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Membranous Cells in Nasal-Associated Lymphoid Tissue: A Portal of Entry for the Respiratory Mucosal Pathogen Group A Streptococcus¹

Hae-Sun Park,* Kevin P. Francis,[†] Jun Yu,[†] and P. Patrick Cleary^{2*}

Human tonsils are suspected to be an antibiotic-impervious human reservoir for group A streptococcus. An intranasal infection model in mice and a bioluminescent-tagged strain were used to investigate this possibility. Viable streptococci were predominantly found both intra- and extracellularly in nasal-associated lymphoid tissue (NALT), a human tonsil homologue. *Ulex europaeus*-1, a membranous (M) cell-specific lectin, identified cells harboring streptococci at the epithelial surface of NALT and blocked bacterial colonization of this tissue. These results suggest that M cells in NALT transport this Gram-positive pathogen across the epithelial layers in a manner similar to those in Peyer's patches, which permit enteric pathogens to invade deeper tissues from the gastrointestinal tract. *The Journal of Immunology*, 2003, 171: 2532–2537.

Group A streptococcus (GAS)³ is the causative agent of a wide variety of human diseases, including pharyngitis, impetigo, necrotizing fasciitis, and sepsis. The organism is known to persist at the pharyngeal mucosa and tonsils, and can be shed in saliva, even following intense antibiotic therapy and without overt disease. Although known as an extracellular pathogen, GAS produces multiple invasins that allow the bacteria to efficiently enter a variety of cell lines and primary cultures of tonsillar epithelial cells (1–3). Österlund et al. (4) reported that tonsils from children with recurrent tonsillitis contained intracellular streptococci and suggested that this secondary lymphoid organ may be a reservoir for recurrent infections. Occasionally this organism is associated with rapidly progressing sepsis and, more rarely, with toxic shock when no evident portals of entry have been identified (5, 6). The mechanism by which it breaches mucosal barriers remains undefined.

Tonsils, collectively termed Waldeyer's ring, are prominent components of mucosal-associated lymphoid tissue and play an important role in Ag uptake for initiation of respiratory mucosal immunity (7). The lymphoepithelium with invaginated crypts are lined with epithelial cells intimately associated with lymphoid cells. Ags are taken up by specialized cells of the lymphoepithelium that functionally and ultrastructurally resemble membranous (M) cells in the Peyer's patches and colonic lymphoid follicles (8). M cells endocytose Ags and deliver them to underlying macrophages, dendritic cells, and lymphocytes. M cells also serve as a gateway for various enteric pathogens, such as *Salmonella* and

Shigella (9). However, the molecular mechanisms for selective adherence of microorganisms to M cells are poorly understood.

Intranasal infection of mice has been used as a model for studying immunity to and pathogenesis of GAS (10, 11). Although rodents lack Waldeyer's ring, they have a pair of lymphoid lobes, termed nasal-associated lymphoid tissue (NALT), located along the lateral nasopharyngeal wall. NALT is known to resemble human tonsils in function (12–14) and to contain M cells along the epithelium covering of NALT (15, 16). Using a mouse intranasal infection model we investigated the possibility that tonsils or NALT, which are purported to contain M cells, could function in a manner analogous to Peyer's patches in the gastrointestinal tract and serve as a conduit to underlying tissue for respiratory pathogens.

Materials and Methods

Bacterial strains and culture media

GAS strain 591 (serotype M49) was obtained from Dr. A. Podbielski (Universität Rostock Medizinische Fakultät, Rostock, Germany). Strain 90-226 (serotype M1) was originally isolated from the blood of a septic patient (17). GAS strains were grown in THY medium (Todd-Hewitt Broth with 0.5% yeast extract) at 37°C in 5% CO₂ unless otherwise stated. For vital labeling, streptococci were incubated with the intracellular covalent coupling dye CFSE (Molecular Probes, Eugene, OR) at 5 μM for 20 min at 37°C.

