M Cell–Targeted Ocular Immunization: Effect on Immunoglobulins in Tears, Feces, and Serum

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PURPOSE. This study investigates whether antigen-sampling M cells, present in the follicle-associated epithelium (FAE) above organized conjunctiva-associated lymphoid tissue in rabbits, bind and retro-transport secretory IgA (sIgA) from the tear film. The hypothesis that IgA-mediated uptake of antigens promotes local and systemic production of immunoglobulins was tested.

METHODS. sIgA binding and retro-translocation by M cells was characterized by immunocytochemistry. Immunoglobulin concentrations in tears, feces and serum were measured using enzyme-linked immunoassays (ELISA) after topical and systemic immunization with either goat IgG anti-rabbit IgA or nonspecific goat IgG.

RESULTS. Endogenous sIgA was found associated with the apical membrane of conjunctival M cells. Exogenous anti-IgA immunoglobulins were translocated across M cells. Significant levels of sIgA against goat IgG were present in tears of pre-immune animals. Topical application of either goat IgG specific for rabbit IgA or nonspecific goat IgG led to similar increases in antigen-specific IgA in tear, feces, and serum. The antigen-specific IgG response in tears mirrored the serum response for both immunogens consistent with transudation of this immunoglobulin. The IgM response in tears and serum was weak for both immunogens. Systemic immunization did not sustain or enhance the local mucosal IgA responses.

CONCLUSIONS. Conjunctival M cells bind and translocate sIgA from the tear film. Topical conjunctival immunization leads to generation of antigen-specific immunoglobulins from both local and distant mucosae and in serum. Natural antibodies, present in the tear film before immunization, may have contributed to similar immune responses to goat anti-rabbit IgA and nonspecific goat IgG. (*Invest Ophthalmol Vis Sci.* 2010;51:1533–1539) DOI:10.1167/iops.09-4491

Lymphoid follicles are found in close association with the epithelium of the conjunctival mucosae in humans, rabbits, Guinea pigs, dogs, pigs, and many other mammals.¹ The conjunctival follicle associated epithelium (FAE), as in other mucosae, contains a unique cell type, the M cell, which can selectively bind and translocate proteins, latex beads, and bacteria.^{2–4} In other mucosae, M cell translocation delivers soluble and particulate antigens, as well as pathogens, to antigen-presenting cells (APC) in the underlying follicle.⁵ The APC are then poised to present the digested material to lymphoblasts

within the follicle. The primed lymphoblasts, both in the follicle or after traveling to a draining lymph node, undergo proliferation and differentiation into IgA-secreting plasma cells and activated T-cells. These effector cells migrate back to both the original and distant mucosal sites to participate in the effluent immune response.

In the intestine, M cells have been found to bind secretory IgA (sIgA) present in luminal fluid and retro-transport these antibodies into the underlying lymphoid tissue where they can be endocytosed by APC cells.^{6,7} This characteristic has been exploited in the mouse to show that oral administration of reconstituted sIgA consisting of mouse polymeric IgA plus human secretory component evokes both a mucosal and systemic immune response to the human secretory component in the absence of any mucosal adjuvant.⁸

To further demonstrate that conjunctival M cells are equivalent in function to M cells in other mucosae, their ability to bind and translocate sIgA from the tear film is shown in the present article. A goat IgG with a binding specificity for rabbit IgA was then used to test the hypothesis that heterologous antibody binding to IgA would be the equivalent of IgA binding to its antigen and mimic the natural complexes that form when any specific rabbit sIgA binds to its target antigen in situ. It was anticipated that the binding might trigger conformational changes in the sIgA, which when combined with the relatively large size of the resulting goat IgG-rabbit sIgA macromolecular complex, would promote its preferential uptake via M cells and lead to a stronger immune response than a corresponding control immunization with nonspecific goat IgG. In addition, the ability of ocular immunization to trigger IgA secretion in a distant mucosal site, the colon, and to raise serum immunoglobulin levels was investigated.

MATERIAL AND METHODS

Animals, Topical Application Procedure, and Surgical Technique

New Zealand White female rabbits, between 12 and 15 weeks old, were obtained for our initial morphology experiments (Harlan Laboratories, Indianapolis, IN) and for the immunization studies (Myrtle's Rabbitry, Thompson's Station, TN). Ketamine (35 mg/mL) and xylazine (5 mg/mL) were used for anesthesia. Beuthanasia (100 mg/kg) was used to euthanize the rabbits before tissue collection. 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.

