Bacterial Transcytosis across Conjunctival M Cells

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Purpose. Antigen-sampling M cells have been identified in conjunctival tissue overlying lymphoid follicles in rabbits and guinea pigs. Conjunctival M cells in the guinea pig display α(2-3) sialic acid on their surfaces, as evinced by selective labeling by *Maackia amurensis* leukoagglutinin (MAL)-I. *Haemophilus influenzae* strains OM12, which expresses the HMW1 adhesin for α(2-3) sialic acid, and Rd KW20, which lacks HMW1, were used to test the hypothesis that conjunctival M cells translocate large microbes.

Methods. Fluorescein-labeled bacteria were instilled into the conjunctival sac for up to 130 minutes. Confocal laser scanning microscopy and electron microscopy were used to visualize bacterial distribution.

Results. M cells, but not nonfollicular epithelial cells in the palpebral region, selectively bound and translocated bacteria. By 66 minutes, 423 ± 165 bacteria/mm^2 of follicle-associated epithelial (FAE) surface were found in three-dimensional reconstructions extending 15.4 μm below the surface. By 127 minutes, the number of bacteria increased to 579 ± 44/mm^2 of FAE surface and they had moved 50% deeper into the follicle. Coadministration with MAL-I reduced OM12 transport by 61%. Similarly, Rd KW20 uptake was 71% less at 63 minutes and 58% less at 121 minutes, indicating that OM12 uptake is at least partially mediated by binding to α(2-3) sialic acid.

Conclusions. Conjunctival M cells are a port of entry for large microbes and may play a role in initiation of mucosal immune responses against commensal or transient ocular bacterial species and may allow the entry of pathogens. (*Invest Ophthalmol Vis Sci.* 2007;48:2172–2177) DOI:10.1167/iovs.06-1202

The epithelium overlying conjunctiva-associated lymphoid tissue (CALT) in the guinea pig and rabbit has been shown to contain antigen-sampling M cells.[1,2] The unusual features of M-cell fine structure, including distinctive microvilli, a large number of intracellular membranous vesicles, and a thin apical bridge of cytoplasm overlying intercellular pockets of lymphocytes and macrophages, all participate in the cell's principal function of sampling and transcytosis of antigens across the cell for delivery to antigen-presenting cells. Antigen sampling by M cells is the first step in the mucosal immune response that leads to the subsequent production of anti-inflammatory secretory IgA.

In addition to their role in initiating adaptive immune responses, M cells in other locations have been found to be exploited by opportunistic bacterial and viral pathogens as a means of invasion.[3] Once across the epithelial barrier, the pathogens evade phagocytosis and destruction by antigen-presenting cells and go on to induce local or systemic disease.

It is important to establish whether microbes can cross conjunctival M cells, since this may be either the first step in generation of antimicrobial IgA present in the tear film[4–6] or be a previously unrecognized entry point for ocular pathogens. The observation that M cells in the guinea pig conjunctiva express α(2-3) sialic acid on their surface made this an attractive model to study uptake of microbes which bind this carbohydrate epitope.[2] Pathogens and microbes known to bind α(2-3) sialic acid include reovirus,[7] influenza virus,[8] *Helicobacter pylori*,[9] *Mycoplasma pneumoniae*[10] and some strains of nontypeable *Haemophilus influenzae* (NTHi).[11] In this study, we used NTHi to test the hypothesis that M cells can translocate large microbes by analysis of the conjunctival surface before and after in vivo incubation with NTHi bacteria.

Methods

Guinea Pigs

Thirteen outbred, male guinea pigs 5 to 7 weeks of age (Harlan, Indianapolis, IN) were used in the study. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and University of Missouri Animal Care and Use Committee guidelines.

