Nasal associated lymphoid tissue & M cells, a window to persistent streptococcal infections

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Background & objectives: Intranasal infection of mice has been used as a model of streptococcal pharyngitis, as nasal associated lymphoid tissue (NALT) in this animal is structurally and functionally analogous to human tonsils. The present study was carried out to determine whether group A streptococci preferentially colonized or invaded NALT.

Methods: Lux+ strain Sp3 was created and exponential phase bacteria were introduced intranasally into BALB/C female mice, total photon emission from selected areas was quantified, sections of NALT tissues were used for immunofluorescent staining and M cell staining.

Results: Intranasal infection of mice with bioluminescent group A streptococci or unlabeled streptococci demonstrated that NALT was a primary target of this pathogen. Streptococci readily gained access to the blood stream from this site of infection. Immunofluorescence microscopy studies showed that M cells, dispersed along the mucosal epithelium adjacent to NALT, were preferentially infected and likely to provide the window through which streptococci reached the underlying tissue.

Interpretation & conclusion: The present study suggests that this upper respiratory Gram positive pathogen uses a mechanism similar to that of enteric pathogens in the intestine to gain access to underlying tissue, lymphatics and blood.

Key words Group A streptococcus - invasion - M cells - mouse model - spesis

The cellular and molecular mechanisms employed by Gram positive mucosal pathogens to persist in the oral cavity and to breach mucosal epithelia are poorly understood. Group A streptococci (GAS) have been a common cause of highly virulent blood infections with high mortality, and can lead to toxic shock. The past twelve years have witnessed a resurgence of systemic infections and in as many as 25 per cent of cases the portal of entry to the blood stream is unapparent and unknown. This observation led us to consider the possibility that pre-existing streptococci in the throats of symptom-free carriers periodically seed the blood and then become lodged at an imperceptible injury, such as a bruise. We earlier discovered that these streptococci were able to efficiently invade epithelial cells, suggested a mechanism by which the organism could traverse mucosal or epidermal barriers to reach the blood stream. More recently the cellular and molecular mechanisms that mediate ingestion of streptococci by epithelial cells have been defined. Cue et al demonstrated that fibronectin (Fn) bound to M protein bridged streptococci to a 5b1 integrins to promote cytoskeletal changes that resulted in uptake by A549 lung and primary keratinized, tonsillar epithelial cells. Others showed that the high affinity Fn-binding protein SbfI can also induce internalization of streptococci by similar mechanism. Unexpectedly, different strains or M types exhibited different requirements for invasion of mammalian cells; some required serum, a probable source of Fn, while others did not. This strain variability was in part...
determined by the M protein. The surface bound C5a peptidase was also required for cellular invasion by both group A (Sudha and Cleary, unpublished data) and group B streptococci, though this protein was less efficient than M or SfbI proteins.

A comparison of isolates from blood and throats of carriers showed that epithelial cells more efficiently ingested the latter than the former. This questioned our original suggestion that invasion of epithelial cells, accompanied by transcytosis was a primary pathway to deeper tissues. It was demonstrated that strains from carriers were more readily ingested by epithelial cells. For this reason we considered other mechanisms by which GAS could circumvent the mucosal obstacle to systemic dissemination. Intranasal infection of mice has been used as a model of streptococcal pharyngitis and vaccine studies, even though this mammal lacks Waldeyer’s ring of tonsillar lymphoid tissue. The mice, in fact have two lobes of lymphoid tissue beneath the floor of the external nares and above the hard palate (Fig.). It was suggested that this nasal associated lymphoid tissue (NALT) was structurally and functionally analogous to human tonsils. Therefore, it was sought to determine whether group A streptococci preferentially colonized or invaded NALT.

Material & Methods

GAS strain 591 (serotype M49) was obtained from Dr Andreas Podbielski (Universität Rostock Medizinische Fakultät, Germany). The Lux+ construct of strain 591 has been described previously. Strain 90-226 (serotype M1) was originally isolated from the blood of a patient with sepsis. GAS strains were grown in Todd-Hewitt broth with yeast (THY) medium at 37°C in 5 per cent CO₂. For vital labelling, streptococci were incubated with 5 µM of carboxy fluorescein diacetate succinimidyl ester (CFSE) for 20 min at 37°C.

The first experiment was performed to determine whether streptococci enter the bloodstream following intranasal inoculation. Six mice were used for each experiment. The inocula contained 1x10⁹ cfu log phase 90-226 M1 streptococci in 30µl of buffer, a volume sufficiently small to avoid introduction of streptococci into lungs. Mice inhaled the inoculum, placed in drops just beneath the nares. The pipette tip does not touch the nasal mucosa. Blood samples were taken by retroorbital bleed at 6 and 24 h after inoculation and cultured for bacteria.

