Local Production of Secretory IgA in the Eye-Associated Lymphoid Tissue (EALT) of the Normal Human Ocular Surface

Erich Knop, Nadja Knop, and Peter Claus

PURPOSE. Secretory IgA (SlgA) is a critical local defense mechanism of mucosal immunity. Although the conjunctiva, as part of the ocular surface, has a mucosa-associated lymphoid tissue, the production of SlgA by local plasma cells and its transport is unequivocally accepted to occur only in the upstream lacrimal gland (LG). The molecular components were therefore investigated by immunohistochemistry (IHC) and their local production verified by RT-PCR.

RESULTS. Plasma cells were present in the diffuse lymphoid tissue of all investigated specimens and showed an intense immunoreactivity for IgA. This immunoreactivity was absent when the antiserum was preadsorbed with the protein. The luminal epithelium, with the exception of goblet and basal cells, was strongly positive for the epithelial transporter molecule secretory component (SC) in the conjunctiva and interconnecting excretory duct similar to the LG. PCR products for IgA, the monomeric IgA-joining molecule (J-chain) and SC were regularly found in all conjunctival zones and in the LG in gel electrophoresis and were sequenced.

CONCLUSIONS. The local production of SlgA is for the first time verified by RT-PCR in the human conjunctiva and in the LG. This finding points to an active role of the conjunctiva in secretory immune protection of the ocular surface and supports the presence and importance of EALT at the normal ocular surface. (Invest Ophthalmol Vis Sci. 2008;49:2322–2329) DOI:10.1167/iovs.07-0691

Secretory IgA (SlgA) forms a first line of defense at mucosal surfaces which also include the ocular mucosa, consisting of the ocular surface proper (conjunctiva and cornea) and its continuously connected mucosal adnexa composed of the lacrimal gland (LG) and lacrimal drainage system, that together form an anatomical and functional unit. The ocular surface and lacrimal drainage system represent a mucosa, similar to that of the intestine and airways, along with a large associated gland, the lacrimal gland (LG). The ocular mucosa is directly and constantly exposed to the external environment, which puts it at risk of microbial invasion and allergic disease. To counter these environmental insults, the mucosa is supported by an array of defense mechanisms.

In addition to the innate defense, which is composed of nonspecific cells and antimicrobial molecules including lysozyme, lactoferrin, and defensins, components of the specific immune system also occur at the ocular surface. Lymphocytes and plasma cells are known to reside at the ocular surface, but their significance is unclear.

In a historical misconception, they were considered as "inflammatory cells." It is now known that mucosal organs contain a part of the specific immune system, termed mucosa-associated lymphoid tissue (MALT). When this concept was applied to the ocular surface, it led to the description of a conjunctiva-associated lymphoid tissue (CALT) in the rabbit and in other species.

Evidence of a mucosal immune system at the human ocular mucosa has increased over the years. Lymphocyte subpopulations including mucosa-specific ones, along with accessory structures such as specialized high endothelial venules (HEVs) for their migration, have been shown. The universal presence of CALT was verified in a large number of normal human conjunctival wholemounts. Later, lymphoid tissue was shown in the lacrimal drainage system, which is now termed the lacrimal drainage-associated lymphoid tissue (LDALT). Lymphoid populations at the ocular surface and adnexa are therefore statistically normal and have a physiologic function. Their accepted presence in conjunctiva and lacrimal drainage system along with the LG has led to the concept of the eye-associated lymphoid tissue (EALT) according to the established nomenclature of mucosal immunology and representing a new component of the mucosal immune system of the body.

SECRETORY IMMUNITY is one of the best-defined defense mechanisms in the mucosal system. It consists of the production of immunoglobulins by local plasma cells and their transport through the overlying epithelium to build up a protective layer at the mucosal surface. Mucosal immunoglobulins mainly consist of polymeric (pIgA), which is secreted as a 390-kDa dimer linked by the peptide joining chain (J-chain). pIgA is released into the connective tissue and binds to a 120-kDa transmembrane protein (poly-Ig receptor; pIgR) at the basolateral membrane of the epithelial cells which promotes the transport of its ligand pIgA through the transcytotic pathway. At the luminal surface, pIgA is cleaved together with an extracellular 80-kDa domain of its transporter, termed secretory component (SC), resulting in secretory IgA (SlgA).
IgA is not restricted to luminal actions that prevent adhesion and invasion of antigens.\textsuperscript{1,2} It can also provide intracellular neutralization of virus particles inside the epithelial cells\textsuperscript{29} and has an excretory function that “cleans” the tissue via the vectorial transport of IgA-bound antigens.\textsuperscript{30} Conversely, IgA has also been characterized as a vehicle for selective antigen uptake by follicular M-cells, which may play a role in immune regulation.\textsuperscript{31} Furthermore, pIgR and SC can bind to bacterial antigens, are integrated into signaling networks, and exert innate immune functions that are assumed to link to adaptive immunity.\textsuperscript{31,32}