Growth and Fluorescent Labeling of Bacteria

Nontypeable *H. influenzae* strain OM12 (R2846) is a middle-ear isolate from a patient with otitis media.[12] Rd KW20 (R652) is a reference *H. influenzae* strain that has been fully sequenced.[13] Rd strains are susceptible to killing by human serum[14] and are generally avirulent in human[15] and animal models.[14] Both strains came from the collection of Arnold L. Smith (Seattle Biomedical Research Institute, Seattle, WA). Bacteria were grown overnight on chocolate agar[16] without bacitracin and resuspended at A_{600} = 2.0 in calcium, magnesium-free PBS-CMF (138 mM NaCl, 2.67 mM KCl, 1.47 mM KH_2PO_4, 8.06 mM Na_2HPO_4 [pH 7.4]). An equal volume of fluorescein isothiocyanate (FITC; Sigma-Aldrich, St. Louis, MO) in the same buffer was added to a 0.5-mg/mL final concentration, and the suspension was gently shaken for 15 minutes at room temperature. The labeling reaction was stopped with the addition of a 0.1 volume of 10 mg/mL BSA in PBS (CMF-PBS plus 0.901 mM $CaCl_2$, 0.493 mM $MgCl_2$ [pH 7.4]). Bacteria were washed three times with PBS containing 0.1% gelatin (PBS-G)[17] and resuspended in PBS-G at an A_{600} = 1.0 (0.8–1.6 × 10^9/mL CFU/mL for OM12 and 1.5–2.1 × 10^9/mL CFU/mL for Rd). After a brief lag, FITC-labeled bacteria grew at the same rate as unlabeled bacteria, and more than 90% were viable as colony-forming units (data not shown).

Surgical Procedure

Guinea pigs were anesthetized with intramuscular injections of ketamine-HCl (40 mg/kg) and xylazine (5 mg/kg). Before bacterial instillation, the guinea pig's head was rested laterally with the nose propped up, so that the orientation of the right eye was facing upward and parallel to the ground, ensuring equal distribution of instillations to the superior and inferior conjunctival sac. FITC-labeled bacteria in PBS-G (A_{600} = 1.0) were instilled into the superior and inferior conjunctival sac (15 μL each) of the right eye to begin the 120-minute incubation. Bacteria were replenished at 10, 20, and 30 minutes after the initial instillation with 10 μL aliquots into each of the superior and inferior

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conjunctiva. At 50 minutes after initial instillation, the guinea pig's head was rotated so that the left eye was now facing upward and the 60-minute incubation was begun with similar instillations at 50, 60, 70, and 80 minutes. At 110 minutes, the animals were euthanatized with an intravenous injection of beuthanasia (100 mg/kg) and both eyes quickly removed, starting with the right eye. After removal of the lens, the conjunctival sac was cut at the lateral canthi and the wholemount pinned out on a wax backing with the mucosal surfaces facing upward.[18] Wholemounts were rinsed thoroughly with PBS-CMF and then fixed for at least 2 hours in 2% freshly depolymerized paraformaldehyde in HWB (70 mM NaCl, 30 mM HEPES, 2 mM $CaCl_2$ [pH 7.4]). Variations in dissection speed resulted in the average time between the initial instillation to the time the tissue was placed in fixative of 66.0 ± 2.0 and 126.7 ± 4.9 (mean ± SD) minutes. For blocking experiments, 15-μL aliquots of 0.5 mg/mL biotinylated *Maackia amurensis* leukoagglutinin I (MAL-I; Vector Laboratories, Burlingame, CA) were administered to both the superior and inferior conjunctival sac 15 minutes before beginning the same series of instillations as just described, with the addition of 0.5 mg/mL MAL-I-biotin to each aliquot of FITC-labeled bacteria. All follicles used in the present study were isolated from the fornix region. FAE analyzed in this set of experiments was exclusively sampled from the inferior fornix and control tissue was harvested from the inferior palpebral region. There did not appear to be a difference between superior and inferior forniceal follicles, but the superior side was not systematically analyzed.

Immunohistochemistry

Tissue from the inferior forniceal and palpebral conjunctiva was blocked for at least 30 minutes with 0.1% BSA in HWB, and then stained sequentially with 20 μg/mL MAL-I-biotin in 0.1% BSA in HWB for at least 1 hour followed by 1 μg/mL streptavidin-Alexa 568 (Invitrogen-Molecular Probes, Eugene, OR) and 3 μM 4′,6-diamidino-2-phenylindole (DAPI [4′,6′-diamino-2-phenylindole]; Invitrogen-Molecular Probes) in 0.1% BSA in HWB for at least 1 hour. Tissues were stored in 2% paraformaldehyde in HWB at 4°C until examination. For imaging, tissues were mounted between two coverslips in the presence of an antifade agent, 1% 1,4-diazabicyclo[2.2.2]octane (Aldrich Chemical, Milwaukee, WI) in 200 mM Tris buffer (pH 8.6).

