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Targeted Delivery of Antigen to Hamster Nasal Lymphoid Tissue with M-Cell-Directed Lectins

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The nasal cavity of a rodent is lined by an epithelium organized into distinct regional domains responsible for specific physiological functions. Aggregates of nasal lymphoid tissue (NALT) located at the base of the nasal cavity are believed to be sites of induction of mucosal immune responses to airborne antigens. The epithelium overlying NALT contains M cells which are specialized for the transcytosis of immunogens, as demonstrated in other mucosal tissues. We hypothesized that NALT M cells are characterized by distinct glycoconjugate receptors which influence antigen uptake and immune responses to transcytosed antigens. To identify glycoconjugates that may distinguish NALT M cells from other cells of the respiratory epithelium (RE), we performed lectin histochemistry on sections of the hamster nasal cavity with a panel of lectins. Many classes of glycoconjugates were found on epithelial cells in this region. While most lectins bound to sites on both the RE and M cells, probes capable of recognizing α-linked galactose were found to label the follicle-associated epithelium (FAE) almost exclusively. By morphological criteria, the FAE contains >90% M cells. To determine if apical glycoconjugates on M cells were accessible from the nasal cavity, an M-cell-selective lectin and a control lectin in parallel were administered intranasally to hamsters. The M-cell-selective lectin was found to specifically target the FAE, while the control lectin did not. Lectin bound to M cells in vivo was efficiently endocytosed, consistent with the role of M cells in antigen transport. Intranasal immunization with lectin-test antigen conjugates without adjuvant stimulated induction of specific serum immunoglobulin G, whereas antigen alone or admixed with lectin did not. The selective recognition of NALT M cells by a lectin in vivo provides a model for microbial adhesin-host cell receptor interactions on M cells and the targeted delivery of immunogens to NALT following intranasal administration.

Intranasal (i.n.) immunization has been explored extensively in rodent models as a method to induce antibody production in serum and mucosal secretions (34, 54, 63). Immune protection from oral and respiratory pathogens may be most effectively elicited following i.n. vaccination (5, 27, 57, 61). The induction of antibody responses following i.n. immunization of rodents likely occurs in organized lymphoid tissue found at the base of the nasal cavity known as nasal lymphoid tissue (NALT) (33, 53). The cellular constituents of NALT in rats include both B and T lymphocytes, macrophages, and follicular dendritic cells (32). The organization of NALT, however, often does not include germinal centers, bringing the role of NALT in immunological memory into question (33). In rats and rabbits, similar lymphoid aggregates termed bronchus-associated lymphoid tissue (BALT) are present deeper in the respiratory tract at the branch points of airways (6, 43). BALT may serve as the primary antibody inductive site for humoral protection of the bronchi and lower airways.

Very little is known about the role of the epithelial lining of the nasal cavity in the sampling of immunogens following i.n. administration. A more complete understanding of the nasal cavity epithelium may lead to improved strategies for i.n. immunization. Within the rodent nasal cavity, distinct types of epithelia are found in different regions. Keratinized stratified squamous epithelium dominates the most proximal regions (2). Further into the nasal cavity, following a transition zone, respiratory epithelium (RE) containing pseudostratified ciliated columnar cells and goblet cells is found only on the ventral surfaces of the cavity lining the entrance to and the length of the nasopharyngeal duct (NPD) (59). The NPD empties at the nasopharynx into the pharynx. Accordingly, i.n. administered antigens, if given in high doses, can be found in the intestinal tract (20, 60). Because rodents do not breath through their mouths, all air must pass through the NPD into the pharynx. The nasal inductive sites (NALT) located on the ventral surface of the nasal cavity near or within the NPD are in the ideal position to maximize exposure to airborne antigens.

As found in other mucosal inductive sites, a specialized follicle-associated epithelium (FAE) containing M cells is found overlying NALT (53). M cells are responsible for sampling and transporting antigens to the underlying lymphoid tissue (reviewed recently in reference 40). While adherent particulate antigens are efficiently sampled by M cells overlying NALT and are not taken up by ciliated cells, soluble antigens may traverse the RE (33). This observation suggests that microorganisms may be sampled exclusively by M cells in the nasal cavity.

The biochemical features of M cells that distinguish this lineage from other cellular residents of the epithelium are largely unknown. The relative infrequency of M cells and the difficulty of obtaining enriched M-cell populations have so far precluded the generation of cell lines that exhibit the M-cell phenotype. Morphological criteria, such as short microvilli and a basolateral membrane invagination containing lymphocytes and macrophages, are often used to identify M cells. Other criteria include the differential expression of brush border hydrolases (41, 51) and vimentin in rabbit M cells (16, 23).

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TABLE 1. Lectins and antibodies used in this study

Lectin or antibody	Specificity[a]	Epitope(s)[a]	Reference
MAL I (*Maackia amurensis* I)	Terminal galactose	Galβ(1-3)GlcNAc; Neu5Acα(2-3)Gal	29
Jacalin (*Artocarpus integrifolia*)		Galβ(1-3)GalNAc; Galα(1-6)Gal	49
PNA (*Arachis hypogaea*)		Galβ(1-3)GalNAc	56
ECA (*Erythrina cristagalli*)		Galβ(1-4)GlcNAc	26
GS I-B_4 (*Griffonia simplicifolia* I isolectin-B_4)		Galα(1-3)Gal	38
Anti-blood group B (clone 3E7)		Galα(1-3)[Fucα(1-2)]Gal	7
DBA (*Dolichos biflorus*)	Terminal GalNAc	GalNAcα(1-3)Gal	47
WFA (*Wisteria florabunda*)		GalNAcα/β	4
WBA I (*Psophocarpus tetragonolobus* I)		GalNAcα	25
STA (*Solanum tuberosum*)	GlcNAc	$(GlcNAc\beta)_{2-3}$	35
WGA (*Triticum vulgaris*)		$(GlcNAc\beta)_{2-5}$; Neu5Ac	3
UEA-II (*Ulex europaeus* II)		$(GlcNAc\beta)_2$	55
SNA (*Sambucus nigra*)	Sialic acid	Neu5Acα(2-6)Gal/GalNAc	50
MAL II (*Maackia amurensis* II)		Neu5Acα(2-3)Gal/GalNAc	28
PSA (*Pisum sativum*)	Mannose	Manα	31
UEA-I (*Ulex europaeus* I)	Fucose	Fucα(1-2)Gal	45
LTA (*Lotus tetragonolobus*)		Fucα(1-2)Galβ(1-4)[Fucα(1-3)]GlcNAc	44
OPA (*Aleuria aurantia*)		Fucα(1-6/3)GlcNAc	12
EEA (*Euonymus europaeus*)	Complex	Galα(1-3)[Fucα(1-2)]Galβ(1-3/4)GlcNAc; Galα(1-3)Galβ(1-4)GlcNAc	46
WBA II (*Psophocarpus tetragonolobus* II)		Fucα(1-2)Gal; Galβ(1-3)GlcNAc	1
SJA (*Sophora japonica*)		Galβ(1-3/4)R; GalNAcβ(1-6)Gal; Galα(1-3)Gal	4

[a] GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Neu5Ac, *N*-acetylneuraminic acid. A slash means or.