Construction of Sp3-2 bioluminescent strain

Strain 591, grown to early exponential phase in THY with 20 mM glycine, was transformed by electroporation with the plasmid pXen-5 (formerly named pAUL-A Tn4001 *luxABCDE Km^R*) as described previously (18). The transformants were further selected on chocolate agar plates containing 200 μg/ml kanamycin for chromosomal integration of Tn4001 *luxABCDE Km^R*. The colonies were screened using Xenogen's IVIS camera system and LivingImage software (Xenogen, Alameda, CA) to assess their bioluminescence phenotype. The genomic DNA lying upstream of the Tn4001 *luxABCDE Km^R* integration site was obtained by inverse PCR (19). Chromosomal DNAs from transformants were digested with *EcoRV* and *Clal*, blunt-ended, and then self-ligated. The upstream region of the integration site was PCR amplified using primers IR2 (5'-CGT TTC ATT ACC TCT GTT TGA G-3') and XCF (5'-GGG AAT TCT CGA GGA GGA TGG CAA ATA TGACTA A-3'). Resulting PCR products were directly sequenced using the primer IR2. Southern blot and an inverse PCR data showed that the highly luminescent strain, designated Sp3-2, contained only one copy of the *lux* transposon (data not shown), and that the transposon was inserted in the second open reading frame (hypothetical

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³ Abbreviations used in this paper: GAS, group A streptococcus; M cells, membranous cells; NALT, nasal-associated lymphoid tissue; UEA-1, *Ulex europaeus* agglutinin.

protein, accession no. NP_268809) of a possible four-gene operon of unknown function. Biochemical profiles and growth rates of the bioluminescent recombinant strain were indistinguishable from those of the parent, strain 591.

In vivo monitoring of GAS infection in live mice

Bacteria were grown to exponential phase and then suspended in HBSS to a final concentration of 5×10^8 CFU/20 μ l. GAS was introduced intranasally into mice under anesthesia (BALB/c females, 8–11 wk old, immunocompetent) by placing 10 μ l of bacterial suspension on the each side of the nares and allowing the mice to inhale the inoculum. Neither physical damage of the nasal mucosa nor direct introduction of bacteria into lungs was apparent. A total of eight mice were used for strain Sp3-2 infection, and three mice were used for the parental strain 591. Mice were imaged for 5 min using Xenogen's IVIS CCD camera system (Xenogen, Alameda, CA) at 0, 4.5, 24, and 48 h postinoculation. Both dorsal and ventral images were taken, and the total photon emission from selected areas was quantified using the LivingImage software package (Xenogen). At the end of the experiment (72 h after inoculation), mice were sacrificed, and various tissues were aseptically removed and imaged *ex vivo* using the IVIS. The tissues were also homogenized in HBSS and plated on BHI agar plates after serial dilution to determine CFU.

Histology and immunohistochemistry of NALT

NALT tissues were collected as previously described (12). Briefly, euthanized mice were decapitated. With their heads immobilized, the lower jaws including tongue were removed. The remaining head was either frozen using Tissue-Tek O.C.T. embedding medium (Miles, Elkhart, IN) in a Tissue-Tek Cryomold or paraffin-embedded using standard methods after overnight fixation with 10% paraformaldehyde in PBS. For immunofluorescent staining, 5- μ m-thick frozen sections were cut, air-dried, and fixed in acetone at 4°C. Sections were then rehydrated in PBS and incubated for 30 min in Fc blocking solution (culture supernatant from the 24G2 hybridoma containing anti-FcR Abs and 2% normal donkey serum). Sections were washed in PBS and then incubated with rabbit anti-GAS Abs (Fitzgerald, Concord, MA) and subsequently with Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). For M cell staining, sections were incubated for 20 min with FITC-labeled *Ulex europaeus* agglutinin (UEA-1) (Sigma-Aldrich, St. Louis, MO) at 60 μ g/ml in PBS and counterstained with 4',6-diamido-2-phenylindole hydrochloride (Molecular Probes, Eugene, Oregon) at 10 μ g/ml in PBS.

Intranasal administration of lectins

UEA-1 or *Griffonia simplicifolia* I isolectin-B₄ was administered to mice by an intranasal route in two 15- μ l doses (300 μ g/ml of HBSS) in the same way as bacterial infection. After 1 h, mice were infected with $3\text{--}5 \times 10^8$ bacteria. The NALT tissues were removed at 2 or 24 h postinfection, homogenized in PBS, and plated on blood agar to determine CFU after dilution.