Confocal Microscopy

Follicles were located by using fluorescence microscopy to identify MAL-I-labeled M cells. A total of 10 different regions of FAE from the inferior fornix were sampled by collecting one stack of images from the center of each randomly chosen follicle by using a laser scanning confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc., Thornwood, NY). Each stack covered a cross-sectional area of 112 × 112 μm and contained 36 optical sections that were collected at a 0.44-μm step interval, for a total depth of 15.4 μm. Stacks began at the MAL-I-stained apical surface of the epithelium. Control images were obtained in the same manner by collecting stacks for 10 randomly chosen regions of inferior palpebral conjunctiva. For control tissues, the apical surface was also determined by MAL-I staining, because it labels the superficial mucin in goblet cells in the non-FAE regions.

Images were collected at a resolution of 1020 × 1020 which the software (Carl Zeiss MicroImaging, Inc.), based on the Nyquist theorem, determined was optimal for the 40× water-immersion objective (NA = 1.2; PlanApochromat; Carl Zeiss MicroImaging, Inc.) used in this study. All images were collected with bidirectional scanning at a scan speed of 7 and frame averaging set to 4. The 30-mW 488-nm laser was used at 1.92% power with a gain of 700 and offset of −0.2. The 1-mW, 534-nm laser was used at 100% power with a gain of 700 and offset of −0.1. The green channel pinhole was set at 1.0 Airy units and the red channel set at 0.89 Airy units, so that the two channels had matching optical section thicknesses of ~0.9 μm.

Quantification of Bacteria

A standardized method for counting bacteria was used for all images. This method used an image-processing routine (Fovea Pro 4.0 image processing software; Reindeer Graphics, Inc., Asheville, NC) which separated bacteria that were just barely touching and reduced stray pixel noise while retaining any signal greater than or equal to 5 × 5 pixels. The protocol created a binary image of the green channel by using a standard threshold of 20 to 255 for all images. An opening operation, which is an erosion followed by dilation, was then performed using the Euclidean distance map (EDM) morphologic operation. The EDM opening was chosen because it is more isotropic, preserving shape information better than other morphologic routines that reduce stray noise.

By scrolling through each section of a stack of images, bacteria which were found in at least two consecutive planes were counted. Clusters of bacteria or two contiguous bacteria which were not resolved were counted as one. The depth of each bacterium was estimated by recording the distance between the deepest plane in which the bacterium was detected and the first plane in the stack of images.

Electron Microscopy

Conjunctival tissue for scanning electron microscopy (SEM) was prepared by incubating a wholemount in 10 mL of an ice-cold solution of 2.2×10^9/mL OM12 in PBS for 60 minutes. After the tissue was rinsed in ice-cold PBS, it was fixed in ice-cold PFG (2% paraformaldehyde + 2.5% glutaraldehyde in HWB) for 2 hours. The tissues were then immunolabeled with 100 μg/mL MAL-I-biotin, followed by 10-nm gold-conjugated goat anti-biotin and gold particle enhancement, as described previously.[2] Tissues were osmicated, dehydrated, and critical point dried before viewing using secondary electron and backscattered electron (SE or BSE) imaging (S-4700 FESEM; Hitachi, Tokyo, Japan).[2] SE and BSE signals were overlaid by using image-management software (Photoshop; Adobe Systems, San Jose, CA).

Tissue for transmission electron microscopy (TEM) came from an animal inoculated with OM12, as described earlier, for 61 minutes before fixation in PFG for 2 hours. Tissues were postfixed with an OTO protocol (1% osmium in HWB, followed by 1% thiocarbohydrazide in water, then 1% osmium in water; all steps for 1 hour each with extensive water rinses between steps) based on the method of Willingham and Rutherford.[19] After en bloc staining with 0.25% uranyl acetate in 25% ethanol, tissues were dehydrated and embedded (Em-Bed 812; Electron Microscopy Sciences, Fort Washington, PA).

Statistics

Summary data are reported as the mean ± SD for results referring to a single eye, and mean ± SE for comparing means from multiple animals in different treatment groups. Student's *t*-test was used to determine statistical significance; statistics were calculated with commercial software (StatView SE; Abacus Concepts, Berkeley, CA).