Because of the plethora of local functions inside the tissue, it can be assumed that a sole luminal supply of IgA via the tear film from the upstream lacrimal gland, which represents the presently established opinion on secretory immunity at the ocular surface,\textsuperscript{10,11,33,34} is not sufficient for immune defense. The molecular components of the human ocular secretory immune system have been insufficiently described to date and remain controversial. Although conjunctival plasma cells have been regularly observed by histology, IgA and SC has not been remain controversial. Although conjunctival plasma cells have been regularly observed by histology, IgA and SC has not been

Materials and Methods

Tissues

Complete tissues of the LGs and conjunctiva were obtained from the Department of Anatomy from body donors who had macroscopically normal ocular surfaces. The time after death was between 12 and 36 hours, and the bodies were stored cold. The donors had given previous informed consent, and the study protocol compiled with the Declaration of Helsinki and was approved by institutional review committee. The donors were of old age, and the distribution of the sexes was relatively even, with a slight predominance of women (Table 1). Tissue from 16 donors was used for IHC examination of the conjunctiva and from 7 of these also for examination of the LGs; conjunctiva and LG tissue from 6 additional randomly chosen donors was used for RT-PCR.

Table 1. Tissue Data

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Donors</th>
<th>Mean Age (SD)</th>
<th>Sex (F:M)</th>
<th>Used (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>Conjunctiva</td>
<td>16</td>
<td>75.4 y (15.3)</td>
<td>10:6</td>
</tr>
<tr>
<td></td>
<td>LG</td>
<td>7</td>
<td>77.7 y (20.0)</td>
<td>4:3</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Conjunctiva and LG</td>
<td>6</td>
<td>76.3 y (8.9)</td>
<td>4:2</td>
</tr>
</tbody>
</table>

Histology and IHC

Fourteen of 18 conjunctival sacs and all 9 LGs were fixed in 4% paraformaldehyde and embedded in paraffin according to standard procedures. Four of 18 conjunctival sacs were embedded unfixed in OCT compound (Ted Pella Inc., Redding, CA) and snap frozen in liquid nitrogen. Serial sections were cut at a thickness of 5 μm from paraffin blocks (HM 3555 microtome; Microm, Walldorf, Germany) and 10 μm from cryo blocks (CM1500 microtome; Leica, Wetzlar, Germany). Cyro sections were fixed with cold acetone for 10 minutes after they were cut and then air dried. Consecutive parallel sections were stained with hematoxylin and eosin (HE).

After mild enzymatic pretreatment (0.1% trypsin for 5 minutes) for antigen retrieval, IHC was performed with the highly sensitive ABC technique\textsuperscript{36} using the following primary antibodies. Mouse monoclonal anti-IgA against the heavy chain (α-chain) of both IgA isotypes, IgA1 and IgA2 (M0728, dilution ×160; Dako, Hamburg, Germany) and rabbit polyclonal anti-SC against the secretory component from human colostrums (A0187, dilution ×1000; Dako) were incubated at 4°C overnight. Primary antibodies were detected with biotinylated secondary antibodies from the goat (Jackson/Dianova, Hamburg, Germany) and visualized by streptavidin-coupled peroxidase, both incubated for 30 minutes at room temperature. Diaminobenzidine (DAB; Hoechst, Ingelheim, Germany) was used as a chromogen. For immunofluorescence, FITC and Cy3 (Jackson/Dianova) were used, coupled either to streptavidin as before or directly to the secondary antibody, whereas cell nuclei were labeled with DAPI (4',6'-diamino-2-phenylindole; Sigma-Aldrich, Munich, Germany).

Polymerase Chain Reaction after Reverse Transcription

Primer Construction. Primers were designed according to sequence data from the National Center for Biotechnology Information (NCBI; National Institutes of Health, Bethesda MD) database (www.ncbi.nlm.nih.gov) and controlled for specificity (NCBI Blast). Primers were constructed for SC, human poly-Ig receptor transmembrane secretory component: IgA, Homo sapiens CH gene encoding immunoglobulin, constant region, heavy chain, α-2 subunit; and J-chain, Homo sapiens immunoglobulin J polypeptide. The respective primer sequences are shown in Table 2: primer oligonucleotides were manufactured by MWG Biotech (Ebersberg, Germany).

