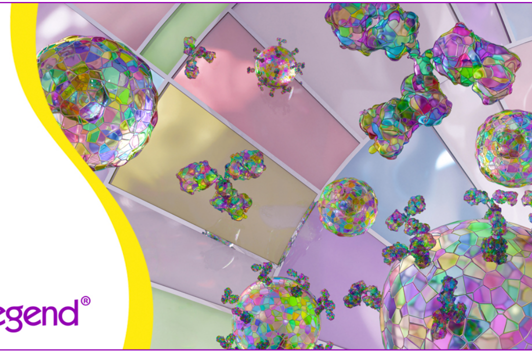

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Lacto-*N*-fucopentaose III Found on *Schitosoma mansoni* Egg Antigens Functions as Adjuvant for Proteins by Inducing Th2-Type Response[1]

Mitsuhiro Okano,[2]*† Abhay R. Satoskar,* Kazunori Nishizaki,† and Donald A. Harn, Jr.*

We have recently demonstrated that induction of Th2 responses by *Schistosoma mansoni* egg Ag is largely due to carbohydrates on the Ag functioning as adjuvants. Lacto-*N*-fucopentaose III (LNFPIII), a polylactosamine sugar, is the predominant carbohydrate found in *S. mansoni* egg Ag. Therefore, using neoglycoprotein, we investigated whether LNFPIII induces in vivo Th2 response and functions as an adjuvant. Following intranasal immunization with LNFPIII linked to human serum albumin (HSA) (HSA-LNFPIII), BALB/c mice mounted a strong Th2 response and produced significantly higher levels of total IgE as well as HSA-specific IgG, IgG1, and IgE. HSA-LNFPIII was over 1000-fold more potent in inducing Ab production as compared with HSA alone. Although LNFPIII itself did not function as an epitope for either IgG or IgE, its conjugation with protein was essential for the adjuvant activity. Moreover, fucose residue on LNFPIII was crucial for induction of Ab production. Nasal lymphocytes from mice immunized with HSA-LNFPIII produced IL-4, IL-5, and IL-10, but not IFN-γ following in vitro stimulation with HSA or HSA-LNFPIII. In addition, these activated nasal lymphocytes also showed a significant increase of B7-2 expression on B220-positive cells. Furthermore, not only intranasal but also both i.p. and s.c. immunization with HSA-LNFPIII induced significant production of HSA-specific Abs compared with the immunization with HSA alone, suggesting that the activity of LNFPIII was not restricted on particular route of immunization. These results demonstrate that Lewis type carbohydrate LNFPIII can function as an adjuvant by their ability to induce a Th2 response. ***The Journal of Immunology,* 2001, 167: 442–450.**

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Several pathogens express complex carbohydrates on their surface that are capable of eliciting immune responses. Carbohydrates have been shown to function as T/B cell epitopes (1, 2), act as ligands for selectins (3), regulate the process of neovascularization (4), activate the alternate complement pathway and modulate immunity against viruses (5). Moreover, carbohydrates expressed on various cancer cells are also regarded as onco-developmental Ag (6).

We recently demonstrated that *Schistosoma mansoni* egg Ag (SEA)[3] induces a strong Th2-associated cytokine and Ab responses in BALB/c mice following intranasal immunization (7). Conversely, we also found that deglycosylation of SEA by periodate treatment significantly abrogates its ability to induce a Th2 response (8). These findings indicate that carbohydrates on SEA are required for in vivo induction of Th2 response in this model. Recent studies have demonstrated that lacto-*N*-fucopentaose III (LNFPIII) is the predominant carbohydrate component of SEA that activates B and B-1 cells to produce IL-10 and PGE_2 (9, 10). LNFPIII also binds ICAM-1, P-, L-, and E-selectins and plays a critical role in embryonic development and differentiation (11). Moreover, LNFPIII is also expressed by cancer cells and considered as a risk factor for tumor metastasis (12). Nevertheless, this oligosaccharide is also found in human milk (13).

The purpose of this study was to investigate the role of LNFPIII as an adjuvant by its ability to induce a Th2-like response. To approach this question, we immunized BALB/c mice intranasally, i.p., and s.c. with a protein Ag human serum albumin (HSA) conjugated to LNFPIII (HSA-LNFPIII). Our results demonstrate that LNFPIII is a potent inducer of a Th2 response and can also act as an adjuvant by inducing Ab production against coupled protein Ag.

Materials and Methods

Animals

Seven- to 9-wk-old female BALB/c and C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were maintained in the specific pathogen-free facility at Harvard School of Public Health according to the guidelines for animal research.

Antigens

HSA (fraction V) and BSA (fraction V) were purchased from Sigma (St. Louis, MO). LNFPIII, lacto-*N*-neotetraose (LNnT, a nonfucosylated homologue of LNFIII), LNFPIII conjugated to HSA (HSA-LNFPIII) and LNnT conjugated to HSA (HSA-LNnT) were purchased from Accurate Chemical and Scientific (Westbury, NY). Both LNFPIII-HSA and LNnT-HSA contain ~13 mol of carbohydrates conjugated to 1 mol of HSA. Lacto-*N*-fucopentaose I (LNFPI) conjugated to HSA (HSA-LNFPI) was also obtained from Accurate Chemical and Scientific. LNFPIII conjugated to BSA (BSA-LNFPIII) was purchased from Dextra Laboratories (Reading, U.K.).

Immunization

Groups of four mice were immunized intranasally with HSA, HSA-LNFPIII, HSA-LNnT, or HSA-LNFPI in Dulbecco's PBS (Life Technologies, Grand Island, NY) at several protein concentrations, or Dulbecco's PBS

*Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115; and †Department of Otolaryngology, Okayama University Medical School, Okayama, Japan.

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[2] Address correspondence and reprint requests to Dr. Mitsuhiro Okano, Department of Otolaryngology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. E-mail address: mokano@cc.okayama-u.ac.jp

[3] Abbreviations used in this paper: SEA, *Schistosoma mansoni* egg Ag; LNFPIII, lacto-*N*-fucopentaose III; HSA, human serum albumin; HSA-LNFPIII, HSA conjugated to LNFPIII; LNnT, lacto-*N*-neotetraose; HSA-LNnT, LNnT conjugated to HSA; BSA-LNFPIII, LNFPIII conjugated to BSA; TMB, tetramethylbenzidine.