Results

Follicles appeared as large protuberant domes when viewed with a stereoscope. MAL-I staining was restricted to the apical surface of M cells on FAE domes and to intracellular mucin stores in goblet cells in the non-FAE regions. The polygonal staining pattern of the apical surface of M cells was easily distinguished from the more rounded or oval-shaped staining of goblet cells. As previously reported, goblet cells were generally absent from the FAE region, which is consistent with the need for an attenuated mucus layer over the antigen-sampling M cells.[2]

FITC-labeled OM12 was difficult to see with fluorescence stereomicroscopy but were routinely observed in FAE regions with confocal microscopy (Fig. 1). At 66 ± 1.5 minutes, an average of 423 ± 165 bacteria/mm^2 of FAE surface were found in three-dimensional volumes measuring 112 × 112 μm in the *x*–*y* dimension and 15.4-μm deep (Table 1). A 15.4-μm depth was selected for the *z*-stacks after a preliminary screening of random follicles failed to find any bacteria deeper than this. In

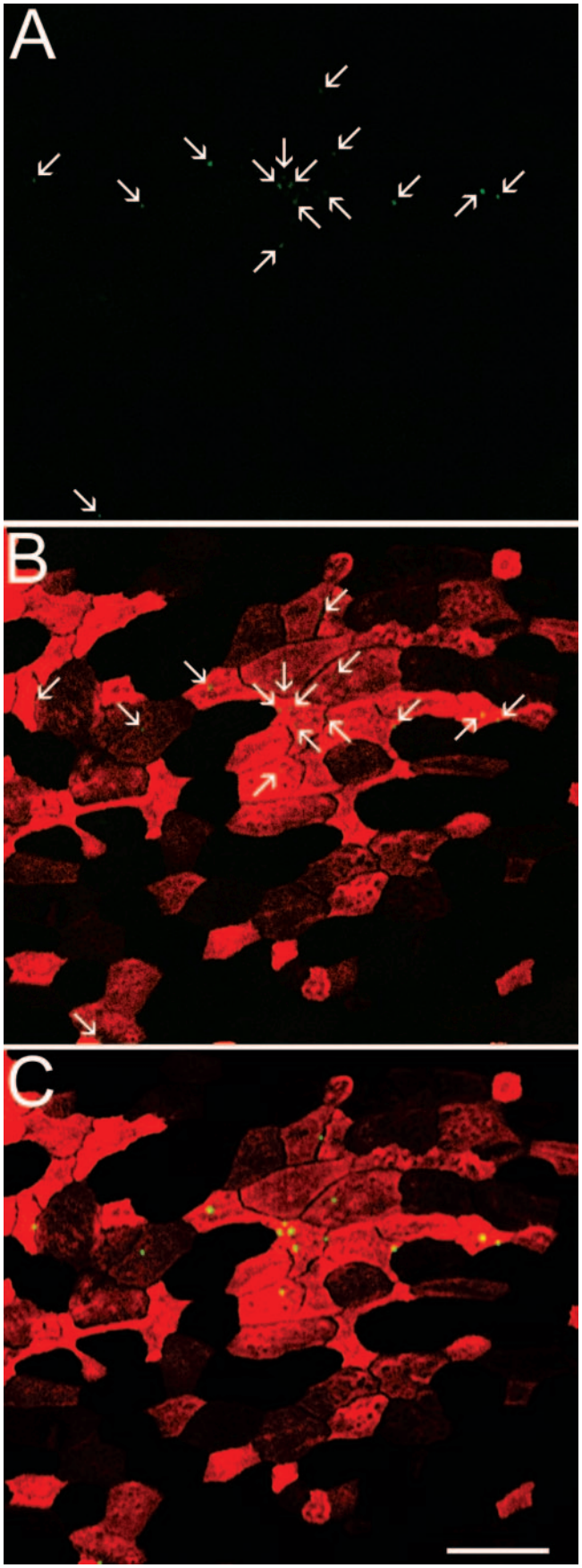

FIGURE 1. Three alternative displays of a single *z*-stack of 36 optical sections of an FAE region after a 66-minute in vivo exposure to 1.6×10^9 FITC-labeled OM12 bacteria. (**A**) A two-dimensional maximum projection of the *green* channel (OM12 bacteria) from one *z*-stack collected under standard conditions. *Arrows*: FITC-labeled bacteria. No image-processing steps have been performed on images shown in (**A**) and (**B**) other than the projection and cropping the image by 10% in the *y*-axis to allow the figure to fit the page. (**B**) FITC-labeled bacteria in the *green* channel shown superimposed on the MAL-I labeling in the *red* channel. Bacteria can be seen but are sometimes difficult to recognize against the intense MAL-I labeling of M-cell surfaces. (**C**) A projection of the same *z*-stack after performance of a standard EDM opening image-processing step to reduce noise and intensify the *green* channel signal. The bacteria are readily detected against the *red* MAL-I staining after this processing step and can be seen to be exclusively associated with MAL-I-labeled M cells. Scale bar, 20 μm.