Results

Anatomical distribution of GAS following intranasal infection

A bioluminescent strain of GAS was stably engineered to permit continuous assessment of the anatomical distribution of GAS in live animals. This strain was made by introducing a modified *lux* operon onto the chromosome of the clinical isolate GAS 591 (serotype M49) using pAUL-A Tn4001 *luxABCDE Km^R* (18) as a delivery vehicle. A transformant, Sp3-2, showing strong bioluminescence, yet otherwise indistinguishable from the parent in growth rate and biochemical profile, was obtained and used to study persistence and *in vivo* distribution of GAS in mice.

We infected mice intranasally with 5×10^8 CFU of Sp3-2 bacteria and monitored *in vivo* distribution of this pathogen over time by taking both dorsal and ventral images of living mice at 0, 4.5, 24, and 48 h postinfection using an IVIS CCD imaging system (Fig. 1A). The level of bioluminescence from bacteria in the infected mice was measured by total photon emission per unit area per time interval using the LivingImage software package. Immediately after intranasal inoculation a strong bioluminescent signal was observed in the nose region of all eight Sp3-2-inoculated mice,

but had diminished to background levels 4.5 h later in all but two mice. By 24 h postinoculation, bioluminescence was again clearly evident in the noses of all mice, indicating that regrowth of the streptococci had occurred. These bioluminescent signals increased significantly over the next 24 h (48 h postinoculation) in each mouse and persisted in this region for at least 72 h postinoculation (data not shown). The production of readily detectable bioluminescence indicates that bacteria were proliferating in mice during infection. Weak bioluminescence was also observed in the chest area of most mice immediately after inoculation. However, this signal was not detected at 48 h postinfection with the exception of one mouse. The inoculum size in this particular experiment was 50 μ l; therefore, it is possible that streptococci were aspirated into the lungs. As expected, control animals, inoculated with the GAS 591 *lux⁻* parental strain, did not produce significant bioluminescent foci after infection (Fig. 1A, *left panel*). Blue-purple signals, representing very low intensity light, were detected. This background light is associated with autoluminescence from mice regardless of infection. Nevertheless, viable bacterial counts from dissected tissues showed no significant differences between parental- and bioluminescent derivative-infected mice.

Examination of various tissues dissected from infected mice showed that NALT was the primary source of bioluminescence, although some bioluminescence was emitted from residual mucosal tissue and NALT remaining in the dissected head (Fig. 1B). In contrast, cervical lymph nodes, spleen, lung-heart, and liver were free of bioluminescence. The mouse dissected and shown in Fig. 1B did not produce bioluminescence from the chest area. Flow cytometry of the single-cell suspension of excised NALT showed that the ratio of CD3⁺ T cells to B cells was 1.2:1, as previously reported (12, 20).

Early clearance and regrowth of GAS following intranasal infection was confirmed using the nonbioluminescent wild-type (serotype M1) strain 90-226. In all subsequent experiments the *lux⁻* M1 strain 90-226 was used to infect mice, and the volume of the intranasal inoculum was reduced to 20 μ l, a volume that avoids introduction of bacteria into lungs (21, 22). The number of viable bacteria recovered from excised tissue changed with time. Only a few thousand colonies (CFU) were present in the NALT at 1–2 h postinoculation and reached a peak of $1\text{--}8 \times 10^6$ CFU by 1 day after infection. Thereafter, streptococci progressively diminished in number over the next 7 days (Fig. 2). These dynamics agreed with those from experiments using the *lux⁺* strain. Streptococci were also found inconsistently in cervical lymph nodes and spleens, but in numbers several orders of magnitude lower than in NALT (Table I). These data demonstrate that NALT is the primary site of streptococcal infection after intranasal inoculation.