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(Life Technologies) as a control in a total volume of 20 μl. Two weeks later, mice were boosted by intranasal immunization using the same dose of Ag used for priming. Later the mice were challenged daily intranasally using 1/10 dose of Ag from day 22 to 28. Serum samples were obtained by tail bleeds for Ab determination after the prime, boost, and sixth challenge at day 13, 21, and 28, respectively. Twelve hours after the last challenge, mice were sacrificed using carbon dioxide, and nasal lymphocytes were isolated by enzyme extraction with collagenase (Boehringer Mannheim, Indianapolis, IN) as described previously (14).

In addition, groups of four mice were immunized i.p. or s.c. with 10 μg (protein) of these Ags in the presence or absence of alum (1 mg) and boosted 2 wk later via same immunization routes and similar Ag dose. One week following the boosting sensitization, serum was collected for Ab determination by ELISA.

Ag-specific IgM, IgG, IgG1, IgG2a, and IgA determination

Serum levels of Ag-specific Ab were determined by ELISA as described previously (7). In brief, ELISA plates (Corning Glass, Corning, NY) were coated with 100 ml of Ag (2 μg/ml) overnight at 4°C in carbonate-bicarbonate buffer (pH 9.6), then blocked with 10% PBS containing 10% FCS for 2 h at 37°C. Individual serum samples were then plated in duplicate in 2-fold serial dilution beginning at 1/100 and incubated for 2 h at 37°C. Plates were washed with PBS and 0.05% Tween 20 and bound Ab were detected by incubation with peroxidase-conjugated goat anti-mouse Ig for 1 h at 37°C. Optimum dilutions of Ab to mouse IgM, IgG, and IgG subclasses (Boehringer Mannheim) and IgA (BioSource International, Camarillo, CA) were 1/10,000, 1/10,000, 1/2,000, and 1/2,000 respectively. After addition of tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the reaction was stopped by addition of 50 μl phosphoric acid (5.0%) and the absorbance at 450 nm was read using an automated plate reader (Molecular Devices, Menlo Park, CA). Results were expressed as endpoint titers where the endpoint equals the final serum dilution yielding an absorbance twice background.

Serum total and Ag-specific IgE

Total and Ag-specific IgE in sera were determined by ELISA as described previously (7). Briefly, ELISA plates were coated with 100 μl rat anti-mouse IgE (5 μg/ml) in carbonate-bicarbonate buffer overnight at 4°C. Plates were blocked and washed as described above, then samples of serially diluted serum or mouse IgE standard were plated in duplicate and incubated for 2 h at 37°C. Biotinylated monoclonal rat anti-mouse IgE (PharMingen, San Diego, CA) (0.5 μg/ml) or biotinylated Ag (1 μg/ml) was then added to wells for 2 h at 37°C for detection of total and Ag-specific IgE, respectively. After washing the plates with PBS and 0.05% Tween 20, peroxidase-conjugated streptavidin (1/1000 dilution; Sigma) was added to the wells and incubated for 1 h at 37°C. The color was developed by adding TMB substrate and the reaction was stopped as described above. For biotinylation, HSA (1.8 mg/ml) in sodium bicarbonate buffer (pH 8.5) was incubated with biotin (long arm) *N*-hydroxy succumide ester (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. The reaction was stopped by adding 5 μl of ethanolamine (Sigma) and dialyzed overnight with PBS containing 0.05% sodium azide.

Competitive inhibition ELISA

The competitive ELISA for Ag-specific IgG that we previously described was followed (15). Briefly, ELISA plates were coated with HSA-LNFPIII overnight at 4°C. Plates were washed with PBS/0.05% Tween 20, and blocked with 10% FCS/PBS at 37°C for 1 h. Serum samples were diluted 500-fold and mixed with different doses of HSA or monovalent LNFPIII as inhibitors. Following incubation for 15 min at room temperature, samples were added to the Ag-coated plate in duplicate and incubated for 2 h at 37°C. Plates were washed with PBS/0.05% Tween 20, and peroxidase-conjugated goat anti-mouse IgG was added to the wells for 1 h at 37°C. Levels of bound Ab were determined by addition of TMB substrate and stopped by addition of acid as described. Results were presented as the average OD at 450 nm ± SEM of the percent of response from sera of four individual mice with inhibitors added divided by the response without inhibitors.

In vitro culture of nasal lymphocytes

Nasal lymphocytes from naive or Ag-primed BALB/c mice were isolated by enzyme extraction with collagenase (14). Cell suspensions containing 2×10^6 lymphocytes per milliliter were cultured with or without Ag (5 μg/ml) for 48 h at 37°C in RPMI 1640 medium (Life Technologies) containing 10% FCS, 5×10^{-5} M 2-ME (Sigma), and 100 U/ml and 100 μg/ml penicillin/streptomycin (Sigma) in flat-bottom 48-well plates (Corning Glass). Cell supernatants were collected and stored at −80°C until assayed. Pelleted cells were resuspended then stained with mAbs coupled to FITC or PE for flow cytometry analysis.

Cytokine determination

Levels of IL-4, IL-5, IL-10, and IFN-γ in the above culture supernatants were measured by captured ELISA as described previously (8). Briefly, Maxisorp multititer 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 2 μg/ml of capture Ab (rat anti-mouse IL-5, IL-10, and IFN-γ from PharMingen and rat anti-mouse IL-4 from Endogen (Woburn, MA) in Tris-HCl buffer at pH 9.0. The plates were blocked for 1 h at 37°C with 10% FCS in PBS after which murine recombinant cytokine standards IL-4 (0–860 pg/ml; Endogen), IL-5 (0–5,000 pg/ml; PharMingen), IL-10 (0–25,000 pg/ml; PharMingen), or IFN-γ (0–20,000 pg/ml; PharMingen) and the culture supernatants were added in duplicate and incubated overnight at 4°C. The plates were washed three times in PBS/Tween 20 and incubated for 1 h at 37°C with 1 μg/ml appropriate biotinylated detection Ab. (rat anti-mouse IL-5, IL-10, and IFN-γ from PharMingen, and rat anti-mouse IL-4 from Endogen). To detect biotinylated Abs, streptavidin-linked alkaline phosphotase (1/2,000 dilution in 10% FCS in PBS; PharMingen) was added and incubated for 45 min in the dark at 37°C. After a final washing in PBS/Tween 20, *p*-nitrophenylphophotase substrate (Sigma) in glycine buffer was added to each well. The absorbance was read at 405 nm using an automated microplate reader (Molecular Devices), and the concentration of the samples was calculated using the standard curve.