Recent studies have revealed that mouse intestinal M cells can be distinguished from neighboring enterocytes by the expression of distinct oligosaccharide epitopes revealed by lectin binding. Mouse Peyer's patch M-cell membranes were found to selectively display glycoconjugates bearing terminal α(1-2)-linked fucose (8, 13, 17). Furthermore, lectins and carbohydrate-specific antibodies directed against various epitopes bearing α(1-2)-linked fucose labeled subpopulations of M cells in a given FAE, demonstrating differences among cells of this lineage (17). Glycoconjugate expression by M cells in the large intestine of mice also distinguishes these cells from enterocytes but by a different class of carbohydrate epitopes bearing terminal α(1-3)-linked galactose (9, 17). These findings demonstrated the regional heterogeneity of M-cell glycoconjugates in different intestinal compartments while underscoring a common theme of selective expression of carbohydrate epitopes by mouse intestinal M cells.

The functional role of M-cell-specific glycoconjugates is unknown, but it is possible that they serve as receptors for adhesins of enteric pathogens which exploit M-cell transepithelial transport to gain access to the lamina propria (39, 42). An area of considerable applied interest is the use of M-cell-directed lectins to target the delivery of mucosal vaccines to inductive sites in the gut (10, 17).

In light of recent interest in i.n. immunization as a strategy to induce protective humoral immunity to a variety of mucosal pathogens, we have investigated the pattern of glycoconjugate expression by M cells overlying NALT in the hamster. We wished to determine if NALT M cells could be characterized by distinct glycoconjugate receptors which might influence uptake and immune responses to adherent antigens. We describe here the differential expression of carbohydrate epitopes by NALT M cells and provide a model for the targeted delivery of vaccine molecules to nasal lymphoid tissue.

MATERIALS AND METHODS

Preparation of nasal tissue containing NALT. Six- to 8-week-old female Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories (Wilmington, Mass.) and fed a standard laboratory diet ad libitum. Animals were anesthetized with isoflurane inhalant (Aerrane; Ohmeda PPD Inc., Liberty Corner, N.J.) and euthanized by cervical dislocation. The lower jaw was removed, and by using the hard palate as a guide (64), the snout was removed with a transverse cut behind the back molars with a large scalpel. After removal of skin and excess soft tissue, phosphate-buffered saline (PBS) (pH 7.2) was flushed through the external nares to wash out any blood within the nasal cavity. This was repeated briefly with ice-cold fixative (3% formaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate, pH 7.2) before immersing the snout in fixative at 4°C for 5 h with one change of fixative. Snouts were rinsed twice (10 min each) in cold 0.1 M cacodylate buffer (pH 7.2) and decalcified in 0.4 M EDTA (pH 7.2) on a rocker table at 4°C for 10 to 14 days with several changes of EDTA solution to sufficiently soften bone for trimming (37). After two washes with PBS (10 min each), tissue was trimmed under a dissecting microscope. After the NPD was located at the base of the posterior face of the nasal cavity, the snout was frontally sectioned to isolate the lower portion containing the NPD. Samples of the dorsal regions of the cavity were also retained. After the hard palate was placed downward, ~2-mm-thick slices were made from posterior to anterior, noting the corresponding hard palate landmark for each slice. Samples were taken posterior to the incisive papilla (59, 64) and were further trimmed to localize the septal window and NPD. All tissues were dehydrated through a graded ethanol series followed by propylene oxide and embedded in Epon-Araldite 6005. Trimmed blocks were sectioned so as to be 0.5 μm thick and stained with 1% toluidine blue to determine tissue orientation.

Lectin histochemistry of nasal tissue. The lectins and antibodies used in this study, along with their specificities, are listed in Table 1. Lectin-biotin or -fluorescein isothiocyanate (FITC) conjugates were purchased from Vector Laboratories (Burlingame, Calif.). Unconjugated lectins and lectin-horseradish peroxidase (HRP) conjugates were obtained from Sigma Chemical Co. (St. Louis, Mo.). Monoclonal mouse immunoglobulin M (IgM) specific for human