Tissue Processing for Fluorescent Microscopy

Ten μ L drops of 1 mg/mL goat anti-rabbit IgA conjugated to fluorescein (Bethyl Laboratories, Montgomery, TX) were added under the superior and inferior conjunctiva six times at 10-minute intervals. The tissue was then isolated, fixed for 2 hours in 2% paraformaldehyde in HWB (70 mM NaCl, 30 mM HEPES, 2 mM CaCl_2 , pH 7.4) + 300 nM 4',6-diamidino-2-phenylindole (DAPI). The inclusion of fluorescent nu-

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clear dyes such as DAPI simplifies identification of follicles using fluorescent stereomicroscopy.⁹ Isolated follicles, along with unexposed control follicles, were dehydrated with a series of increasing concentrations of ethanol, infiltrated in butyl-methylmethacrylate (BMMA) resin, and polymerized at 4°C. For post-embedding immunocytochemistry, a goat anti-rabbit IgA (Bethyl Laboratories) was biotinylated using an NHS-PEO₄ biotinylation kit according to the manufacturer's instructions (Pierce, Rockford, IL). Sections of 0.5 μ m were etched for 10 minutes in acetone before antigen retrieval by exposure to 100 mM glycine (pH 9.6) at 98°C for 30 minutes, blocked with 1% bovine serum albumin (BSA) in HWB for 1 hour, and stained overnight with 20 μ g/mL biotinylated goat anti-rabbit IgA, followed by 4 hours in 5 μ g/mL streptavidin-Alexa 488 (Invitrogen, Carlsbad, CA) + 300 nM DAPI.

Electron Microscopy

For electron microscopy studies, 100 μ L of 0.5 mg/mL biotinylated mouse anti-rabbit IgA (BD Pharmingen, San Diego, CA) was instilled under conjunctival surfaces for 40 to 60 minutes. After removal, tissues were rinsed in PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) and fixed in 2% paraformaldehyde + 0.2% glutaraldehyde + 300 nM DAPI in HWB for 2 hours. After rinsing extensively in 50 mM glycine in HWB, follicle regions were dissected out with the aid of a fluorescent stereomicroscope.

For scanning electron microscopy (SEM), isolated follicles were incubated in 1% acetylated bovine serum albumin (BSAc; Electron Microscopy Sciences, Hatfield, PA) for 45 minutes to block nonspecific binding and then incubated on a drop of a 1:10 dilution of goat anti-biotin IgG-conjugated to 10 nm colloidal gold (Electron Microscopy Sciences) for 2 hours. After rinsing in deionized water, the size of the gold particles was increased using a kit according to the manufacturer's recommendation (GoldEnhance EM; Nanoprobes, Yaphank, NY). 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). SE and BSE signals were overlaid by using image-management software (Photoshop; Adobe Systems, San Jose, CA).

For transmission electron microscopy (TEM), tissues were dehydrated and embedded in LR Gold resin (Electron Microscopy Sciences). Ultrathin sections were blocked for 30 minutes in Aurion goat gold conjugate blocking solution (Electron Microscopy Sciences) and then incubated for 2 hours on 25 μ L drops of 6.5 μ g/mL mouse anti-biotin antibodies in 0.1% BSAc, followed by 2.5 hr on a drop of a 1:40 dilution of goat anti-mouse IgG conjugated to 10 nm colloidal gold (Electron Microscopy Sciences). Sections were counter-stained with uranyl acetate and lead citrate.

Immunization

An affinity-purified goat IgG against rabbit IgA (Bethyl Laboratories) was used as the M cell targeted immunogen. This "specific goat IgG" (SGI) was expected to bind to sIgA in the tearfilm and be preferentially transported across the M cell. A pool of nonspecific goat IgG (NGI; Bethyl Laboratories) served as the control immunogen. A 22-mer unmethylated CpG oligodeoxynucleotide (ODN-2007) was used as the sole adjuvant in the topical ocular immunizations (Cell Sciences, Canton, MA).¹⁰ Aliquots of each immunogen (1 mg/mL) plus ODN-2007 (1 mg/mL) dissolved in PBS were kept at -78°C until application.