Flow cytometry

Nasal lymphocytes (1–2 × 10^5) were incubated on ice for 15 min with the following FITC- or PE-conjugated mAbs (PharMingen): anti-CD45R/B220 (RA3-6B2), anti-B7-1 (16-10A1), and anti-B7-2 (GL-1) or isotype-matched controls. Then cells were washed with cold HBSS containing 0.05% sodium azide. Flow cytometry analysis was performed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA). Dead cells were excluded from analysis using propidium iodide (Molecular Probes, Eugene, OR) staining. Lymphocytes were gated according to forward and side scatter and at least 10,000 events were acquired and analyzed.

Statistical analysis

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

Results

Ab production following intranasal immunization with protein conjugated to LNFPIII

Following intranasal immunization with HSA-LNFPIII, BALB/c mice displayed a significant increase in HSA-specific IgG production as compared with those immunized with HSA alone (Fig. 1*A*). This effect of LNFPIII was observed following primary immunization, and was further enhanced with boosting. Furthermore, anti-HSA IgG response was largely IgG1 following the prime, and remained so after boosting. Small amounts of HSA-specific IgG2a and IgG2b were seen after boosting, but IgM or IgA were not detectable at any time point throughout the course of the experiment. (Fig. 1*B*). In addition, BALB/c mice immunized intranasally with HSA-LNFPIII also displayed significantly higher levels of total IgE ($p < 0.05$) as well as HSA-specific IgE ($p < 0.005$) as compared with those immunized with HSA or saline (Fig. 1, *C* and *D*). In contrast, immunization of the mice with HSA-LNnT, the nonfucosylated homologue of HSA-LNFPIII, failed to increase total or HSA-specific IgE and IgG above baseline (Fig. 1, *A*, *C*, and *D*). C57BL/6 mice also produced significant amounts of HSA-specific IgG and IgE following intranasal immunization with HSA-LNFPIII, but not saline, HSA alone, or HSA-LNnT, although the amounts were significantly lower than those produced in BALB/c mice (Fig. 1, *E* and *F*). Moreover, HSA-LNFPI also failed to induce a significant production of Ab against HSA in mice when used as a sensitizing Ag (data not shown). In terms of Ag dose capable for the induction of Ab production, HSA-LNFPIII

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was able to induce a significant production of HSA-specific IgE and IgG in doses 1.0–10 μg (weight of HSA) as compared with 1000 μg of HSA alone required to induce equivalent HSA-specific IgG response. Moreover, even at such a high dose, HSA alone failed to induce a significant IgE production (Fig. 2, *A* and *B*).

Next we sought to determine whether the effect of LNFPIII on humoral immune response and assess the generality in terms of protein that is conjugated to the carbohydrate. BALB/c mice were immunized with saline, BSA, or BSA-LNFPIII in an identical manner to the intranasal immunization with HSA-LNFPIII. The mice displayed a significant increase in BSA-specific IgG and IgE production as compared with those immunized with saline or BSA alone (Fig. 3, *A* and *B*).

LNFPIII acts as an adjuvant but not an epitope for produced Ab

To determine whether HSA-LNFPIII-immunized mice produce Ab against LNFPIII, we performed competitive inhibition ELISA as described before. HSA could completely inhibit, in a dose-dependent fashion, IgG binding to HSA-LNFPIII-coated plates. In contrast, LNFPIII itself could not inhibit IgG binding at all (Fig. 4*A*). Furthermore, IgG from mice immunized with HSA-LNFPIII