striking contrast, no bacteria were found in 10 similar randomly selected *z*-stacks of palpebral tissues after a 66-minute in vivo exposure. Although *z*-stacks from the bulbar region were not systematically acquired and quantified, visual evaluation failed to detect any bacteria in this region. The number of OM12 per individual follicle varied widely in a single eye, with some follicles having no bacteria and others as many as 1754 bacteria/mm^2 at 66 minutes. These variations may have resulted from subtle differences in the rate the bacteria penetrated the tear film and gained access to the epithelial surface rather than differences in the receptors between follicles since both intra- and interanimal variability was greatly reduced at 127 minutes (Table 1). In *z*-stacks of FAE tissue collected 125 ± 4.7 minutes after in vivo instillation, the number of OM12 increased 37% to 579 ± 44 bacteria/mm^2 of FAE surface (Table 1). Control palpebral regions occasionally contained an isolated bacterium but overall had less than 3% of the number found in the FAE region. The control values may overestimate bacteria uptake in this region because it was not possible to distinguish bacteria caught in mucus-filled crevices on the surface or being transported via lymphatic vessels from the follicle region to draining lymph nodes. Pre- and simultaneous incubation of the α(2-3) sialic acid binding lectin MAL-I with OM12 resulted in a significant 61% decrease ($P = 0.0027$) in uptake, to an average of 229 ± 143 bacteria/mm^2 of FAE surface at 124 ± 4 minutes (Table 1).

Bacteria moved progressively deeper into the follicle region with time (Fig. 2). At 66 minutes after instillation, the average depth of OM12 was 4.12 ± 0.5 μm and none of the bacteria were deeper than 10 μm. At 125 minutes after instillation, the average depth of bacteria had increased to 6.08 ± 1.4 μm and 5.5% of all bacteria was deeper than 10 μm.

The Rd KW20 *H. influenzae* strain, which does not express the sialic acid binding HMW1 adhesin, was not as efficiently taken up by conjunctival M cells (Table 1). At 63 ± 2.1 minutes, only 122 ± 116 Rd KW20 were found per square millimeter of FAE surface, which is 29% of the level of OM12 present at a comparable time point. Likewise, at 121 ± 2.1 minutes, there was an average of 243 ± 178 Rd KW20/mm^2 of FAE, which is only 42% of the OM12 uptake.

Observation by SEM failed to find any bacteria associated with the FAE or non-FAE surface after 15 to 120 minutes of incubation in vivo. Only occasional clusters of extracellular bacteria trapped in small bits of remaining surface mucus were seen. When conjunctivae were excised and incubated on ice in the presence of OM12, some bacteria were found to be associated with the apical surface of the epithelia. In all cases, these bacteria were bound to cells within the FAE that had M-cell characteristics: sparse, irregular microvilli of various lengths that were labeled with MAL-I (Fig. 3).

In TEM cross sections, OM12 bacteria were found in FAE cells with M-cell morphology and in interepithelial lymphoid cells (Fig. 4).

TABLE 1. Bacterial Binding to Forniceal FAE and Palpebral Control Regions

Bacterial Strain	Incubation Conditions	Palpebral Control	Forniceal FAE	Control vs. FAE Paired *t*-Test *P*
OM12	65 min	0 ± 0*	462 ± 451*	0.0014
OM12	66 min	0 ± 0	686 ± 620	0.0014
OM12	68 min	0 ± 0	120 ± 147	0.0014
OM12	121 min	27 ± 40	566 ± 386	0.0017
OM12	123 min	8 ± 25	662 ± 415	0.0005
OM12	130 min	16 ± 34	510 ± 263	0.0010
OM12	120 min + MAL-I		175 ± 149	
OM12	123 min + MAL-I		391 ± 510	
OM12	128 min + MAL-I		120 ± 126	
Rd KW20	61 min		8 ± 25	
Rd KW20	62 min		239 ± 269	
Rd KW20	65 min		120 ± 272	
Rd KW20	119 min		255 ± 276	
Rd KW20	122 min		415 ± 240	
Rd KW20	123 min		60 ± 93	

Data are the mean ± SD for 10 follicles or control regions except for the 123-min Rd KW20 sample, in which only 8 distinct follicles could be imaged.