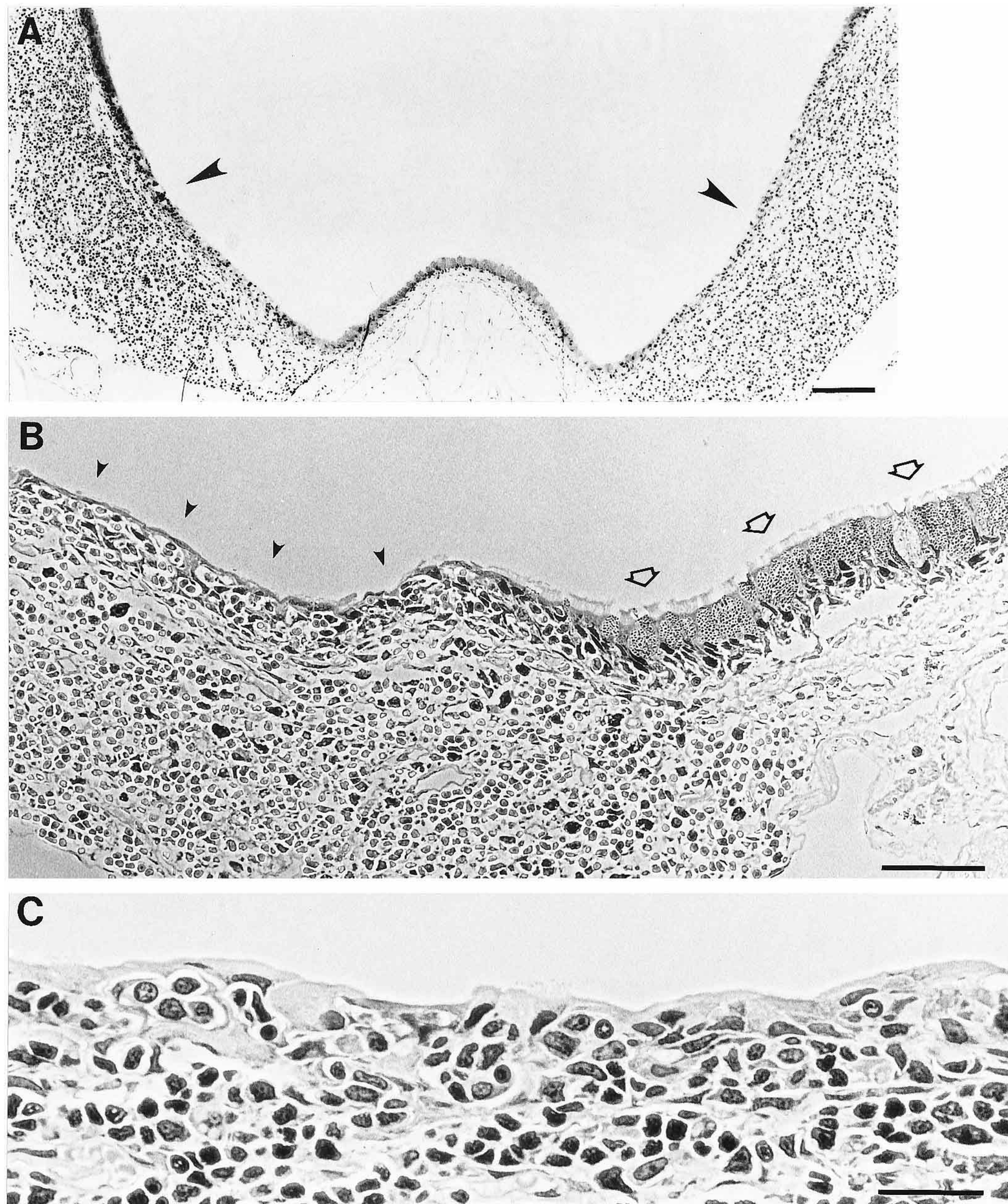

FIG. 1. Localization and morphology of hamster NALT. (A) Aggregates of lymphoid tissue are found at ventrolateral locations (arrowheads) at the entrance to and within the NPD (first palatal ridge shown; 0.5-μm-thick section, toluidine blue stain). Bar, 100 μm. (B) The RE (open arrowheads) with ciliated columnar cells and goblet cells dominates this region of the nasal cavity. The epithelium overlying NALT (small solid arrowheads) is largely devoid of these cells. Bar, 50 μm. (C) Higher-magnification view of the cellular constituents of the FAE. Most cells have flattened surfaces and an abundance of intraepithelial lymphoid cells; both are typical features of M cells. Bar, 20 μm.

blood group B antigen was purchased from DAKO Corp. (Carpinteria, Calif.). Streptavidin-FITC or -tetramethylrhodamine (TRITC) was purchased from Molecular Probes (Eugene, Oreg.). FITC- or TRITC-conjugated goat anti-mouse IgM was acquired from American Qualex (La Mirada, Calif.). The fluorochrome-conjugated antibodies did not react with hamster tissue without their cognate primary antibody (not shown).

Labeling of semithin sections was performed as described previously (17). Epoxy resin was removed with melting solution (2 g of potassium hydroxide, 10 ml of methanol, 5 ml of propylene oxide) (36). Unstained sections on glass slides were covered with the solution for 4 to 5 min, rinsed with 100% methanol and distilled H_2O, and air dried. Unreactive aldehydes in tissue were quenched with 50 mM NH_4Cl in PBS for 10 min, followed by blocking of nonspecific protein binding with 0.2% gelatin (type A-porcine skin) in PBS for 30 min in a humid chamber. Lectin conjugates and the mouse monoclonal antibody were diluted to 10 and 2 μg/ml, respectively, in gelatin-PBS. Tissue sections were incubated with lectins or antibody for 60 min at room temperature. Slides were washed three times (5 min each) with gelatin-PBS and then incubated for 30 min with either streptavidin-fluorochrome or secondary goat anti-mouse IgM–fluorochrome diluted to 2 μg/ml in gelatin-PBS. Slides were briefly washed in PBS and distilled H_2O, and coverslips were mounted with Moviol (Calbiochem Corp., San Diego, Calif.) containing 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma). Photography was performed with a Zeiss Axioskop microscope equipped for epifluorescence and Kodak T-Max 400 film.

Specificity of lectin binding was determined by preincubating lectins with their cognate monosaccharide or a control irrelevant monosaccharide at 0.2 M for 2 h before incubation of lectins with tissue sections. The lectins and monosaccharides used for control experiments included GS I-B_4 (D-galactose; Sigma), SJA (D-GalNAc; Sigma), and Jacalin (D-galactose; Sigma).