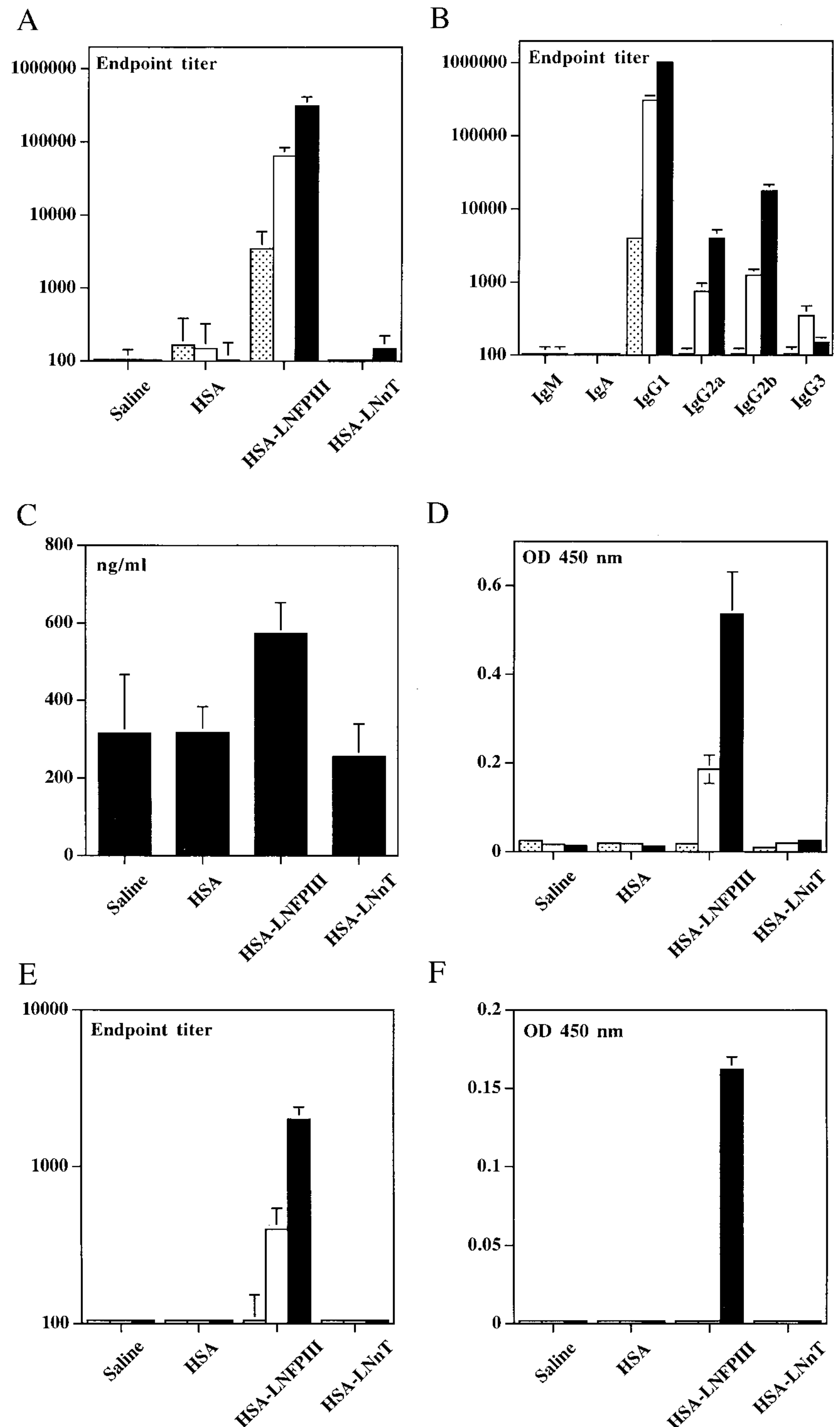

FIGURE 1. In vivo Ab production after the intranasal immunization with saline, HSA or sugar-conjugated HSA in BALB/c (*A–D*) and C57BL/6 (*E* and *F*) mice. Serum samples were taken after prime (▨), boost (□), and challenge (■) as described in *Materials and Methods*. Ab of IgG (*A* and *E*), IgM, IgA, and IgG subclasses (*B*) specific for HSA were measured by indirect ELISA and estimated by endpoint titer. IgE titers specific for HSA (*C* and *F*) and the volume of serum total IgE (*D*) were determined by sandwich ELISA. Levels of HSA-specific IgE were presented as the absorbance at 450 nm from duplicate wells of 1/4 serum dilution. Results show the mean ± SEM of four individual serum per group. Data are representative of three (*A–D*) and two (*E* and *F*) separate experiments.

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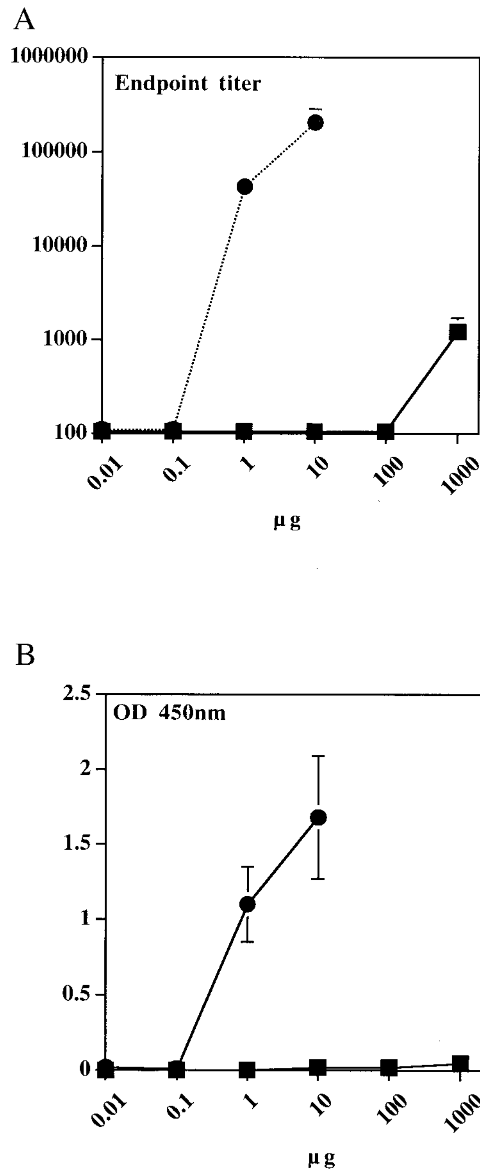

FIGURE 2. Analysis of Ag dose capable for the induction of Ab production. BALB/c mice were immunized intranasally with serial amounts of HSA (■) or HSA-LNFPIII (●) described in Fig. 1. Amounts of Ag were determined by BCA protein assay. After 6th challenge, sera were collected, and HSA-specific IgG (*A*) and IgE (*B*) were analyzed. Results show the mean ± SEM of endpoint titer (*A*) or absorbance at 450 nm of 4× diluted serum (*B*) from four individual serum per group. Data are representative of two separate experiments.

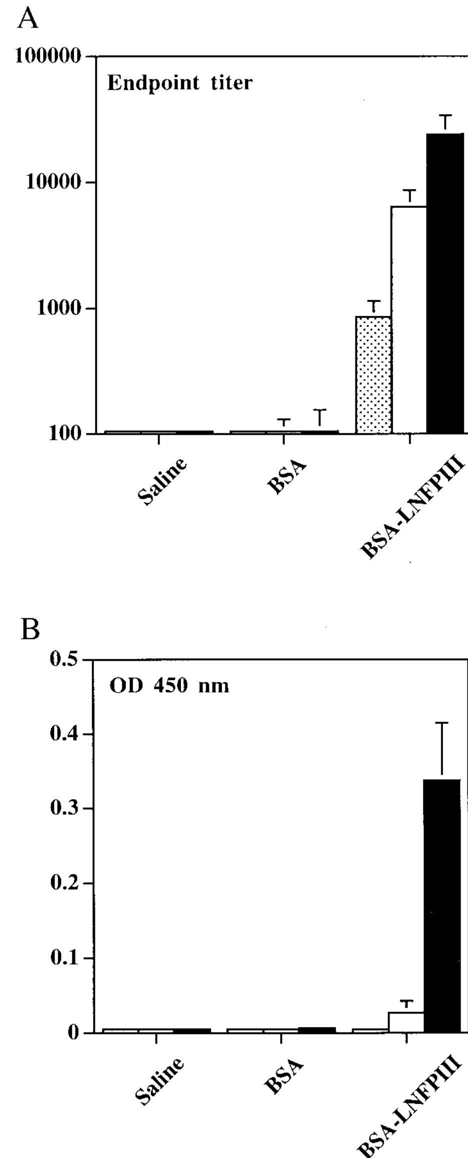

FIGURE 3. In vivo Ab production after the intranasal immunization with saline, BSA or BSA-LNFPIII. Plasma samples were taken after prime (▨), boost (□), and challenge (■) in an identical manner as described in Fig. 1. Ab of IgG (*A*) specific for BSA was measured by indirect ELISA and estimated by endpoint titer. IgE titers specific for BSA (*B*) were determined by sandwich ELISA and presented as the absorbance at 450 nm from duplicate wells of 1/4 serum dilution. Results show the mean ± SEM of four individual serum per group. Data are representative of two separate experiments.

bound to HSA, HSA-LNnT, and HSA-LNFPIII, but not BSA-LNFPIII, indicating that immunization with HSA-LNFPIII induced Ab response against HSA (data not shown). Similarly, IgE from mice immunized with HSA-LNFPIII did not react with biotinylated BSA-LNFPIII or biotinylated Lewis^x, which is the fucose-containing triasaccharide of LNFPIII (9), indicating that this carbohydrate was not an epitope for IgE (Fig. 4*B*).