* Bacteria/mm^2 of FAE in three-dimensional volumes measuring 112 × 112 × 15.4 μm.

DISCUSSION

Bacteria were found to be transcytosed across M cells above conjunctival lymphoid follicles and to move deeper into the follicle with time. These findings demonstrate that conjunctival M cells, like those in other mucosal locations, are a site of entry for large microbes and may be an entry point for bacterial and viral pathogens. Nontypeable (nonencapsulated) *H. influenzae* are commensal bacteria of the human upper respiratory tract, but under appropriate conditions are responsible for many mucosal diseases including conjunctivitis, otitis media, sinusitis, and bronchitis.[20] *H. influenzae* has not been reported to cause experimental conjunctivitis in the guinea pig although the species has been used for studying NTHi-mediated otitis media. The guinea pig was chosen as a model system to study how microbes may cross the mucosal barrier, because conjunctival M cells in this species are known to express α(2-3) sialic acid selectively on the apical surface and the well-characterized ability of some NTHi strains to bind this carbohydrate made it a convenient probe. The intent of the present study was not to investigate bacterial conjunctivitis in the guinea pig, but to demonstrate that conjunctival M cells are a potential port of entry for microbes. Numerous studies have found potentially pathogenic bacteria such as NTHi, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in the conjunctiva of normal individuals with no overt signs of infection.[21–23] In addition, nonpathogenic commensal bacteria such as *Propionibacterium*

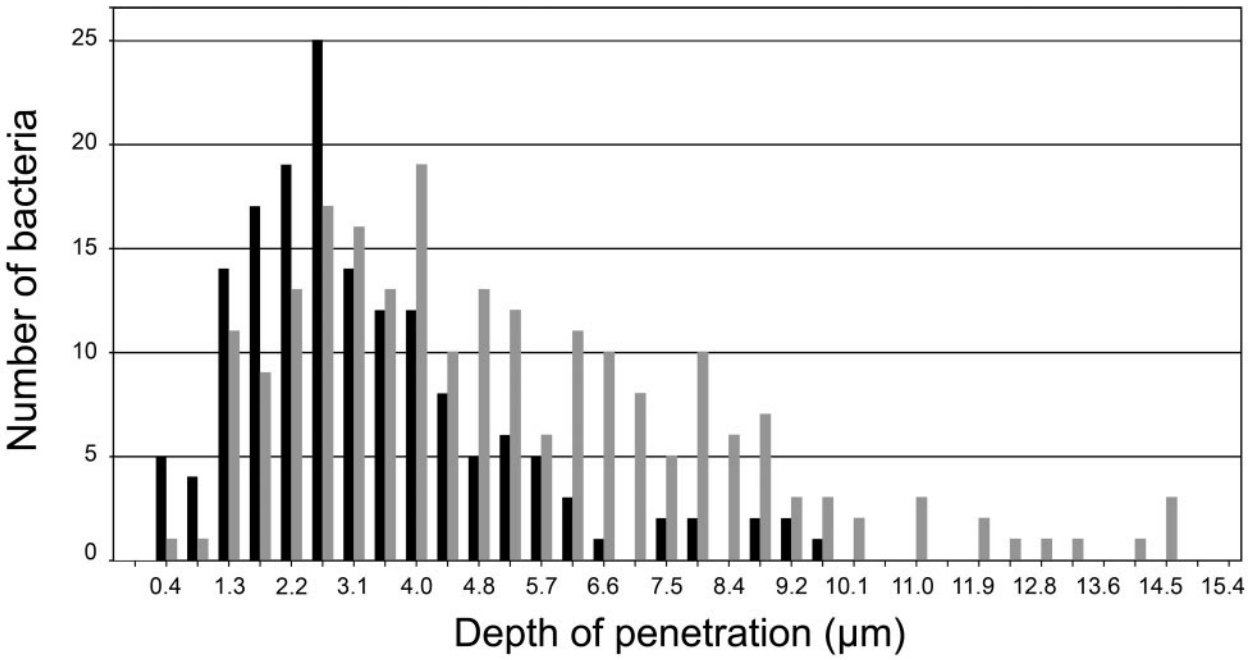

FIGURE 2. After instillation of 1.6 × 10^9 FITC-labeled OM12 bacteria into the inferior conjunctiva sac, 15.4 μm deep *z*-stacks of FAE tissue were collected by confocal microscopy. The penetration depth of each bacterium was determined by measuring the lowest optical section plane that it appeared in after a 66-minute (■) or 127-minute (▨) in vivo incubation. Bacteria were found to move deeper into the follicle with increasing time.