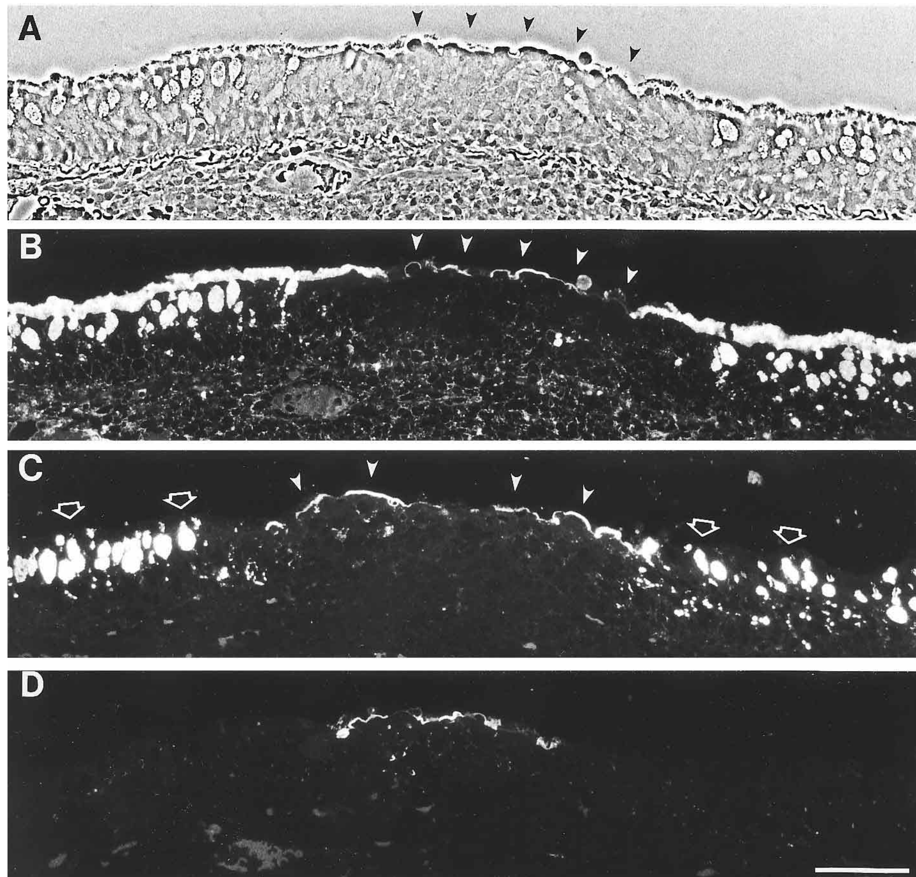

FIG. 2. Distribution of epithelial lectin-binding sites in FAE and RE. Sections of hamster nasal tissue containing NALT were incubated with lectins which recognize distinct classes of oligosaccharide epitopes, and their binding distribution was analyzed by fluorescence microscopy. (A) A phase-contrast image of epithelium with FAE at center (arrowheads). (B) The same section stained with the sialic acid-specific lectin SNA shows strong labeling of ciliated cell surfaces as well as mucin granules of goblet cells of the RE flanking the relatively sparsely stained FAE (arrowheads). Follicle cells are uniformly stained with lower intensity. (C) Neighboring sections were labeled with the GalNAc-specific lectin DBA, which strongly labels goblet cell mucin granules (open arrowheads) but not ciliated cell surfaces of the RE. The thinner M-cell surfaces (small solid arrowheads) are strongly stained in many cases. The FAE in this section is broader than in panels A, B, and D. (D) Staining of sections with the α-linked galactose-specific lectin GS I-B_4 shows epithelial binding restricted to a subpopulation of M cells in the FAE and to a few rare ciliated cells scattered in the RE. Bar, 50 μm.

i.n. administration of lectins. Biotin conjugates of GS I-B_4 and WGA lectins were reconstituted to 1 mg/ml in PBS. Each lectin conjugate (100 μg) was administered to hamsters by the i.n. route without anesthesia in two 50-μl doses spaced 5 min apart. Doses were delivered with a micropipette and were divided equally between both nostrils. After 1 h, animals were euthanized and tissues were processed as described above. Lectin-biotin conjugates were detected in extracted semithin tissue sections with fluorochrome-labeled streptavidin. To determine the relationship between the distribution of resident lectin-binding sites and the fate of administered lectin-biotin conjugates, sections were labeled with lectin-FITC conjugates and with streptavidin-TRITC simultaneously.

i.n. immunizations with lectin-HRP conjugates. Preimmunization samples of blood, feces, and saliva were taken from all hamsters. HRP and HRP conjugates of GS I-B_4 and WGA were reconstituted in PBS and used as antigens. Groups of five hamsters were given 25 μg each of GS I-B_4–HRP, WGA-HRP, HRP alone, GS I-B_4 admixed with HRP (25 μg each), or PBS (control) in 50-μl volumes without adjuvant, as described above. Immunizations were given as four weekly doses on days 0, 7, 14, and 21 followed by collection of postimmunization samples on day 28.

HRP-specific antibody titers in sera, feces, and saliva were measured by indirect enzyme-linked immunosorbent assay. Microtiter plates (Corning-Costar, Cambridge, Mass.) were coated with 100 μl of HRP at 1 μg/ml in 0.1 M carbonate-bicarbonate buffer (pH 9.8) overnight at 4°C. After the samples were washed twice with PBS-Tween (PBS, 0.05% Tween 20) and blocked with 2.5% nonfat dry milk in PBS-Tween for 1 h at 37°C, twofold serial dilutions of samples in duplicate were prepared and incubated for 1 h at 37°C. A 1:100 starting dilution was employed for sera, and a 1:10 starting dilution was used for saliva and fecal extracts. Following four washes with PBS-Tween, bound antibody was detected after a 1-h incubation of either goat anti-hamster IgG-alkaline phosphatase (AP) conjugate (Southern Biotech Associates, Birmingham, Ala.) or rabbit anti-hamster IgA (prepared at OraVax, Inc., using hamster colostral IgA as immunogen) followed by goat anti-rabbit IgG-AP (Southern Biotech). After six washes, ρ-nitrophenyl phosphate substrate (1 mg/ml; Sigma) in 0.1 M diethanolamine buffer (pH 9.6) containing 0.5 mM $MgCl_2$ was incubated on plates at room temperature for 20 min, and optical densities were recorded on a Vmax plate reader (Molecular Devices) at 405 nm.

RESULTS

Localization of hamster NALT. In order to prepare nasal tissue containing NALT, excised hamster snouts were fixed, washed, and decalcified in EDTA at 4°C for a minimum of 10 days. This slow decalcification method (a modification of the method described in reference 37) yields excellent preservation of fixed tissue and is superior to the more rapid decalcification in acid. Examination of sections of the ventral region of the nasal cavity allowed visualization of the septal window and the anterior portion of the NPD, which has a diameter of ~1.5 mm. Situated at discrete ventrolateral locations in the septal window and the NPD were paired lymphoid aggregates (NALT) (Fig. 1A). By using the hard palate as a landmark, NALT extended from approximately the first palatal ridge to the posterior molars. The epithelium overlying the lymphoid tissue was almost completely devoid of the

TABLE 2. Lectin- and antibody-binding patterns in hamster nasal tissue containing NALT

Specificity	Lectin[a] or antibody	Staining frequency and intensity[b]				Follicle cells
		FAE		RE		
		Apical	Subcellular[c]	Ciliated cells	Goblet cells	
GalNAc	DBA	++3	++1	−	+++3	+3
	WFA	++2/3	++2	++2/3	+++2	+3
	WBA I	++3	++3	++++1	+++3	+1
GlcNAc	WGA	+++2/3	+++2/3	++3	+++3	++3
	STA	+++3	+++3	+++3	+++2	+++3
	UEA II	++3	++1	+++2	+++2	−
Sialic acid	SNA	++3	−	+++3	+++3	+++2
	MAL II	+++2/3	+++2/3	+++2	−	+++2/3
Mannose	PSA	+++2/3	+++2/3	+++2/3	+++2/3	+++2/3
Fucose	UEA I	−	++1	−	++3	−
	LTA	−	−	−	−	−
	OPA	+++2	+++2	+++2	+++2/3	+++2
Galactose	MAL I	−	−	++1/2	−	++2
	PNA	++3	++1	+1	++3	−
	Jacalin	+++3	+++3	+++3	+++2/3	+++3
	ECA	++2	−	++2	++2	−
	GS I-B_4	++2/3	++1/2	+/−3	−	+2
	Anti-blood group B	++2/3	++1	−	−	−
Complex	WBA II	−	−	−	−	−
	EEA	++2	++1/2	−	−	−
	SJA	++2	++1/2	−	+++2	−

[a] See Table 1 for lectin abbreviations.
[b] Staining frequency: +++, all cells; ++, subpopulation of cells; +, infrequent cells; +/−, rare cells. Staining intensity: 3, strong; 2, moderate; 1, weak; 2/3, moderate to strong; 1/2, weak to moderate.
[c] Subcellular: basolateral and/or intracellular vesicular membranes.

ciliated columnar cells and goblet cells which comprise the RE in this region (Fig. 1B). Instead, the FAE was largely (>90%) composed of cells with flattened apical surfaces containing an abundance of intraepithelial lymphoid cells (Fig. 1C). These morphological characteristics combined with their exclusive location overlying NALT are consistent with the M-cell phenotype characterized in various mucosal tissues.