Intracellular GAS in NALT

While GAS has long been regarded as an extracellular pathogen, it was recently shown to efficiently promote its own uptake by a variety of mammalian cells (23–25). To assess whether GAS exists in the intracellular state *in vivo*, antibiotics protection assays on single-cell suspensions of NALT were performed (Table I). NALT from the infected mice were removed at 3 days after infection, and single-cell suspensions were made. Cells were incubated in tissue culture medium in the presence of antibiotics for 2 h. After washing the cells, diluted samples were plated for CFU. To ascertain the sensitivity of extracellular bacteria to antibiotics in cell suspensions, $2\text{--}5 \times 10^6$ streptococci were coincubated with NALT cells from an uninfected mouse. The inoculum was completely eliminated following 2 h exposure to antibiotics. However, 1–10% of bacteria associated with NALT from intranasally infected mice

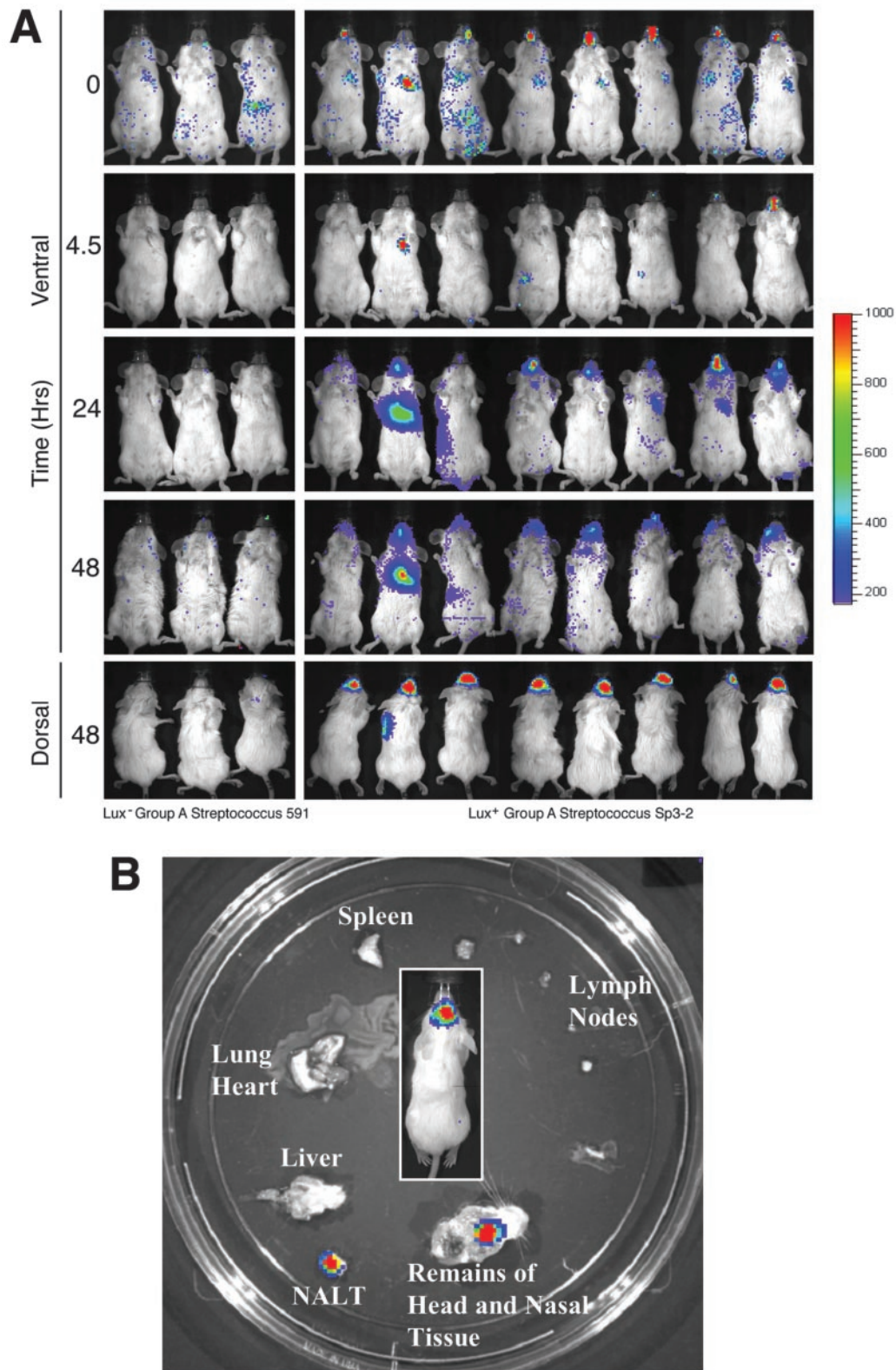


FIGURE 1. A, Bioluminescence from infected mice. Bioluminescence was recorded over time from mice infected with either the GAS strain 591 (*left columns*) or the *lux*⁺ derivative Sp3-2 (*right columns*). Mice were inoculated with bacteria and imaged at the indicated time points after inoculation. Photon emission from each mouse was quantified using the LivingImage software package and color-coded. B, Bioluminescence of various tissues dissected from the mouse (*center*) infected with *lux*⁺ strain Sp3-2 at 72 h after inoculation.

survived under this condition, indicating that some streptococci invade host cells within this lymphoid tissue. This result is comparable to previous reports using human epithelial cell monolayers

or primary human tonsillar epithelial cells (3, 25) and may account for the frequent failure of antibiotic therapy to eliminate it from the throats of children with recurrent tonsillitis (4).

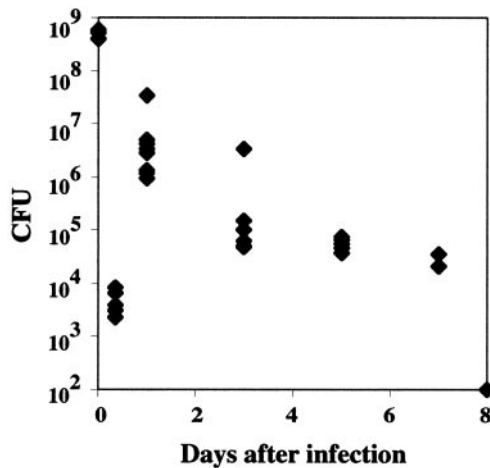