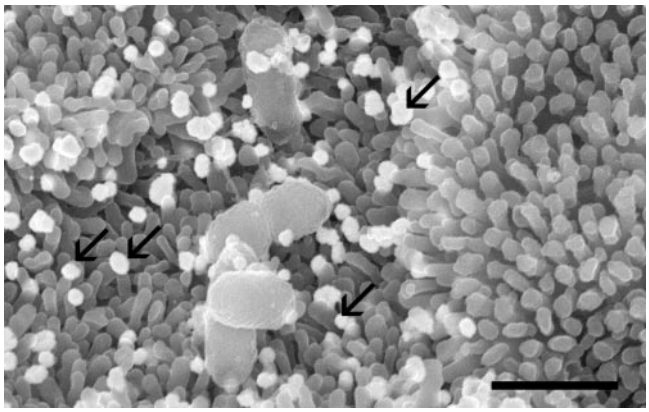

FIGURE 3. Preferential adherence of OM12 bacteria to the apical surface of conjunctival FAE cells expressing α(2-3) sialic acid on their surface. Excised conjunctival tissue was exposed to NTHi for 60 minutes on ice. After rinsing and aldehyde fixation, M-cell surfaces were labeled with biotinylated MAL-I. Gold-conjugated antibodies against biotin and gold enhancement was used to generate large colloidal gold deposits (*arrows*) on the surface of M cells. Bacteria were found exclusively on the apical surfaces of cells that were also labeled with MAL-I. Scale bar, 1 μm.

spp and *Staphylococcus epidermidis* are often found in the conjunctiva.[6,21,23] Bacterial colonization is prevented by innate immune factors in the tear film, such as lysozyme, lactoferrin, and lactoperoxidase, as well as adaptive immune responses generating bacteria-specific IgA. IgA prevents colonization by binding to bacterial adhesins and preventing bacteria adhesion to the mucosal surfaces. IgA-mediated agglutination of bacteria may also play a role in limiting colonization. IgA antibodies against NTHi, *S. epidermidis*, *P. aeruginosa*, *Escherichia coli*, and herpes simplex virus have all been found in the tears of asymptomatic human volunteers.[4–6] The present studies suggest a mechanism for the initiation of the local mucosal immune response against commensal or transient ocular bacterial species.

Adherence and subsequent colonization are essential first steps in NTHi pathogenesis, and these bacteria have evolved multiple adhesins.[24] One of the better characterized adhesins, HMW1, a high-molecular-weight protein expressed on the surface of 51% to 80% of clinical isolates,[24,25] mediates adhesion to epithelial cells via α(2-3) linked sialic acid.[11] *Maackia amurensis* agglutinin (MAA), which generally refers to an unfractionated mixture of MAL-I and MAL-II, has been shown to reduce OM12 binding to Chang epithelial cells by 74%.[11] In the present study, MAL-I reduced OM12 uptake by 61%. These observations indicate that OM12 binding to guinea pig conjunctival M cells is partially mediated by HMW1. It is likely, however, that OM12 expresses other adhesins that assist in its binding to M cells. In addition, it is likely that M cells have other receptors that have broad specificity for both Gram-positive and Gram-negative bacteria. Two recent papers highlight a possible role for Toll-like receptors (TLRs) expressed on M cells in the intestinal tract.[26,27] TLRs are a family of pattern recognition receptors which recognize conserved motifs of bacterial and viral pathogens. Further studies are needed to determine whether TLRs and other pattern recognition receptors may play a role in M cell sampling in the conjunctiva.