Lectin staining of hamster NALT. To determine whether the NALT M-cell lineage could be distinguished from other cell types of the hamster RE by selective expression of lectin-binding sites, a panel of lectins (Table 1) was applied to sections of hamster nasal cavity tissue containing NALT. As shown in Table 2, the RE demonstrated an abundance of lectin-binding sites. Although both ciliated and goblet cells were labeled by many lectins of various specificities, clear differences in the expression of certain lectin-binding sites were found. For example, the sialic acid-specific lectin SNA strongly stained the long cilia of ciliated cells, as well as mucin granules of goblet cells, M-cell apical membranes, and follicle cell membranes (Fig. 2B). However, the GalNAc-specific lectin DBA showed strong staining of goblet cell mucin granules but did not label ciliated cells (Fig. 2C). DBA also stained the apical surfaces of a subpopulation of M cells as well as follicle cells infrequently. In contrast, the α-linked galactose-specific lectin GS I-B_4 demonstrated epithelial binding largely restricted to the FAE, where a subpopulation of M cells were labeled (Fig. 2D). Only rarely did ciliated cells show GS I-B_4 binding. Within the follicle, GS I-B_4-positive cells were found infrequently.

Another sialic acid-specific lectin, MAL II, showed an abundance of binding sites on apical membranes of many ciliated cells as well as M cells of the FAE. Strong staining of lymphoid cells within and adjacent to the follicle was consistently observed, whereas goblet cell mucins were negative (Fig. 3A). Other probes reported to recognize glycoconjugates containing terminal α(1-3)-linked galactose were similarly tested. A lectin (SJA) with complex binding characteristics which can recognize terminal α-linked galactose stained a subpopulation of M cells as well as goblet cell mucins (Fig. 3B). A monoclonal antibody specific for blood group B antigen demonstrated a binding pattern similar to that of GS I-B_4. This antibody stained a subpopulation of M cells in the FAE, while no staining of RE was observed (Fig. 3C). EEA lectin, which can bind to terminal α-linked galactose, selectively labeled a subset of M cells in the FAE (not shown). No other class of probe demonstrated binding which was restricted to the FAE (Table 2). These results indicate that the hamster NALT M-cell lineage can be distinguished from other epithelial cells in this region of the nasal cavity by the expression of glycoconjugates possessing terminal α(1-3)-linked galactose.

i.n. administration of NALT M-cell-selective lectin. To test whether a NALT M-cell-selective lectin could be targeted to NALT M cells following i.n. administration, GS I-B_4 lectin or a control lectin (WGA) which recognized epitopes common to

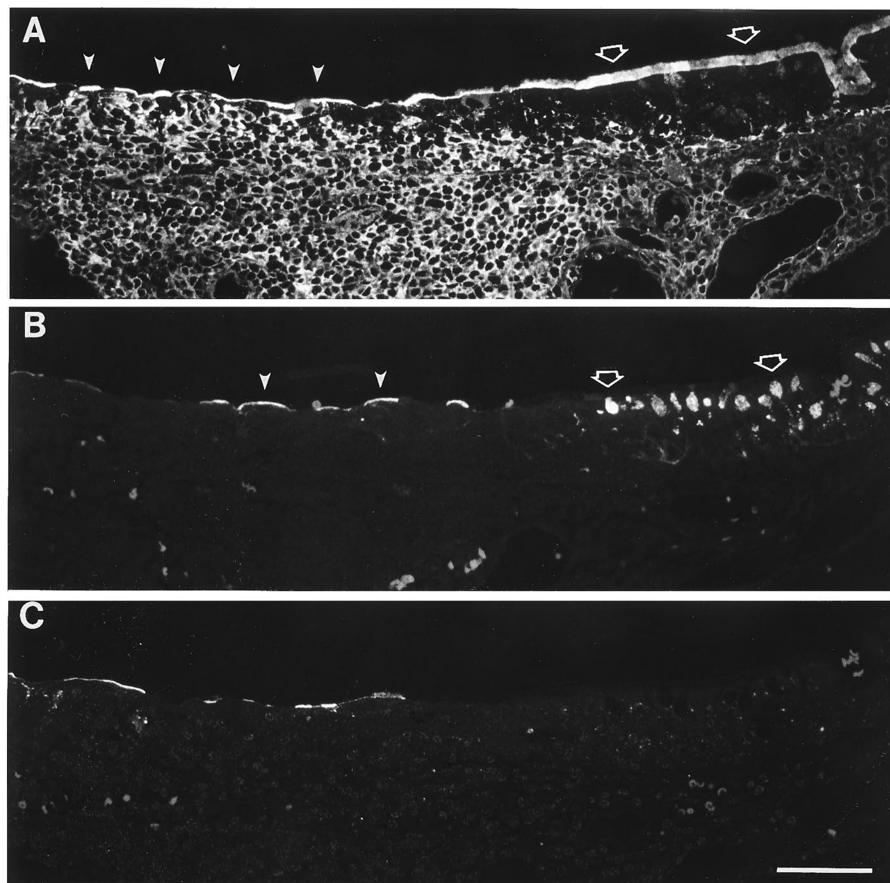

FIG. 3. Distribution of epithelial lectin- or antibody-binding sites in FAE and RE. (A) A nasal tissue section incubated with another sialic acid-specific lectin (MAL II) shows ciliated cell surfaces (open arrowheads) within the RE stained with different intensities, while goblet cell mucin granules are unlabeled. The FAE (small solid arrowheads) also demonstrates nonuniform staining intensity. Lymphoid cells in the lamina propria and follicle are strongly labeled with this lectin. Note the infiltration of lymphoid cells into the FAE. (B) A neighboring section was labeled with a lectin (SJA) capable of recognizing α-linked galactose as well as other epitopes. SJA-binding sites are found in goblet cell mucins (open arrowheads) and on the membranes of a subpopulation of M cells (small solid arrowheads). (C) Another section incubated with anti-blood group B antigen shows binding sites exclusively on certain M cells in the FAE. No RE staining is observed. Bar, 50 μm.

all cell types in the RE was given to hamsters i.n. without anesthesia. For this purpose, biotin conjugates of GS I-B_4 or WGA were administered i.n. to allow subsequent detection of biotin in tissue. The lectin-biotin conjugates were incubated for 1 h, the animals were sacrificed, and the nasal tissue was processed as described above. Examination of the septal window and NPD of these hamsters revealed no obvious epithelial changes related to lectin incubation. Despite an abundance of available binding sites, as seen by WGA-FITC staining of NALT-containing tissue sections (Fig. 4A), animals incubated with WGA-biotin showed little lectin bound to the epithelium in this region, as detected by streptavidin-TRITC (Fig. 4B). However, apical binding to goblet cells at low to moderate density was observed in many cases. The relatively large number of WGA-binding sites in the nasal epithelium at large likely resulted in the majority of WGA-biotin being bound in the most proximal regions of the nasal cavity.