FIGURE 2. Colonization of NALT by intranasally administered streptococci. Viable counts of GAS 90-226 recovered from homogenized NALT 2 h and 1, 3, 5, 7, and 8 days following inoculation of bacteria.

Immunohistochemical detection of streptococci in NALT

Histologic examination of frontal sections of mouse heads demonstrated that NALT is situated in the mucosa of both lateral walls at the nasal floor on the posterior side of the palate (Fig. 3A). Fig. 3A shows the histology of NALT within the whole nasal cavity. NALT is covered by the ciliated columnar epithelium of the nasal cavity and is separated from the stratified squamous cells by a thick layer of collagen. The presence of high walled endothelial venules within the NALT and proximal vessels is observed (Fig. 3B). In sections from infected mice, large numbers of Gram-positive cocci were observed within the NALT tissue. While most were distributed in the subepithelium, some bacteria appeared to be associated with cells at the surface epithelial layers that are morphologically distinctive (Fig. 4A). Sections from uninfected mice did not contain Gram-positive streptococci within NALT (not shown). To confirm that the Gram-positive cocci within NALT are derived from the original inoculum of GAS, we infected mice with fluorescent dye CFSE-labeled bacteria (green) and stained NALT sections subsequently with an Ab specific for streptococci (red), and sections were viewed by confocal microscopy. In this double-label experiment, numerous streptococcal clumps were seen in NALT from infected mice (Fig. 4C), while control sections from uninfected mice did not show bacteria (data not shown). These bacterial clumps, representing either individual or microcolonies of bacteria, were no longer green at their peripheries, but were seen as red, indicating bacterial growth that results in significant dilution of the prelabeled CFSE (Fig. 4, C and D). Serial sections of NALT from the mice sacrificed at 1 day postinoculation showed that most streptococci were localized in the subepithelial dome, with a few bacteria on the epithelial surface.