The guinea pig conjunctiva is not the only site with M-cell α(2-3) linked sialic acid glycoconjugates. The attachment and translocation of reovirus by M cells in the rabbit Peyer's patch has been shown to be mediated by α(2-3) linked sialic acid–containing glycoconjugates. The sialic acid–binding lectins MAL-I and -II inhibit binding by reovirus to these cells.[28] Because MAL-II bound to the surface of all rabbit intestinal cells, both inside and outside the FAE, these investigators concluded that the sialic acid epitope may be on a membrane component common to all intestinal epithelial cells but that larger ligands such as reovirus can only interact with it on M cells, because they have a reduced glycocalyx. Although the glycocalyx on guinea pig conjunctival epithelial cells is less extensive than in the intestinal tract, it still forms a dense coat extending 300 nm out from the microvilli and microplicae of the surface.[29] Less is known about the glycocalyx above the follicle-associated epithelium of the conjunctiva, but M-cell microvilli are sparse and pleiomorphic, which would be likely to lead to a reduced, less uniform glycocalyx.[2] It is important to note that in the guinea pig conjunctiva, the M cell is the only epithelial cell type that expresses α(2-3)-linked sialic acid on the apical surface, and therefore it may be associated with an M cell-specific glycoprotein or glycolipid.[2]

NTHi may not be the only Gram-negative bacteria that cross the conjunctiva via M cells. The guinea pig conjunctiva has been used for many years to test for bacterial invasiveness, by using the so-called "Sereny test." The Sereny test is a routinely used assay for *Shigella* virulence in which *Shigella flexneri* are inoculated into the conjunctival sacs of guinea pigs and the degree of keratoconjunctivitis is evaluated.[30,31] Because *S. flexneri* are unable to penetrate the apical membranes of polarized cells,[32] it is reasonable to assume they are crossing conjunctival M cells and triggering inflammation in a manner identical with their entry across the intestinal mucosa via Peyer's patch M cells.[33] Of note, two other bacterial species that test positive in the Sereny test, *Salmonella typhimurium*[34] and *Yersinia enterocolitica*,[35] are also known to cross the intestinal mucosal barrier via M cells.[36]

The absence of mucosal lymphoid follicles and their associated M cells in the conjunctiva of mice and rats has delayed the recognition of this important cell type in ocular mucosa. Mice and rats do have extensive nasal-associated lymphoid tissue (NALT) and drainage from the nasolacrimal duct that may take the place of CALT. The absence of CALT in mice and rats suggests that they may be poor models for the study of human ocular allergy or microbial uptake, since human CALT is more similar to that of the rabbit and guinea pig. In humans,

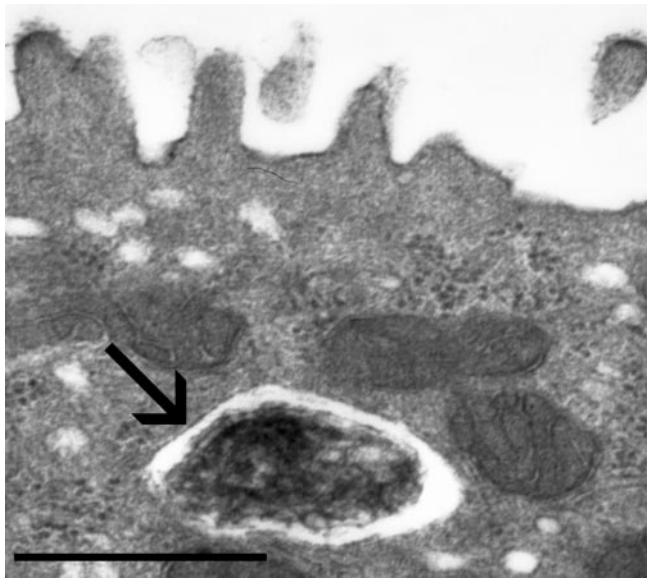

FIGURE 4. Conjunctival M cells containing a vesicular bound bacterium (*arrow*) 61 minutes after instillation of OM12 into the inferior conjunctival sac. The M cell can be recognized by its sparse, pleiomorphic microvilli. Scale bar, 0.5 μm.

CALT is initially absent in full-term infants, but follicles are routinely observed by 1 year of age. An average of 33 follicles/eye is found in children 6 to 10 years of age.[37] Consistent with other follicular tissue, CALT involutes with age but more than half the eyes in elderly patients have an average of 10 follicles.[38] M cells have yet to be characterized as part of the normal human CALT, but it would be surprising if they were not, based on their presence in the guinea pig and rabbit.

Future studies are needed to determine whether clinically important human ocular pathogens such as herpes simplex virus use conjunctival M cells as a port of entry. In addition, it may be possible to use M-cell targeted immunogens as vehicles to elicit local mucosal immune responses against microbial pathogens or ocular allergens.

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