Examination of nasal cavity tissue incubated with GS I-B_4–biotin revealed virtually no lectin binding to the RE of the septal window and NPD (not shown). A few rare ciliated cells which did exhibit bound lectin were found to express GS I-B_4 binding sites. When tissue sections containing NALT were incubated with GS I-B_4–FITC, lectin binding was restricted to the FAE (Fig. 5B). The abundance of GS I-B_4–FITC binding sites was clearly variable between individual cells in the FAE. When the same sections were probed for biotin with streptavidin-TRITC (Fig. 5C), we observed that the distribution of lectin administered i.n. correlated closely with the pattern of resident GS I-B_4-binding sites, demonstrating that the lectin was capable of targeting its cognate receptor(s) in vivo. A high density of lectin bound to the surface of the majority of M cells in the FAE. At higher magnifications, it was found that at the 1-h time point tested, GS I-B_4 lectin was bound to apical surface receptors on M cells as well as within endosomal vesicles (Fig. 6C). Compared with the pattern of GS I-B_4 binding sites in the same section (Fig. 6B), many of the apical and subcellular sites were bound with incubated lectin. No lectin was observed below the FAE at this time point. In summary, a lectin which showed NALT M-cell-selective binding in sections of hamster nasal tissue was able to target those same sites when administered by the i.n. route.

i.n. immunization with lectins. The ability of lectins to enhance the immunogenicity of a conjugated test antigen following i.n. immunization was assessed in the hamster model. Naive animals were immunized with HRP conjugates of GS I-B_4 or WGA and were compared to animals given HRP alone or HRP admixed with GS I-B_4 to assess the requirement for conjugation. Anti-HRP serum IgG antibodies were observed in animals immunized with either lectin-HRP conjugate, whereas

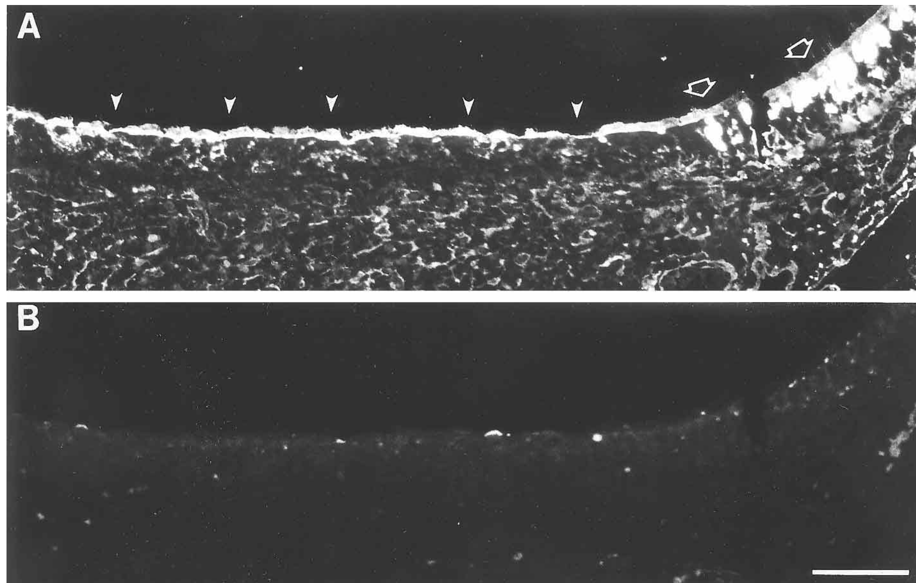

FIG. 4. In vivo distribution of WGA lectin following i.n. administration. WGA-biotin conjugate was given to hamsters i.n. without anesthesia. One hour later, nasal tissue from euthanized hamsters was prepared as described in Materials and Methods. (A) A section of nasal tissue containing NALT was incubated with WGA-FITC conjugate to demonstrate the distribution of binding sites. Many ciliated cells and virtually all goblet cells of the RE (open arrowheads) are recognized by this lectin. The FAE was also uniformly stained on cellular surfaces (small solid arrowheads). Many lymphoid cells express WGA-binding sites as well. (B) Incubating the same section with streptavidin-TRITC revealed the distribution of WGA-biotin administered i.n. Very little WGA-biotin is observed on the surface or within the epithelium of tissue which contains NALT. Infrequently, the lectin conjugate is seen preferentially bound to certain cells despite the ubiquitous distribution of epithelial cell surface-binding sites. Bar, 50 μm.

no detectable HRP-specific IgG responses were seen in hamsters given HRP alone, HRP admixed with GS I-B_4 lectin, or buffer alone (Fig. 7). No salivary IgG specific for HRP was detectable. Similarly, no HRP-specific IgA antibodies were detected in sera, saliva, and fecal extract samples (data not shown). Clearly, rendering HRP adherent by virtue of conjugation to lectins which bind to nasal cavity epithelium in vivo improved its immunogenicity when administered by the i.n. route.

DISCUSSION

We have demonstrated that the NALT M-cell lineage in hamsters is distinguished from other cells of the RE by the expression of glycoconjugates bearing terminal α(1-3)-linked galactose. The hamster RE was found to display an abundance of carbohydrate epitopes which can be categorized with carbohydrate-specific probes such as lectins and monoclonal antibodies. Although binding sites for certain lectins were uniformly distributed among cells of the RE, the differential expression of many epitopes was observed. For example, the GalNAc-specific lectin DBA strongly labeled goblet cell mucin granules, while the sialic acid-binding lectin MAL II showed uniform labeling of ciliated cell membranes. Thus, in hamsters as in other species, epithelial cell lineages can be distinguished on the basis of glycoconjugate expression patterns (13, 52).