To determine whether the adjuvant effect of LNFPIII was dependent on the covalent linkage of sugar to the protein, mice were immunized with HSA alone or HSA-LNFPIII mixed with several different concentrations of monovalent LNFPIII. Free form of LNFPIII failed to alter the adjuvant effect of HSA-LNFPIII. Moreover, LNFPIII did not induce anti-HSA Ab production when coadministered with HSA in a nonconjugated form (Fig. 5).

Cytokine production by nasal lymphocytes following intranasal immunization with LNFPIII

Mice were primed, boosted, and challenged with saline, HSA, or HSA-LNFPIII as described in *Materials and Methods*. Nasal lymphocytes were isolated 12 h after final challenge, and cultured with or without Ag for 48 h. Cytokine levels in these supernatants were determined by captured ELISA. Following in vitro stimulation with HSA, nasal lymphocytes from with HSA-LNFPIII-immunized mice produced significantly more IL-4 ($p < 0.05$), IL-5 ($p < 0.05$), and IL-10 ($p < 0.01$) as compared with those from saline- and HSA-immunized mice. Interestingly, nasal lymphocytes from mice sensitized with HSA alone or saline could produce IL-5 and IL-10 in response to HSA-LNFPIII in vitro; however, the

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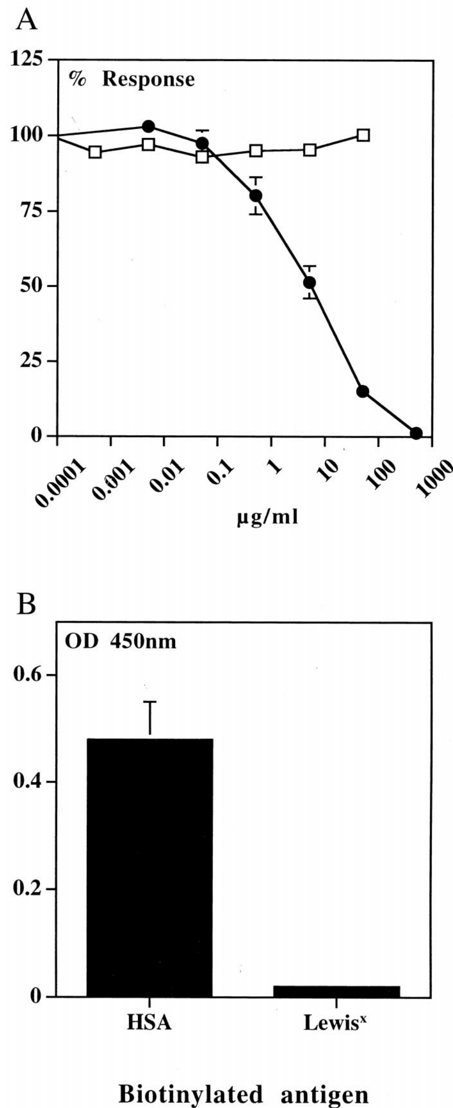

FIGURE 4. *A,* Inhibition ELISA for HSA-LNFPIII-specific IgG using HSA (●) and free LNFPIII (□) as an competitor. Sera used in this study were from mice immunized and challenged with HSA-LNFPIII. Results are given as the average absorbance at 450 nm ± SEM of percent response from sera of four individual mice with inhibitors added divided by the response without inhibitors. *B,* Comparison of binding between HSA and Lewis^x for IgE in murine sera intranasally immunized with HSA-LNFIII. Either biotinylated HSA or Lewis^x was added as a detection reagent in captured IgE ELISA. Results show the mean ± SEM of four individual serum. Data are representative of two separate experiments.

amounts were significantly less than those from mice sensitized with HSA-LNFPIII. In contrast, Ag-stimulated nasal lymphocytes from all three groups of mice failed to produce IFN-γ (Fig. 6).

Selective B7-2 expression on B220$^+$ cells in nasal lymphocytes following intranasal immunization with LNFPIII

Next, we examined whether immunization with HSA-LNFPIII altered expression of costimulatory molecules necessary for T cell activation and differentiation (16). Flow cytometric analysis showed that Ag-stimulated nasal lymphocytes from mice immunized with HSA-LNFPIII showed significantly higher ($p < 0.005$) expression of B7-2 on B220$^+$ cells as compared with cells from mice immunized with HSA alone or saline (Fig. 7, *D–F*). However, expression of B7-1 was similar among all groups tested (Fig. 7, *A–C*).

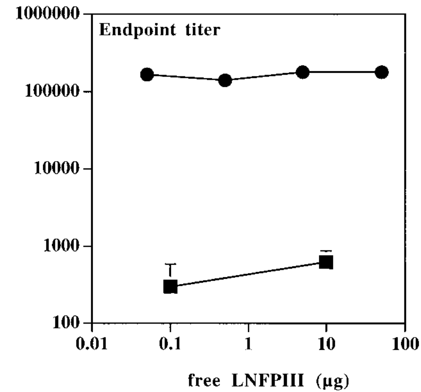

FIGURE 5. Effect of free LNFPIII on HSA-specific IgG Ab production in response to immunization with HSA (■) or HSA-LNFPIII (●). BALB/c mice were primed intranasally with HSA or HSA-LNFPIII (10 μg protein) simultaneously mixed with monovalent LNFPIII. Boost and challenge were performed as described in *Materials and Methods*, and free LNFPIII was given at same dose and 1/10, respectively. After 6 the challenge, sera were taken and endpoint titer of HSA-specific IgG Ab was determined described as above. Results show the mean endpoint titer ± SEM of four individual serum. Data are representative of two separate experiments.

Effect of route of immunization on adjuvant activity of LNFPIII

Further, we investigated whether the adjuvant activity of LNFPIII on the induction of Th2-type responses was dependent on the route of immunization. HSA-LNFPIII induced a significant HSA-specific IgG and IgE production in BALB/c mice when administered i.p. (Fig. 8, *A* and *B*). Furthermore, levels of Abs in HSA-LNFPIII-immunized mice were comparable to those immunized with HSA adsorbed with alum, the classical Th2-inducing adjuvant (17) (Fig. 8, *A* and *B*). Similar results were observed when mice were immunized s.c. although the levels of IgE were lower as compared with those observed after i.p. immunization (Fig. 8, *C* and *D*). Furthermore, mice immunized i.p. or s.c. with HSA-LNFPIII adsorbed with alum displayed an increased production of both specific IgG and IgE as compared with mice immunized with either HSA adsorbed with alum or HSA-LNFPIII by same routes.