Topical administration of the immunogen plus ODN-2007 was delivered on days 0, 14, and 28 by 5 μ L eye drops each to the superior and inferior conjunctival surfaces of both eyes three times at 10-minute intervals for a total immunization of 30 μ g immunoglobulin plus 30 μ g adjuvant per eye each day. The rabbits were lightly anesthetized during the ocular immunization and their snouts slightly elevated to slow draining of the tears into the nasolacrimal duct. For systemic immunization, the immunogens (100 μ g) plus ODN-2007 (20 μ g) in 200 μ L of PBS were mixed with an equal volume of adjuvant (Imject Alum; Pierce) immediately before use and delivered intramuscularly on days

42 and 56. Tear, serum, and fecal samples were collected on days -7, 7, 21, 35, 49, and 63.

Sample Collection

Tears were collected using 16 $\times$ 2 mm polyester rods (Filtrona Fibertec, Colonial Heights, VA).¹¹ The absorbent rods were used to collect tears along the outer lid margin and medial canthus six times for 2 minutes at 5-minute intervals. At the end of each tear collection, the rod was placed in a 400 μ L microfuge tube with a small hole at its base that was then placed inside a 2 mL microfuge tube and centrifuged for 2.5 minutes at 16,000g at 4°C. At the completion of the six collections, the rods were spun for an additional 15 minutes. Total tear volume ranged from 5 to 23 μ L per eye. Tears were diluted five-fold with PBS + 0.1% BSA + 0.01% reagent (Micr-O-Protect; Roche Applied Science, Indianapolis, IN), aliquoted, and stored at -78°C until assayed.

Blood was collected from the marginal ear vein, allowed to clot for 2 hours at room temperature, centrifuged at 3000g for 15 minutes, aliquoted, and stored at -78°C until assayed.

Fecal samples were resuspended in PBS + 0.1% BSA + 0.05% reagent (Micr-O-Protect; Roche Applied Science) at a ratio of 1 mL PBS to 100 mg feces. After homogenization (Tekmar TissueMixer; Tekmar TissueMixer Co., Cincinnati, OH) at 50% power for 2 minutes on ice, the sample was centrifuged at 16,000g for 15 minutes. The supernatant was removed and re-spun for an additional 15 minutes, aliquoted, and kept frozen at -78°C until assayed.

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure specific IgA against goat IgG, 96-well polystyrene plates were coated with 100 μ L of 2.5 μ g/mL goat IgG (Bethyl Laboratories) in 50 mM sodium carbonate buffer (pH 9.6) overnight at room temperature. All time points from a single animal were measured on the same plate. After rinsing with wash buffer (PBS + 0.05% Tween-20) and blocking in 1% BSA in wash buffer for 4 hours, plates were incubated overnight with the standards and tear, fecal, or serum unknowns. Since a purified IgA specific for goat IgG was not available for use as a standard, a stock solution of a mixture of tears from multiple animals post-immunization was used as an arbitrary reference standard for all specific IgA ELISAs. All standards were run in triplicate, while unknowns were run in duplicate. Because the specific IgAs we were assaying might have retained free F_{ab} sites that could have reacted with the detection antibodies, an overnight blocking step with 2.0 μ g/mL nonspecific goat IgG was included at this point. For the detection step, each well was incubated with 100 μ L of 50 ng/mL goat anti-IgA (F_c class-specific) HRP-conjugated antibody (Bethyl Laboratories) overnight. After rinsing, 100 μ L of 1-step ultraTMB (Pierce) was added to each well for 30 minutes. After stopping the reaction by adding 100 μ L of 2 M sulfuric acid, the absorbance at 450 nm was read using a V_{max} microplate reader (Molecular Devices, Sunnyvale, CA). A standard reference curve was fit using a four-parameter logistic-log model; all ELISAs had squared correlation coefficients of >0.95. On average, four different dilutions of each unknown fell within the range of the standard curve and used to calculate the relative concentration; the average coefficient of variation for all time points was 10.3%.

To measure specific IgG, a protocol identical with the one for specific IgA was used except 50 ng/mL HRP-conjugated goat anti-rabbit IgG (F_c class-specific; Bethyl Laboratories) was used as the detection antibody, and affinity-purified rabbit anti-goat IgG (Bethyl Laboratories) was used as the standard.