M cells in NALT are potential entry sites for GAS

From Gram-stained sections we observed streptococci associated with distinctive cells that intercept the ciliated epithelium in luminal surfaces (Fig. 4A). In an attempt to characterize the cells, we performed double-label experiments on the adjacent sections of NALT shown in Fig. 4A. M cells were localized with the lectin UEA-1, which specifically binds to α -L-fucose residues that are typically present on murine M cells (26). Staining with fluorescent-labeled UEA-1 was restricted to cells in the epithelium on the luminal side of NALT (Fig. 4B), which corresponded to those cells in epithelium carrying intracellular bacteria (Fig. 4A). Abundant bacterial clumps, detected by GAS-specific Ab (red), were observed primarily below ciliated columnar epithelium of NALT and were often found in association with the M-like cells detected with UEA-1 (Fig. 4B, inset). Bacteria were rarely observed in association with ciliated epithelial cells that did not stain with UEA-1. These data demonstrate that at least during the early stage of infection, M-like cells in NALT are the primary luminal target for GAS, suggesting their role in bacterial translocation across the mucosal surfaces.

If M cells are the primary entry point for GAS into NALT, administration of UEA-1 lectin may interfere with the infection of this tissue. This possibility was tested by administration of lectin UEA-1 before intranasal infection (Fig. 5). The numbers of viable streptococci in homogenized NALT from mice that were preadministered UEA-1 lectin were significantly reduced 2 h postinfection compared with those that were pretreated with buffer. Differences in CFU in NALT suspensions from these two groups of mice remained the same at 24 h post infection. These experiments suggest that lectin inhibited initial colonization of NALT by GAS rather than interfering with the growth of bacteria. However, another M cell-specific lectin, *Griffonia simplicifolia* isolectin-B₄, showed no inhibitory effect on colonization by GAS in NALT (data not shown), suggesting that GAS interacts with a specific glycoprotein.

Discussion

The resurgence of systemic infections over the past 12 years reminded the health care community that GAS are a common cause of rapidly progressing sepsis. In contrast to enteric pathogens, the mechanisms by which Gram-positive bacteria breach mucosal and epidermal barriers to enter the bloodstream without apparent disruption of epithelial integrity are completely unknown. We previously showed that GAS invade epithelial cells as efficiently as *Listeria* or *Salmonella*, suggesting that invasion, followed by transcytosis, can introduce streptococci to deeper tissues (2, 25). The potential of hyaluronic acid to open tight junctions between epithelial cells when bound to CD44 is another mechanism by which GAS could translocate into underlying tissues (27).

Persistent carriage of GAS in tonsils following antibiotic therapy (4) suggested that this lymphoid tissue might be the source and

Table I. Intracellular survival of GAS in mice^a

Ratio of Culture-Positive Tissue in Infected Mice ^b			Mean CFU (range of % intracellular bacteria)			Total CFU/mg Tissue		
NALT	LN	SP	NALT	LN	SP	NALT	LN	SP
6/8	6/8	3/8	6.8×10^5 (1–10%)	2.2×10^3 (1–2%)	4.5×10^2 (1–2%)	4.7×10^4	76	3

^a NALT, lymph node (LN), and spleen (SP) were removed from mice 3 days after infection with GAS. Single-cell suspensions of each tissue were made in RPMI 1640 and incubated at 37°C in the presence of penicillin (5 μ g/ml) and gentamicin (100 μ g/ml) for 2 h. Cells were washed and plated for counting CFU.

^b Number of positive cultures per total number of mice.

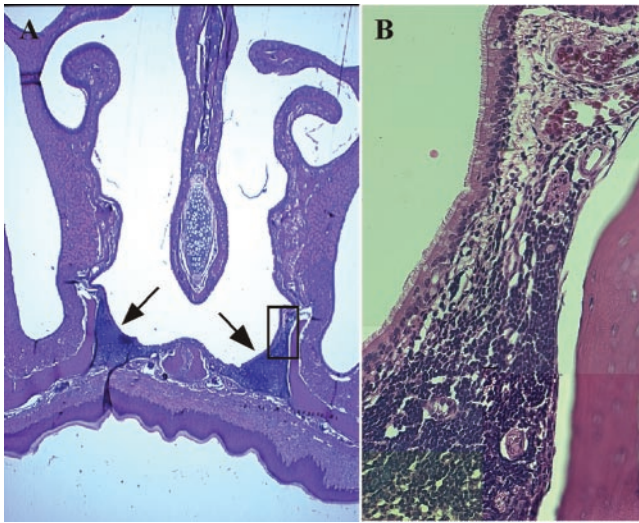


FIGURE 3. Anatomy and general histology of NALT. Mouse heads were paraffin-embedded after removing the lower jaws, using standard methods. Five-micron-thick serial sections were cut and stained with H&E. **A**, Entire head section ($\times 2$), with arrows pointing at NALT. **B**, Enlargement of selected area (\square) of NALT shown in **A** ($\times 40$).