Priming effect of LNFPIII on Ab production following subsequent immunization with nonconjugated protein.

Finally we sought to determine whether the priming with LNFPIII-conjugated protein would lead to an increase in Ab production against protein following the subsequent immunization with nonconjugated protein. BALB/c mice primed with HSA-LNFPIII and subsequently boosted and challenged with HSA alone produced significantly higher amounts of both HSA-specific IgG and IgE following the nasal challenge as compared with those following the primary immunization. In addition, these mice produced significantly higher amounts of specific IgG and IgE following the challenge as compared with mice immunized with HSA alone throughout the study (Fig. 9).

Discussion

The observations presented in this study clearly demonstrate that the lactosamine sugar LNFPIII conjugated to HSA acts as a Th2-type adjuvant and induces HSA-specific IgG1 and IgE Ab production. Moreover, the dose of HSA-LNFPIII (1 μg) required to induce HSA-specific Ab production was 1000-fold lower than HSA (1000 μg) alone. Additionally, even at such a high dose of HSA itself did not induce HSA-specific IgE production. Competitive inhibition studies revealed that HSA but not LNFPIII completely

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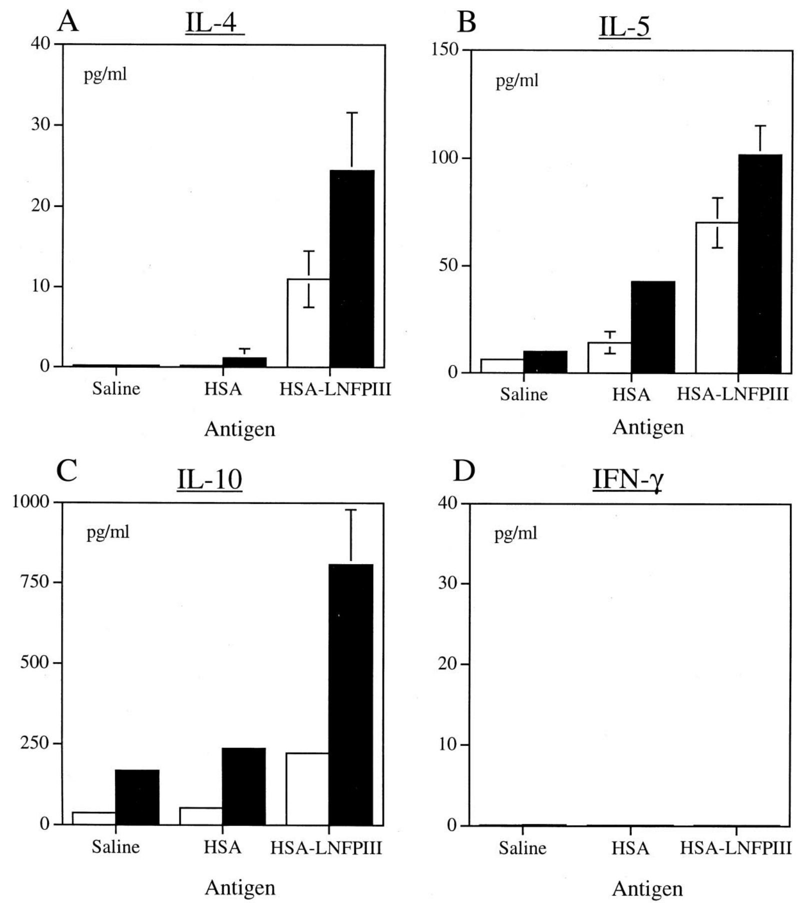

FIGURE 6. Production of IL-4 (*A*), IL-5 (*B*), IL-10 (*C*), and IFN-γ (*D*) by nasal lymphocytes from BALB/C mice immunized intranasally with HSA, HSA-LNFPIII or saline. Nasal lymphocytes were isolated as described in *Materials and Methods* and restimulated in vitro with 10 μg/ml of HSA (□) or HSA-LNFPIII (■) for 48 h. Cytokines were determined by ELISA. Results show the mean ± SEM of four different experiments. Detection limit was 6 pg/ml, 20 pg/ml, 40 pg/ml, and 20 pg/ml for IL-4, IL-5, IL-10, and IFN-γ, respectively.

inhibited Ab binding to HSA-LNFPIII-coated plates in a dose-dependent fashion, indicating that the LNFPIII was not an epitope for Ab. These findings demonstrate that as an adjuvant, LNFPIII clearly functions in a manner different from the conventional "hapten-carrier" conjugate, where protein acts as the carrier to stimulate T cell activation to provide "help" for Ab production against hapten. Furthermore, these results provide further evidence that the process of conjugating LNFPIII to protein Ag such as HSA does not significantly alter its antigenicity.

Several studies have demonstrated that SEA are potent inducers of a Th2-like response that is associated with an increased Th2-type cytokine and IgE production, and eosinophilia (18, 19). LNFPIII is one of the oligosaccharides that is present on SEA and is also found in human milk (13, 20). We recently demonstrated that

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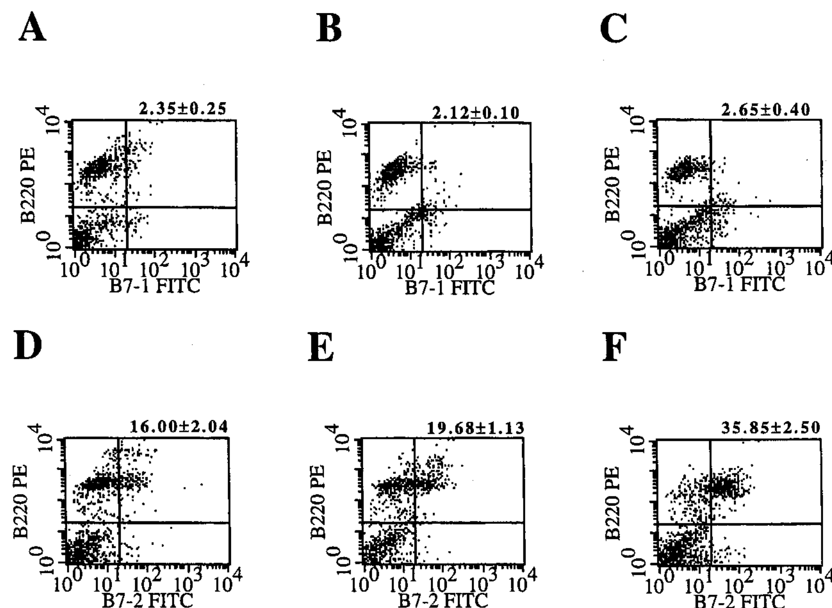