The Lux+ strain Sp3 was created by transposon insertion of the Lux operon into strain 591 as described previously. Thirty micro litre of exponential phase bacteria, suspended in Hank’s balanced salt solution (HBSS), was introduced intranasally into mice (BALB/c females, 8-11 wk old) by placing 15 µl of bacterial suspension on each side of the nares and allowing the mice to inhale the inoculum. Mice were imaged for 5 min using Xenogen’s IVIS™ CCD camera system (Xenogen Inc, Alameda, CA, USA) and the total photon emission from selected areas was quantified using the LivingImage® software package. In some experiments mice were sacrificed, and various tissues aseptically removed and imaged ex vivo using the IVIS™ or homogenized in HBSS and plated on Todd Hewitt agar for cfu.

NALT tissues were obtained by removing the soft palates as previously described. Sections were Gram stained or frozen. For immunofluorescent staining, 5-µm thick frozen sections were cut, air dried, and fixed in acetone at 4°C. Sections were then rehydrated in phosphate buffer saline (PBS) and incubated with rabbit anti-GAS antibodies (Fitzgerald, Concord) and subsequently with Cy™3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Labs, West Grove, PA). For M cell staining, sections were incubated for 20 min with FITC-labeled UEA-1 at 60 µg/ml in PBS and counter stained with 4',6-diamidino-2-phenylindole (DAPI) at 10 µg/ml in PBS.

Results & Discussion

Streptococci were cultured from the blood of all six mice at 6 and 24 h after intranasal inoculation; whereas, 2 of 6 and 3 of 6 blood samples, taken from mice infected with a C5a peptidase deficient strain were culture positive, respectively. Moreover, blood from mice infected with the wild type strain had higher number of streptococci contained (30-50 fold more cfu/ml of blood) compared to other strains. It was presumed that the mutant was less invasive because it was cleared more rapidly by a more intense inflammatory infiltrate. This experiment confirmed that streptococci were capable of reaching the blood stream from the nasal mucosa without physical disruption of the epithelium.
The physical location of streptococci over time was evaluated using a bioluminescent or Lux+ serotype M49 strain of streptococcus with a Lux operon inserted into its chromosome. Streptococci emit light during log phase growth, and luminescence subsides as they enter stationary phase. Following intranasal challenge with this culture, mice emitted light from their noses for up to 7 days. Streptococci were cleared or entered in stationary phase during the first 4 h following infection, producing little light, but returned to yield visible bioluminescence by 24 h. Dissections of infected mice showed that the source of luminescence was NALT and that the draining lymph nodes, spleen, liver, heart, or lungs had too few bacteria to detect with the IVIS100 video system. Small numbers of viable streptococci were found in lymph nodes and spleen when these tissues were homogenized and cultured.

The propensity of streptococci to localize in NALT following intranasal inoculation was confirmed by several experiments, wherein, mice were challenged with the Lux+ M1 strain 90-226. Viable streptococci were present in NALT in numbers 10-100 times greater than in draining lymph nodes or spleen. Moreover, antibiotic protection experiments confirmed that 1-10 per cent of streptococci in NALT were intracellular. Gram stained sections of heads from infected mice confirmed that bacteria were dispersed throughout NALT with few bacteria associated with the ciliated columnar epithelium that lines the nasal canal (Fig.). The presence of streptococcus in NALT was confirmed by immunofluorescence microscopy with anti-group A antibody. M-like cells, sporadically dispersed along the epithelium that separated NALT from the canal were, however, disproportionately associated with greater numbers of streptococci. These cells were confirmed to be M cells by double fluorescence staining of sections with FITC-labeled UEA-1 lectin and rabbit anti-group A carbohydrate and donkey anti-rabbit IgG conjugated to Cy3. Examination of sections by confocal microscopy with computer overlays of red and green images revealed yellow streptococci, contained within fluorescent green cells that corresponded to the M-like cells that were observed in a gram stained adjacent sections.

Although group A streptococcal sepsis is a relatively common serious infection, little is known about the mechanism by which these bacteria traverse mucosal epithelial barriers. Enteric pathogens breach the intestinal mucosal epithelium by specifically interacting with M cells, located in Peyer’s Patches. Because M cells are sporadically dispersed along the tonsillar epithelium, this study entertained the possibility that this lymphoid tissue served as a window for streptococci to disseminate to deep tissue and to the blood stream. Our intranasal mouse infection model made use of the fact that mice have two lobes of secondary lymphoid tissue below their nasal passage which is functionally similar to human tonsils.

In conclusion, intranasal inoculation of mice with M1 streptococci resulted in transcytosis of bacteria into nasal associated lymphoid tissue and subsequent invasion of the blood stream. Streptococci become specifically associated with M cells that are presumed to provide a portal of entry to the underlying lymphoid follicles. Thus, group A streptococci made use of these highly absorbent cells to gain access to lymphoid tissue underlying the upper respiratory tract in a manner that was analogous to the mechanism by enteric pathogen escape the lumen of the intestine to enter the Peyer’s Patches.

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