To measure specific IgM, a protocol similar to the one for specific IgA was used, except 50 ng/mL HRP-conjugated goat anti-rabbit IgM (F_c class-specific; Bethyl Laboratories) was used as the detection antibody. Two ELISA plates, one for tear samples and the other for sera, were run using a 1:1000 dilution, in duplicate, of the unknowns from all the time points of all animals after pilot studies showed only low levels of specific IgM. Since all the samples were run on the same plate, the results are expressed as the resulting optical density minus the background signal of blank wells.

FIGURE 1. In vitro and in vivo staining with anti-rabbit IgA preferentially labels conjunctival FAE. (A) Semi-thin sections of conjunctival FAE were stained with biotinylated goat anti-rabbit IgA followed by streptavidin-Alexa 488. Staining of endogenous sIgA (green) is found on the microvilli of selected cells within the FAE. Intracellular vesicles within the apically stained cells are also labeled. Blood vessels in the follicle region exhibit green autofluorescence (arrow). Nuclei have been counterstained with DAPI (blue). (B) No rabbit IgG is seen bound to the FAE after immunolabeling with a goat anti-rabbit IgG F_c -specific primary antibody followed by donkey anti-goat IgG conjugated to Alexa 488. Additional controls with streptavidin-Alexa 488 in the absence of a biotinylated primary antibody were equally negative. (C) Conjunctival wholemount staining after a 60-minute in vivo exposure to goat anti-rabbit IgA conjugated to fluorescein. Follicles are identifiable as prominent mounds of brightly stained tissue in the fornix region after preferential binding of anti-IgA (green) to FAE cells. Tissue was visualized with fluorescent stereomicroscopy. Scale bar: (A, B) 20 μ m.

Statistics

Statistical software (InStat 3.0; GraphPad Software, La Jolla, CA) was used for analysis. Data are expressed as the mean $\pm$ SD. A repeated measures ANOVA with a Tukey multiple comparison post-test was used to compare pre-immune immunoglobulin levels to the various post-immune time points; a $P < 0.05$ was considered significant. An unpaired Student t -test was used to comparison of immunoglobulin responses to goat anti-rabbit IgA and nonspecific goat IgG at individual time points.

RESULTS

Morphologic Evidence of IgA Binding and Uptake by M Cells

Immunofluorescent labeling of tissue sections with goat anti-rabbit IgA antibodies resulted in labeling of plasma cells and blood vessels within the conjunctival lamina pretrial, but when the region of interest was focused on the apical membranes exposed to IgA in the tear film, labeling was restricted to intense staining of microvilli on scattered cells in the FAE (Fig. 1A). Labeled cells frequently had characteristics such as longer microvilli or intraepithelial pockets filled with lymphocytes or antigen-presenting cells. Similarly, after topical instillation of FITC-conjugated goat anti-rabbit IgA in vivo, intense labeling was restricted to patchy regions of the dome-shaped lymphoid follicles (Fig. 1C). After in vivo administration of mouse anti-rabbit IgA, SEM showed that immunogold labeling was restricted to FAE cells that had microvilli that were longer, more irregular, and less densely packed than those on the surrounding cells (Fig. 2). Earlier studies have demonstrated that these surface characteristics are associated with conjunctival M cells that bind and translocate latex beads.² Similarly, when TEM was used to view conjunctiva exposed to biotinylated mouse anti-rabbit IgA in vivo, gold labeling was found within intracellular vesicles and on the basolateral membrane of cells with typical M cell morphology including irregular microvilli, a thin band of cytoplasm bridging an intraepithelial pocket, and an extensive tubulovesicular network (Fig. 3). Labeling was also found within vacuoles of lymphoid cells in intraepithelial pockets along the lateral borders of the M cells.

Response to Immunization

Specific IgA against Goat IgG. No sign of inflammation or allergic response to topical administration of either immuno-

gen was observed. The concentration of specific sIgA recovered from the left and right eyes of an individual animal had an average difference of only $25.6\% \pm 19.6\%$ so all data are presented here as the average of the two eyes.

The specific IgA ELISA found that even a 1:10,000 dilution of the tears from the pre-immune animals resulted in an OD_{450} reading of 0.25 ± 0.18 above background when using goat IgG as the target antigen.