FIGURE 7. Expression of CD80 (*A–C*) and CD86 (*D–F*) on B220+ nasal lymphocytes. Following 48 h in vitro recall stimulation with the same Ag as immunization; saline (*A* and *D*), HSA (*B* and *E*), or HSA-LNFIII (*C* and *F*), respectively, cell pellets were collected and stained by anti-CD80/CD86 and B220 mAbs coupled to FITC or PE, respectively, for flow cytometry analysis. Values given in the *upper right quadrant* indicate the mean percentage ± SEM of double-positive cells from four different experiments.

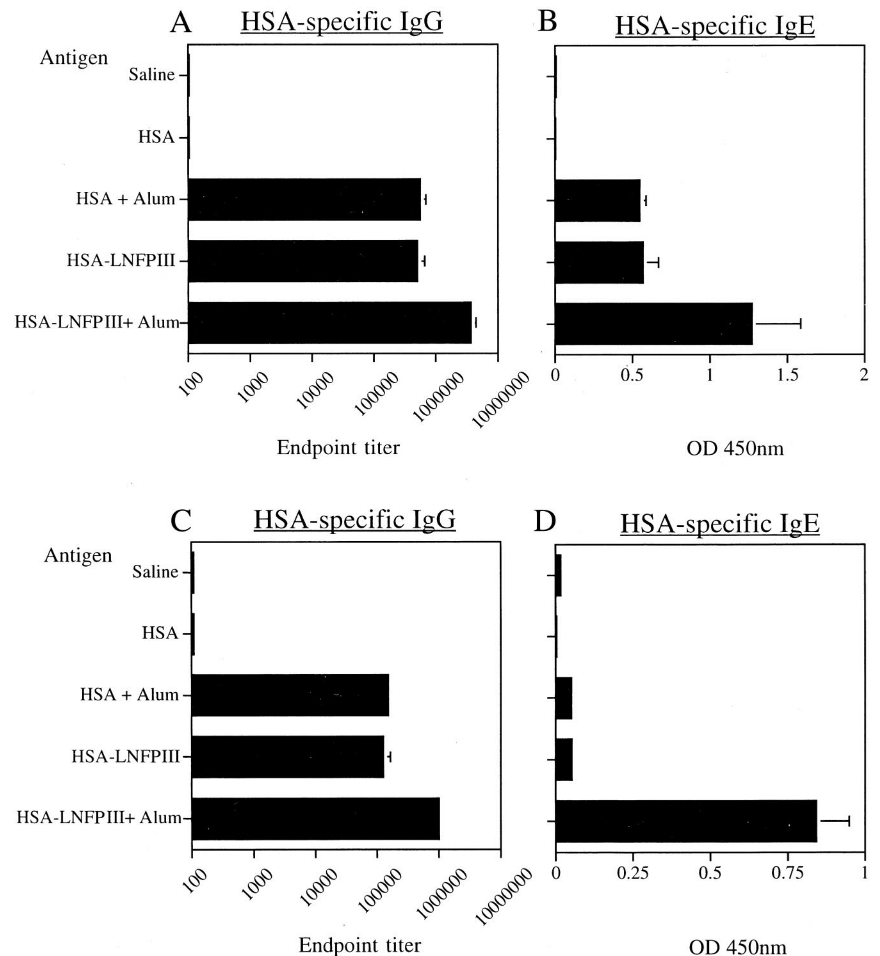

FIGURE 8. In vivo Ab production after i.p. (*A* and *B*) and s.c. (*C* and *D*) immunization with HSA, HSA adsorbed to alum, HSA-LNFPIII, or HSA-LNFPIII adsorbed to alum. Serum samples were taken 1 wk after the boost immunization as described in *Materials and Methods*. Levels of IgG (*A* and *C*) specific for HSA were determined by indirect ELISA and presented as endpoint titer. Levels of HSA-specific IgE (*B* and *D*) were determined by sandwich ELISA and presented as the absorbance at 450 nm from duplicate wells of 1/4 serum dilution. Results show the mean ± SEM of four individual serum per group. Data are representative of two separate experiments.

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the carbohydrates present on SEA not only play a critical role in induction of Th2 response but LNFPIII itself induces IL-10 and PGE2 production by B cells in vitro (9). In the present study, BALB/c mice immunization with HSA-LNFPIII produced significantly higher levels of total IgE and HSA-specific IgE and IgG1 as compared with those immunized with HSA alone. Moreover, nasal lymphocytes from HSA-LNFPIII-immunized mice produced significantly more IL-4, IL-5, and IL-10, but not IFN-γ, in response to re-stimulation with HSA-LNFPIII or HSA. Taken together, these findings suggest that LNFPIII when conjugated to a protein Ag acts as an adjuvant and induces Ag-specific Th2-type Ab and cytokine productions in murine intranasal immunization model. The ability of nasal lymphocytes from HSA-LNFPIII-immunized mice to produce significant amount of Th2 cytokines in response to in vitro antigenic stimulation also suggests that that these nasal lymphocytes may be the effector cells responsible for induction of Th2 type response in this model. Interestingly, adjuvant activity of LNFPIII via intranasal route was also comparable to alum administered via i.p. route, which is the classical Th2-inducing adjuvant but difficult to use in mucosal immunization. Moreover, HSA-LNFPIII adsorbed with alum significantly enhanced Ab production indicating that these two adjuvants have a synergic effect. Nevertheless, we also found that HSA-LNFPIII induced a significant production of HSA-specific Th2-associated isotypes following i.p. as well as s.c. immunization, indicating that the adjuvant activity of LNFPIII is not dependent on the route of immunization.