portal of entry for streptococci to reach lymphatics and, finally, blood. Indeed, NALT was found to be the primary target for persistent infection following intranasal inoculation of mice with streptococci. Within 2 h streptococci were predominantly associated with NALT, and thereafter microcolonies of streptococci were dispersed throughout this tissue. Experiments using both bioluminescent and nonbioluminescent strains showed similar kinetics of clearance and regrowth of streptococcal after intranasal infection. Streptococci persisted in NALT for at least 1 wk. Moreover, streptococci disseminated to the blood and spleen within 24 h after intranasal infection (our unpublished observations), even though the inoculation procedure did not disturb nasal mucosa or intro-

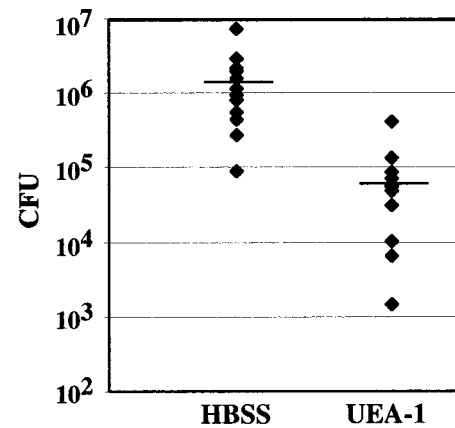


FIGURE 5. Inhibition of streptococcal colonization of NALT by UEA-1. Mice were treated intranasally with either HBSS or UEA-1 1 h before infection. Viable counts of GAS in NALT were measured from homogenized tissues 2 h after infection. The horizontal bars correspond to the geometric mean of CFU for each group. Differences between the numbers of CFU isolated from homogenized NALT were significant ($p < 0.05$) for UEA-1- and buffer (HBSS)-treated mouse groups by t test.

duce streptococci into the lungs. This suggests that NALT provides a window to the bloodstream.

The existence of M cells in human tonsils and mouse NALT has considerable experimental support (16, 28, 29). While the Ag-sampling function of M cells is a vital component of the mucosal immune system, it is becoming increasingly clear that these cells also represent a weak point in the epithelial barrier, exploited by a wide range of pathogens (9). *Streptococcus pneumoniae* was reported to be taken up by M cells in rabbit Peyer's patches (30), but this is not likely to be relevant to respiratory infections with which this pathogen is commonly associated. Our experiments demonstrated frequent and specific association of GAS with M-like cells at the epithelial surface of NALT. Participation of these M-like