Both immunogens triggered similar increases in specific sIgA in tears that peaked at day 35, 1 week after the third ocular immunization (Fig. 4A). Goat IgG anti-rabbit IgA (SGI) elicited a 17.8-fold increase over pre-immune levels and non-specific goat IgG (NGI) caused a 19.1-fold increase. Systemic immunizations were not able to maintain the tear sIgA response. Unexpectedly, there was never a significant difference ($P > 0.15$) between the relative magnitudes of the response to SGI compared with NGI at any time point.

Ocular immunization with both immunogens also caused similar increases in specific sIgA in fecal extracts at day 35 (SGI:

FIGURE 2. Preferential adherence of biotinylated mouse anti-rabbit IgA to the apical surface of conjunctival M cells after an in vivo exposure for 42 minutes followed by detection with a gold-conjugated secondary antibody and gold enhancement. The BSE image has been overlaid on the SE image to show that gold labeling is restricted to FAE cells with the characteristics associated with M cells; longer, more pleiomorphic, and less densely spaced microvilli than surrounding cells. Note the exceptionally long microvillus at the bottom of the image. Scale bar: 2 μ m.

FIGURE 3. Anti-rabbit IgA transcytosis by conjunctival M cells after *in vivo* exposure to biotinylated goat anti-rabbit IgA for 53 minutes. LR Gold sections were immunostained with a mouse anti-biotin antibody followed by a gold-conjugated goat anti-mouse IgG secondary antibody to detect anti-IgA uptake. A thin cytoplasmic bridge of an M cell spans over an intraepithelial pocket filled with lymphoid cells that have a more lightly stained cytoplasm. Anti-IgA gold labeling is seen

within the M cell's tubular-vesicular endosomes and along its basolateral membrane (*white arrows*). Gold-labeling of anti-IgA (*black arrows*) can also be seen along the outer rim of a vacuole (*asterisk*) within the intraepithelial lymphoid cell (Ly). Scale bar: 1 μ m.

11.2-fold; NGI: 11.1-fold; Fig. 4B). Fecal specific sIgA dropped in fecal extracts at day 49 after systemic immunization with SGI but rose after NGI immunization. For both immunogens, the concentration of specific IgA in feces was highly correlated ($r^2 = 0.99$) with the concentration in tears during the first 5 weeks when only topical immunization was being used. When the responses to both ocular and systemic immunization over the whole 9 weeks were compared, the correlation between tear and fecal concentrations dropped slightly ($r^2 = 0.91$ for SGI; $r^2 = 0.95$ for NGI).

Both immunogens elicited similar increases in serum specific IgA at day 35 (SGI: 4.8-fold; NGI: 5.3-fold; Fig. 4C). There was a transient rise in serum specific IgA in response to systemic immunization by either immunogen, albeit stronger for NGI. After the first systemic injection, the response to SGI increased to 8.6-fold and 22.5-fold for NGI on day 49. The second systemic immunization was not able to maintain the increase and the response to both immunogens declined by day 63 (SGI: 6.6-fold; NGI: 10.5-fold). The correlation between tears and serum specific IgA in serum was highly correlated during the first 5 weeks when only ocular immunization was being used ($r^2 = 0.96$ for SGI; $r^2 = 0.99$ for NGI), but dropped off significantly once the responses to the systemic immunizations were added to the comparison ($r^2 = 0.761$ for SGI; $r^2 = 0.80$ for NGI).

Specific IgG against Goat IgG. Tear and serum specific IgG levels were increased by both immunogens after topical ocular immunization and even further after systemic immunization (Fig. 5). For both tears and serum, at any given time point, the IgG response to SGI was never significantly different ($P > 0.05$) than that to NGI. For both immunogens, the concentration of specific IgG levels in tears showed a high degree of correlation ($r^2 = 0.99$) with serum levels over the full 9-week study.

Specific IgM against Goat IgG. Tear specific IgM appeared slightly stronger on days 35 and 49 for both immunogens but only the day 35 response to NGI was statistically significant (Fig. 6A). Ocular immunization with SGI had little effect on specific IgM in serum, and the only statistically significant increase was a 4.2-fold increase on day 49 after the first systemic immunization (Fig. 6B). The specific IgM response to NGI was greater (3.5-fold higher on day 35 and a 13.3-fold increase at day 49).