RNA Preparation and Reverse Transcription. Total cellular RNA from various ocular regions (see the Results section) of 12 eyes of six normal human individuals was isolated (RNeasy Mini Kit; Qiagen, Hilden, Germany). The cDNA was synthesized\textsuperscript{37} in a final volume of 40 μL with the following components: 2.5 μg total RNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 10 mM DTT, 0.5 mM of each dNTP, 3 μg random hexamer primer, 40 U RNase inhibitor, and 200 U Moloney murine leukemia virus reverse transcriptase. The mixture was incubated for 90 minutes at 42°C, heat inactivated for 15 minutes at 70°C, and stored at −20°C.

Table 2. Characteristics of Primers and RT-PCR Products

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primer Sequence</th>
<th>Coded Region (bp)</th>
<th>Intron-Spanning (bp)</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>Forward: 5'-tgc tct tag gtt cag aag cga acc-3'</td>
<td>654</td>
<td>222</td>
<td>433</td>
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<tr>
<td></td>
<td>Reverse: 5'-atg ccc aag tga gtt act tct cgc-3'</td>
<td>242</td>
<td>—</td>
<td>242</td>
</tr>
<tr>
<td>SC</td>
<td>Forward: 5'-aat gct gac ctc caa gtg cta aag-3'</td>
<td>895</td>
<td>—</td>
<td>895</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-atc acc aca cag atg gag cca tcc-3'</td>
<td>258</td>
<td>—</td>
<td>258</td>
</tr>
<tr>
<td>J-chain</td>
<td>Forward: 5'-cac aca cct taa ccc tga ctt ttt-3'</td>
<td>258</td>
<td>—</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-gct gct gtt gta gtc aca gca gac-3'</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

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Reverse Transcription–Polymerase Chain Reaction (RT-PCR). The cDNA was subjected to PCR amplification with specific primers for IgA, SC, and J-chain (Table 2). Primers for the constitutively transcribed (housekeeping) gene glyceraldehyde phosphate dehydrogenase (GAPDH) were used as an internal standard of input cDNA. PCR was performed on a thermocycler (Primus 25; MWG) with 2.5 μg of the RT reaction product in a 25-μL volume with 0.4 U Taq DNA polymerase (Roche, Mannheim, Germany). After a 3-minute denaturation step at 94°C, the reaction proceeded for 27 cycles of 60 seconds at 94°C, 60 seconds at 52°C, and 60 seconds at 72°C for IgA and SC. RT-PCR for J-chain and control runs for the other proteins were also performed with a commercial premixed kit (Taq PCR master mix; Qiagen) and amplified in 35 cycles on a thermocycler (Techne Genius; Biostep, Jahnisdorf, Germany) in the same conditions as before. PCR reaction products were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide and photographed with a gel documentation system (BioDocII; Biometra, Göttingen, Germany).

GAPDH was used as a methodological control for correct performance of the RT-PCR process. The LG tissue, which is a validated documentation system (BioDocII; Biometra, Göttingen, Germany). The omission of cDNA templates (water control).

RESULTS

Histology and IHC of the Human Ocular Secretory Immune System

Lacrimal Gland. Histologic sections of the human LG confirmed the presence of numerous plasma cells located around the secretory acini. They formed a diffuse lymphoid tissue together with other leukocytes, mainly lymphocytes (Fig. 1A). In IHC, the cytoplasm of the plasma cells stained intensely positive for IgA whereas the acinar epithelial cells stained weakly, with increasing intensity toward the apical pole and at the luminal surface (Fig. 1B). SC was absent from the plasma cells but showed a more homogeneous and much stronger staining in the acinar cells than did IgA (Fig. 1C).

Excretory Lacrimal Ducts. The excretory lacrimal ducts that leave the gland and open into the conjunctiva have a cellular sheet of diffuse lymphoid tissue with the same characteristics as in the LGs. The cells in the sheet consisted mainly of plasma cells and lymphocytes (Fig. 2A). The immunostaining characteristics for IgA and SC were also present at the duct. IgA intensely stained in the periductal plasma cells and only weakly inside the ductal epithelium, mainly in the apical cytoplasm and at the luminal surface (Fig. 2B). The staining for SC was intense in the epithelium and mostly restricted to the superficial layer of the two to three-layered pseudostratified epithelium. It also extended downward along the cell outline of the luminal cells, but excluded the basal cells (Fig. 2C) similar to the conjunctiva as shown below.