The FAE overlying NALT, which is comprised of >90% M cells, is morphologically distinct from the surrounding RE. Presumably, the flattened apical surfaces of M cells allow close contact and sampling of particulate antigens which are not cleared by the mucociliary system of the RE. Uptake of particulate antigens may be an exclusive function of NALT M cells (33), as it is for intestinal M cells (40), although the morphological features of respiratory brush cells and nonciliated cuboidal cells suggest particle sampling capability as well (53).

The selective expression of oligosaccharide epitopes by M cells has been demonstrated previously in the small and large intestine of mice (8, 13, 17) and in the rabbit cecum (15, 24) and tonsils (14). In this study, hamster NALT M cells were shown to express terminal Galα(1-3)Gal epitopes that are rarely found on ciliated and goblet cells of the RE. Thus, the expression of certain glycoconjugate receptors appears to be restricted to rodent M cells in various mucosal tissues with distinct physiological functions.

This common theme of selective expression of carbohydrate epitopes by M cells in distinct mucosal tissues in different species suggests a functional role for these glycoconjugates. It may be beneficial to the host for M cells to display a unique surface topology (e.g., shorter, flattened microvilli) as well as distinct biochemical components on their apical membranes. These features may enhance adherence and sampling of microorganisms as part of an immune surveillance mechanism. Unfortunately, invasive pathogens appear to exploit these unique M-cell surface features to breach the epithelial barrier (39).

M-cell-selective lectins have been shown previously to target M-cell surfaces when incubated with mouse Peyer's patches in vivo (10, 17). These observations have been extended by the demonstration of selective targeting of proximal small intestinal Peyer's patch M cells and colonic or rectal M cells in mice with lectins administered by the intragastric and rectal routes, respectively (18). In the present study, we have identified probes which distinguish the hamster NALT M-cell lineage by virtue of a cell-specific pattern of glycosylation. One such probe (GS I-B_4) was shown to target NALT M-cell surfaces and be endocytosed at high levels. Investigators may exploit the M-cell-binding properties of these probes to ferry candidate vaccine molecules directly to M cells in order to clarify the

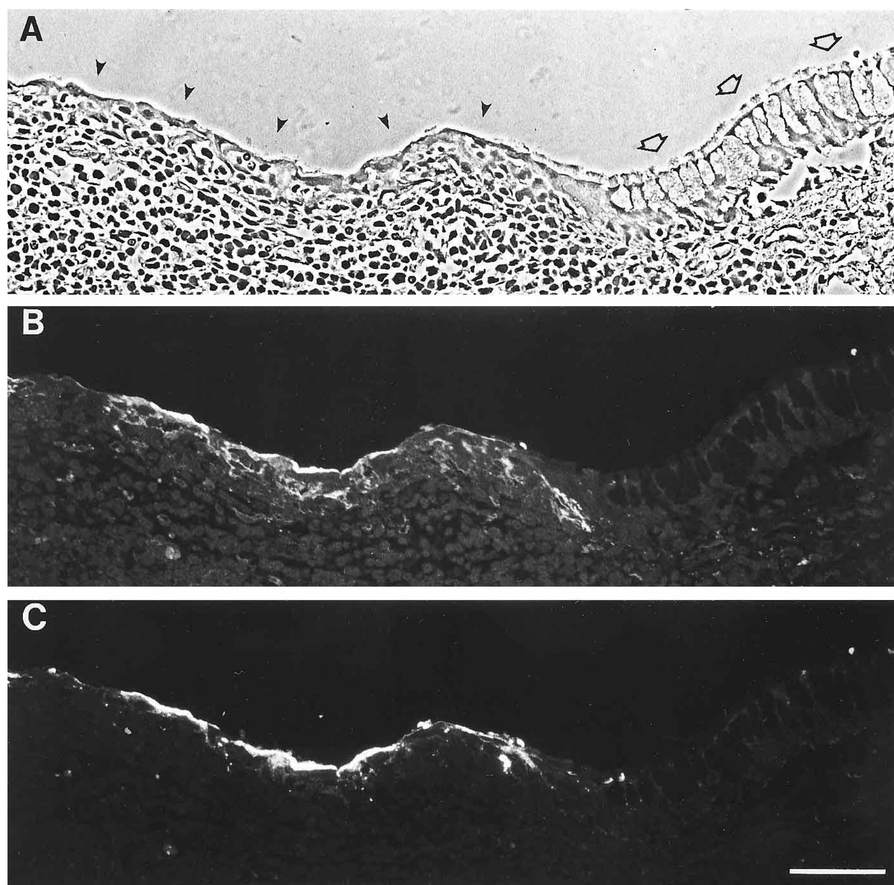

FIG. 5. In vivo distribution of GS I-B_4 lectin following i.n. administration. GS I-B_4–biotin conjugate was administrated identically to WGA-biotin. (A) A phase-contrast image of a section of nasal tissue containing NALT is shown. An adjacent section stained with toluidine blue was shown previously in Fig. 1B. (B) The section shown in panel A was incubated with GS I-B_4–FITC conjugate to demonstrate the distribution of binding sites. This image demonstrates the segregation for GS I-B_4-binding sites between the distinct epithelial domains. The FAE expresses abundant binding sites on M-cell membranes including apical and subcellular domains, while GS I-B_4-binding sites are absent on RE. (C) The same section probed with streptavidin-TRITC reveals the fate of the GS I-B_4–biotin conjugate 60 min after i.n. administration. The lectin distribution following in vivo incubation is virtually identical to the pattern of resident cell surface-binding sites. Bar, 50 μm.

role of M-cell transport in mucosal immunization. By enhancing the efficiency of antigen uptake by M cells, such targeting molecules may improve the efficacy of mucosal vaccination by increasing the protective levels of antigen-specific antibodies in secretions and/or decrease the dose requirements for mucosal immunization.

i.n. immunization may be the most efficient mucosal route to induce antibody responses in serum, as well as local and distant mucosal secretions. Presently, the early events in antigen sampling at the level of the epithelium in the nasal cavity are poorly understood. However, it appears that the physical properties of an antigen may affect the pathway employed for antibody induction. Nonadherent soluble antigens can traverse the RE at large (33), especially when coadministered with cholera toxin (19). Adherent molecules, including cholera toxin B subunit (61–63), which bind to epithelial cells likely improve the efficiency of sampling by resisting mucociliary clearance. These antigens may be subsequently sampled by intraepithelial dendritic cells which are interspersed throughout this epithelium (21) and become antigen-presenting cells following migration from the mucosa to draining lymph nodes (22). The superficial cervical lymph nodes drain the nasal mucosa directly followed by the posterior cervical lymph nodes (30, 58), whereas NALT is drained preferentially by the posterior cervical lymph nodes (30). Administration of particulate antigens and soluble adherent molecules which target NALT M cells likely results in processing and presentation initiated in NALT.