Nasal lymphocytes from mice immunized with HSA produced significantly higher amounts of IL-5 and IL-10, but not IL-4, in response to HSA-LNFPIII as compared with HSA alone. And interestingly, the lymphocytes from mice immunized with saline also produced IL-5 and IL-10 in response to HSA-LNFPIII, suggesting that HSA-LNFPIII has an ability to enhance both IL-5 and IL-10 production in vitro. At least two possibilities can be thought. One, HSA peptides are produced more effectively from HSA-LNFPIII by APC as compared with HSA alone. Second, LNFPIII directly activate naive cells (e.g. macrophages, T cells, and B cells) to produce these cytokines. In fact, we have previously reported that LNFPIII induce IL-10 production by B cells (9, 10). In addition, these results may arise the possibility that LNFPIII or its related carbohydrates may lead to a nonspecific immune responses including polyclonal Ig synthesis, and the investigations are currently undertaken.

There are several mechanisms that may explain the adjuvant activity of LNFPIII. First, LNFIII may simply enhance Ag uptake by APC through binding to one of several APC receptors specific for carbohydrates. This can be explained by the results presented here that only the conjugated LNFIII but not free form of the sugar acts as an adjuvant. For example, macrophages and dendritic cells can express lectin-like receptors such as mannose receptor or mannose-binding lectin receptor, which bind to carbohydrates expressed on pathogens (21, 22). Our result that HSA linked to LNFIII but not nonfucosylated LNnT induced Th2 type Ab and cytokine production in intranasal immunization seems to be consistent with the function of this receptor, and an investigation of the interaction between LNFIII and mannose receptor is now underway. A second explanation is that LNFIII activates cells through binding to P-selectin although this sugar is a weak ligand

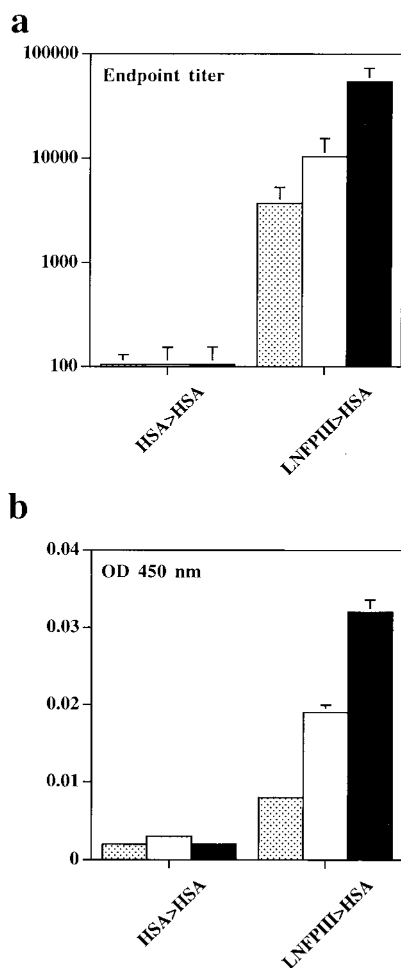

FIGURE 9. Priming effect of HSA-LNFPIII on Ab production following subsequent immunizaiton with HSA. A group of BALB/c mice was primed intranasally with HSA-LNFPIII and subsequently boosted and challenged with HSA alone in an identical manner as described in *Materials and Methods*. As a control, a group of mice was immunized with HSA alone throughout the experiment. Plasma samples were taken after prime (▨), boost (□), and challenge (■). Titers of IgG (*A*) and IgE (*B*) specific for HSA were presented as the endpoint titer and the absorbance at 450 nm from duplicate wells of 1/4 serum dilution, respectively. Results show the mean ± SEM of four individual serum per group. Data are representative of two separate experiments.

for this molecule (3). Third, LNFIII binds to an as yet uncharacterized receptor specific for α1–3 linked fucose. In agreement with this hypothesis is our observation that Lewis^{x}, but not other fucose-containing Lewis-family sugars are capable of direct binding to T and B cells as well as macrophages (our unpublished data). Alternatively, this sugar-protein conjugation may be involved in a selective cleavage of peptides by cathepsins in lysosome, which are known to induce selective immune responses (23).

In the present study, intranasal immunization of mice with HSA conjugated with LNnT (HSA-LNnT), the nonfucosylated homologue of LNFPIII, failed to increase total or HSA-specific IgE and IgG above baseline, suggesting that the fucose residue of LNFPIII is required for such an adjuvant effect. However, LNFI, another homologue of LNFIII that contains fucose α1–2 linked to galactose, also failed the significant Ab production. In addition, we previously found that carbohydrate-containing α1–3 fucose on phospholipase A2 of honey bee venom had no effect of adjuvant activity on Th2-type Ab production (15). Collectively, these findings indicate that the composition, type of conjugation, and subsequent tertiary structures of linked oligosaccharides might be important for adjuvant activity of fucose residue.

The lack of IgM component in the serum anti-HSA is another feature of LNFPIII conjugates on humoral immune responses. In addition, HSA-specific IgG production was produced even after a single immunization (Fig. 1, *A* and *B*). Although the precise mechanisms remain to be elucidated, one possibility can be arisen that LNFPIII conjugate may directly stimulate a memory pool of B cells.

Costimulatory molecules are known to be important for the activation, proliferation and cytokine production of Ag-specific T cells (16). Among these, the B7 family CD80 (B7-1) and CD86 (B7-2) and their counter-receptors on T cells, CD28, and CD152 (CTLA4) are the most extensively characterized and appear to play a role in Th1/Th2 differentiation and/or maintenance (23, 24). In allergic patients who have already been primed with allergens, we and others have found that CD86 is selectively up-regulated in peripheral blood B cells in response to allergen (25). Furthermore, Van Neerven et al. (26) reported that allergen-specific T cell proliferation and cytokine expression require CD28-CD86 costimulation. Our results that B220^{+} nasal lymphocytes from mice immunized with HSA-LNFPIII display a significant increase in expression of B7-2, but not B7-1 indicate that LNFPIII activates B cells led to the up-regulation of B7-2 expression that may favor Th2 development.

In summary, we found that lactosamine sugar LNFPIII when conjugated to a protein Ag is capable of enhancing protein-specific Th2 responses in vivo. Moreover, LNFPIII functioned in a dramatic fashion compared with reports on another carbohydrate adjuvant, pullulan (polymer of α-glucose), which suppressed Ag specific IgE while enhancing Ag-specific IgG (27). In addition, route of immunization did not alter adjuvant activity of LNFPIII, and coadministration of this carbohydrate together with alum synergistically enhanced adjuvant activity of alum. These results suggest that LNFPIII can be used as a novel adjuvant to enhance Th2-type immune responses in vaccines.

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