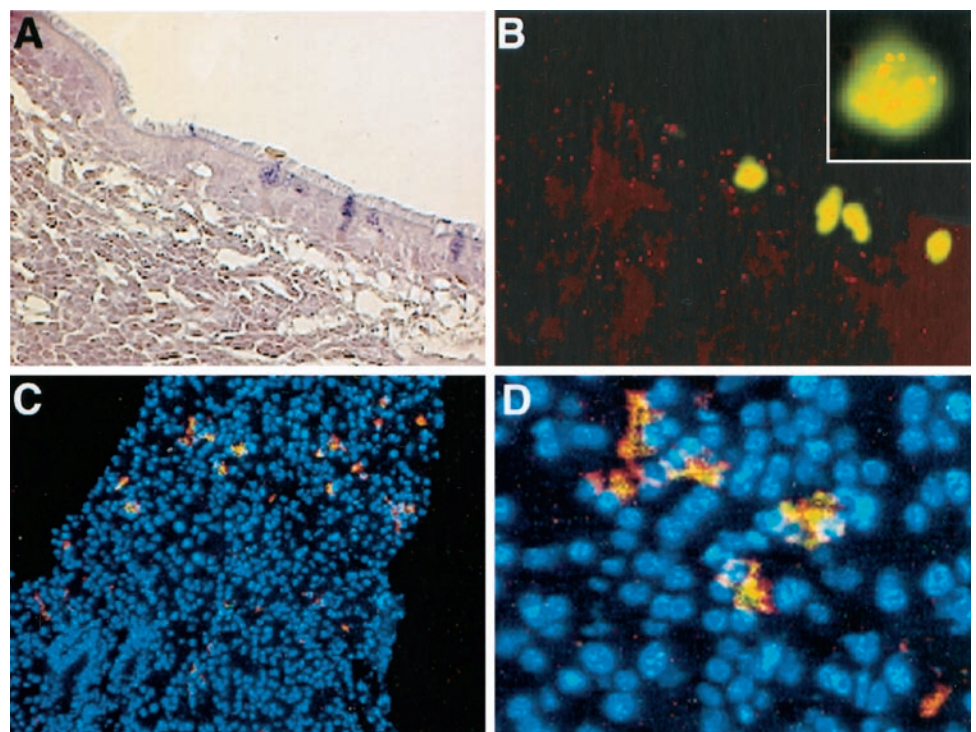


FIGURE 4. Gram stain and immunohistochemistry of NALT 1 day following infection. **A**, Gram-staining of NALT from infected mice. **B**, Double staining of NALT with rabbit anti-streptococcal Ab, Cy3-labeled donkey anti-rabbit IgG, and FITC-conjugated UEA-1. Inset, A cell featured in **B** has been digitally magnified. **C**, Immunofluorescent staining of NALT from mice infected with CFSE-labeled bacteria (green). Frozen sections of NALT were stained with fluorescent (red)-conjugated anti-streptococcus Ab and 4',6-diamidino-2-phenylindole. Images were superimposed using confocal microscopy. **D**, Enlargement of area from section shown in **C**.

cells in transporting streptococci beyond the mucosal surface is supported by the finding that intranasal administration of lectin before infection significantly reduced the number of streptococci isolated from NALT. The mechanisms by which bacteria target and invade M cells remain obscure, with one exception. *Yersinia pseudotuberculosis* was shown to selectively interact with β_1 integrins, expressed by M cells in mouse Peyer's patches (31, 32). It is possible that streptococci either directly or indirectly recognize β_1 integrins on M cells in NALT. The streptococcal M protein was shown to interact with fucose-containing glycoproteins (33) and sialic acid (34) on cultured human cell lines. Moreover, association with β_1 integrins via fibronectin promotes the adherence and invasion of cultured human tonsillar epithelial cells (3). GAS could breach mucosal barriers by several mechanisms. They could pass through or between epithelial cells (27) or, as we suggest, be transported by M cells. Our experiments did not definitively establish that the cells with which streptococci preferentially associate at the mucosal surface are M cells. The M-like cells bound UEA-1 lectin, which can also bind to goblet cells in rats (29), and were clearly morphologically distinct from goblet cells. Pre-exposure of mice to UEA-1 lectin partially inhibited infection of NALT, yet another M cell-specific lectin, GS I-B4, did not alter infection of NALT. This lectin was shown to inhibit uptake of yeast by rat NALT (29). It is likely that particle uptake is receptor specific, and that either streptococci interact with different receptors than yeast or that rat M cells display different receptors. Moreover, experiments did not eliminate the possibility that some streptococci penetrate the mucosal columnar epithelial cells to reach the interior of NALT. Two hours after intranasal inoculation, streptococci were observed on the luminal side of the mucosal surface, but we seldom observed bacteria within these cells, suggesting that M cells are the primary conduit.

Defining the mechanism by which streptococci target M cells is an important area of research, because such studies may permit the development of effective strategies to prevent initial colonization of the host or to interrupt the extracellular-intracellular transition that probably accounts for the failure of penicillin and other antibiotics to eliminate streptococci from the tonsils of children. Therapeutic agents that are able to specifically prevent or retard the process by which bacteria invade M cells are likely to block colonization and allow time for host immune effectors to kill extracellular pathogens innately targeting M cells.

Acknowledgments

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