DISCUSSION

Endogenous sIgA was found selectively bound to apical membranes of M cells but not other epithelial cells in the conjunctiva. Exogenous anti-rabbit immunoglobulin probes were translocated across the M cells into the underlying intraepithelial pockets filled with antigen-presenting cells.

Topical ocular immunization was found to induce secretion of specific immunoglobulins at both local and distant mucosae consistent with the concept of a common mucosal immune system.¹² The morphologic evidence of selective uptake of tracers labeled with anti-IgA suggests that conjunctival M cells played a major role in initiating this immune response. The topically applied immunogens used in the present study, however, would have drained along the nasolacrimal duct. Nasolacrimal ducts in humans are known to have their own organized mucosa-associated lymphoid tissue and, therefore, possibly M cells.^{13,14} Furthermore, any immunogen exiting the nasolacrimal duct could have been refluxed back up into the nasal cavity where it could have interacted with the nasal-associated lymphoid tissue, which is known to contain M cells.^{15,16} Future studies will need to dissect out the role of conjunctiva-associated lymphoid tissue from that of the nasolacrimal duct or nasal cavity and to determine whether nasal immunization is equivalent to topical conjunctival immunization in regards to secretion of specific IgA in tears.

The ability of sIgA to selectively bind to the surface of M cells was first observed in the neonatal rabbit intestine and later extended to the adult rabbit intestine.^{17,18} Weltzin and coworkers subsequently demonstrated that intestinal M cells preferentially translocated IgA-coated gold particles and postulated that this would enhance or sustain the initial immune response.⁶ Mantis and coworkers extended these observations by showing murine Peyer's patch M cells bind IgA but not IgG or IgM, and that human pediatric ileal M cells also bind IgA.⁷ The IgA receptor responsible for M cell binding is unknown but distinct from the classic polymeric Ig receptor or asialoglycoprotein receptor.⁷ Cortes and coworkers were the first to exploit the re-uptake of sIgA to deliver an immunogen. They found that oral administration of rabbit sIgA that had been genetically modified to carry a bacterial epitope elicited specific antibody responses in both serum and saliva of mice.¹⁹

The observation that there was little difference in the immunogenic response to ocular immunization with either SGI or NGI was initially surprising but can be explained by the presence of natural antibodies against goat immunoglobulins present in tears before topical immunization. In addition to highly specific antibodies induced by antigens, it is widely recognized that mammals, including rabbits and humans, make significant amounts of IgA, IgG, and IgM "natural" antibodies that are present before exposure to a specific antigen.²⁰⁻²⁹ Natural sIgA antibodies are better characterized in the intestinal tract but have been demonstrated in tears of infant humans.²⁵ In the present study, the presence of natural sIgA antibodies able to bind goat immunoglobulins is evinced by the relatively high signal present in the ELISA of pre-immune tear samples. We believe the natural antibodies present in tears at the start of the study resulted in uptake of both immunogens

A - specific IgA in tears

B - specific IgA in feces

C - specific IgA in serum

FIGURE 4. ELISA measurements of rabbit IgA against goat IgG in tears (A), feces (B), and serum (C). Responses to topical immunization on days 0, 14, and 28 followed by systemic immunization on days 42 and 56 with goat anti-rabbit IgA (gray bars) or nonspecific goat IgG (black bars). A pool of tears from multiple animals post-immunization was used as an arbitrary reference standard for all specific IgA ELISAs. Statistical significance of each measurement compared with its own pre-immune concentration is noted by an asterisk above the bar. * $P < 0.05$.

and similar strong antigen-induced sIgA responses. Indeed, the presence of polyreactive natural sIgA, combined with the ability of M cells to transcytose sIgA, may be a principal mechanism by which the M cell initially captures novel antigens to induce antigen-specific responses. Cortes has postulated that sIgA binding to its antigen causes a conformational change that increases its affinity for the M cell IgA receptor.²⁴ Our experimental design to use goat anti-rabbit IgA binding to increase

uptake may have not triggered the conformational changes that IgA binding to its antigen would and therefore given SGI no competitive advantage over NGI since both of these goat immunoglobulins would have been equally targeted by same natural antibodies. It must also be realized that our hypothesis predicts that after one or two initial immunizations, even a modest antigen-induced specific IgA response in tears would promote uptake of the antigen during subsequent boost immunizations and make it difficult to compare an "M-cell targeted immunogen" from an "untargeted" protein.