In this study, we i.n. administered lectin-HRP conjugates which were selective in their binding to the FAE overlying NALT (GS I-B_4) or recognized all cells of the RE as well as the FAE (WGA). The HRP-specific antibody responses in postimmunization sera demonstrate that rendering an antigen adherent improves its immunogenicity by the i.n. route in hamsters. Previous studies have reported such an enhancement using lectin-hapten conjugates given by the oral route in mice (11, 48). The induction of antibody responses following immunization with the GS I-B_4–HRP conjugate provides a clear demonstration of the role of M-cell transport in the initiation of mucosal immune responses. Curiously, the GS I-B_4–HRP conjugate elicited HRP-specific serum IgG in the absence of detectable serum IgA or secretory IgA in saliva and feces. This antibody profile was indistinguishable from the response stimulated by WGA-HRP. The addition of cholera toxin adjuvant in future experiments would likely enhance induction of antigen-specific serum antibodies as well as stimulate secretory IgA production. These preliminary immunization experiments were designed to assess the effect of antigen adherence on immunogenicity of a test antigen administered by the i.n. route without adjuvant. This approach is relevant because the clinical

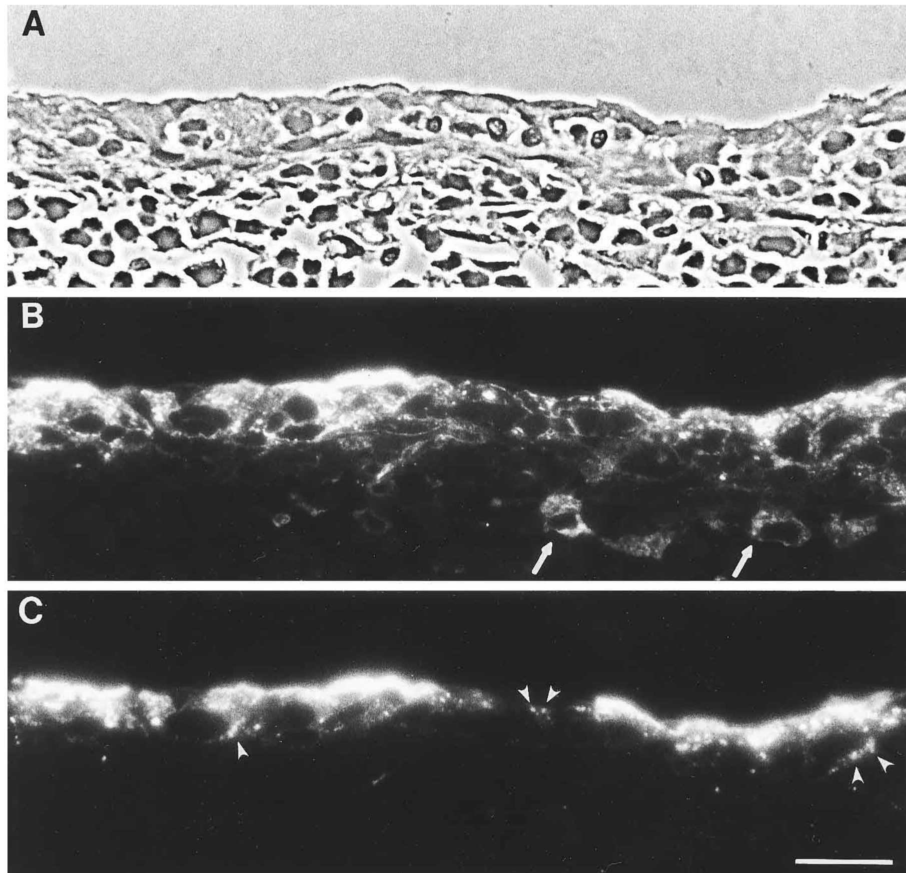

FIG. 6. High magnification of GS I-B_4 binding to FAE in vivo. (A) A phase-contrast image of FAE and subjacent lymphoid cells is shown. (B) The distribution of GS I-B_4-binding sites in this section is displayed. Note the strong apical labeling of certain cells as well as the intracellular vesicular distribution of binding sites. A subpopulation of follicle cells close to the FAE express binding sites for this lectin (arrows). (C) The lectin incubated in vivo is bound to many apical sites in high density. After the 60-min incubation period, substantial endocytosis of lectin is observed as labeled intracellular vesicles (arrowheads). Very little lectin has reached the basement membrane at this time point. Bar, 20 μm.

development of i.n. administered vaccines will likely not include the use of native toxin adjuvants and alternative methods to stimulate immune responses to vaccine antigens are needed.

The probes described in this report, which have a defined binding pattern when administered in vivo, are useful tools for targeting immunogens to specific cell lineages in mucosal epithelia. Such reagents will be necessary for the critical dissec-

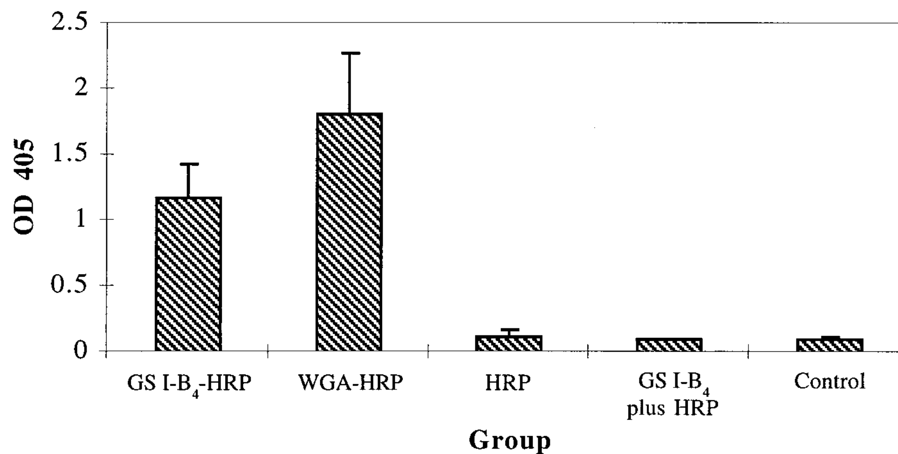

FIG. 7. Serum IgG responses to HRP in hamsters following i.n. immunization with lectin-HRP conjugates, HRP alone, or GS I-B_4 mixed with HRP. Results shown are the mean optical density values ± standard deviations of responses in sera diluted 1:100 for five hamsters per group. OD 405, optical density at 405 nm.

tion of the effect of sampling route(s) on the induction of mucosal immune responses.

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