Three characteristics of natural antibodies may explain the presence of an anti-goat IgG in the pre-immune tears despite the fact that rabbits had never been exposed to any goat proteins. First, natural antibodies tend to be polyreactive or capable of binding multiple epitopes, and the promiscuous nature of their binding may have simply fortuitously included an epitope on goat IgG.^{22,28,29} Secondly, rabbit natural antibodies have previously been shown to bind high-mannose residues on HIV-1 envelope glycoprotein and terminal galactosyl residues on glycolipids, thus raising the possibility that the natural antibodies in tears are reacting with carbohydrate residues on goat IgG.^{21,26} A third alternative is that natural antibodies in tears may represent autoantibodies against rabbit IgG that are cross-reacting with the goat IgG. Numerous studies have demonstrated natural autoantibodies against IgG in the serum of healthy humans and other species.^{20,27-29} The func-

A - specific IgG in tears

B - specific IgG in serum

FIGURE 5. ELISA measurements of specific rabbit IgG against goat IgG in tears (A) and serum (B). Responses to topical immunization on days 0, 14, and 28 followed by systemic immunization on days 42 and 56 with goat anti-rabbit IgA (gray bars) or nonspecific goat IgG (black bars). Affinity purified rabbit anti-goat IgG was used as the standard. Statistical significance of each measurement compared with its own pre-immune concentration is noted by an asterisk above the bar. * $P < 0.05$.

FIGURE 6. ELISA measurements of rabbit IgM against goat IgG in tears (A) and serum (B). Responses to topical immunization on days 0, 14, and 28 followed by systemic immunization on days 42 and 56 with goat anti-rabbit IgA (gray bars) or nonspecific goat IgG (black bars). Each bar represents the average $\pm$ SD of the OD₄₅₀ signal generated by a 1:1000 dilution of tears or serum. Statistical significance of each measurement compared with its own pre-immune concentration is noted by an asterisk above the bar. * $P < 0.05$.

tions of natural autoantibodies, in particular those that are anti-IgG, are not fully understood, but it has been hypothesized that autoantibodies that were anti-IgG Fc would facilitate the clearance of soluble complexes from the circulation.³⁰ In regards to the present study, sIgA against IgG Fc might act to selectively capture and retro-transport any IgG that has entered tears by transudation and then interacted with its target antigen. According to our hypothesis, such a mechanism would then promote the production of sIgA specific for the same antigen as the IgG that was sampled. Autoantibodies against IgG in tears might also inhibit complement reactions from inflaming the surface of the conjunctiva when transudated IgG encounters its target antigen. Characterization of the natural antibodies in tears is outside the scope of the present study but clearly an area needing further study.

Topical immunization had a modest effect on serum IgG levels. The high correlation between specific IgG levels in tears and serum is consistent with tear IgG coming via transudation from serum.^{31–33} The paucity of specific IgM in tears after either topical or systemic immunization indicates that neither the polymeric Ig receptor pathway nor transudation from serum contributes significant levels of this immunoglobulin species to tears.

Intramuscular immunization after nasal immunization has been reported to enhance or maintain mucosal and systemic

antibody responses to protein antigens.^{34,35} In the present study, however, intramuscular immunization was unable to maintain the IgA response in tears that had been initiated by ocular immunization. This difference highlights the subtle influence that the site of mucosal immunization has on the immune response.

The finding that conjunctival M cells bind and translocate sIgA is further evidence that conjunctival M cells are equivalent to their counterparts in intestinal, nasal, and respiratory mucosae. As with M cell-mediated immune responses in the intestine and nasal cavity, topical conjunctival application of a large protein plus CpG ODN adjuvant triggered strong local and distant mucosal immune responses. The regulation of the ocular immune response, and the role conjunctival M cells and organized mucosa-associated lymphoid tissue plays in it, remains incompletely understood. The potential for ocular immunization to modulate inappropriate immune responses in diseases such as ocular allergies or dry eye, as well as provide protection against pathogens such as herpes simplex virus or Chlamydia trachoma, underscores the need for future work in this area.

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