 Conjunctiva. In the human conjunctiva, the lymphoid cells that formed the subepithelial diffuse lymphoid tissue (Fig. 3A) had characteristics similar to those in the LGs and excretory duct. Plasma cells were identified, as in the LGs, by their large basophilic cytoplasm in HE staining that typically contained an eccentric nucleus with heterochromatin clusters and occasionally showed a brighter perinuclear zone corresponding to the Golgi apparatus (Fig. 3A, arrowhead). IgA antiserum intensely stained the plasma cells (Fig. 3B) but also local deposits and occasionally the luminal surface of the epithelium. SC was restricted to the superficial cell layers or frequently only to the most superficial layer of the conjunctival epithelium, similar to the ductal epithelium. Typically, the basal epithelial layer was unstained, and SC was not expressed in the goblet cells (Fig. 3C).

Double-labeling fluorescent IHC with DAPI counterstain of the nuclei (Figs. 4, triple fluorescence) clearly revealed that IgA and SC show a considerable similarity in the conjunctiva and LGs. Numerous IgA-positive plasma cells occurred in the lamina propria, whereas the overlying epithelial cells contained its transporter SC, except for the basal conjunctival layer and the goblet cells. The mixed orange-yellowish color of the apical epithelial cytoplasm and of the luminal surface indicated the presence of both proteins and hence represented SIgA. In the LGs this staining was detected inside luminal spaces of the tubuloacinar gland (Fig. 4A). Deposits of SC and sometimes also IgA were occasionally found in conjunctival epithelial cells (Fig. 4B) and may correspond to accumulation inside cytoplasmic organelles involved in the transcytotic pathway.
Verification of the IgA Antiserum Specificity by Preadsorption

The correct specificity of the IgA antiserum was examined by preadsorption with colostrum IgA protein (Sigma-Aldrich) before staining. The specific staining was blocked when this antiserum was used for IHC. This process was applied to dot blot experiments (Fig. 5A), where IgA was spotted as a target on nitrocellulose, and also to histologic sections (Figs. 5B–5E). A schematic model of conjunctival IgA transport derived from the observed staining pattern is shown in Figure 6.

Verification of the mRNAs by RT-PCR

To verify the local production of IgA, SC, and J-chain at the mRNA level, RT-PCR was performed using tissues from 12 further eyes of six other individuals. Tissue was obtained as small pieces from the LGs and as small mucosal strips from the conjunctiva of approximately 5 × 3 mm at the upper tarsorobital margin (UTO), at the middle area of the upper (UB) and lower (LB) bulbar conjunctiva, and at the upper perilimbal (UL) conjunctiva. These locations are indicated in a schematic drawing of a flat conjunctival wholemount, which also shows the topographical distribution of the respective conjunctival lymphoid tissue (Fig. 7A), and in a representation with opened lids, as seen in the clinical setting (Fig. 7B). The location of accessory LGs is indicated in both figures.

PCR products for IgA, SC, and GAPDH from the LG and UTO conjunctiva of fellow eyes were directly compared in the same amplification procedure (Fig. 7C). In all cases and with bilateral...
symmetry, PCR products of the expected size for IgA and SC formed a distinct, usually broad band in gel electrophoresis; this result also applied to the J-chain. The presence of GAPDH indicated that the PCR reactions were performed correctly. The presence of the PCR product for IgA without the intron-spanning region verified that genomic DNA was not involved in this amplification. The negative control tissue (muscle) did not reveal PCR products for the secretory proteins but still showed those for GAPDH, as expected.

Even though the investigated tarso-orbital location is clearly different from the typical location of accessory LGs as reported in the literature and as observed by us in stained translucent wholemounts, we intended in further experiments to exclude the theoretical possibility that accessory LG tissue with a known first time of the secretory in all could have accidentally contaminated the conjunctival specimens. Therefore, tissue of the middle areas of the upper (UB) and lower (LB) and of the UL conjunctiva was also investigated (Figs. 7D, 7E). IgA, SC, and J-chain were also expressed in all eyes in these locations when samples of the LG were compared with conjunctival specimens. Therefore, this result also applied to the J-chain. The presence of GAPDH, its transporter SC and J-chain were additionally sequenced (MWG Biotech) and the identity confirmed.

**DISCUSSION**

IgA-positive plasma cells were consistently found in the conjunctive tissue along with its transporter molecule SC in the overlying conjunctival epithelium of all normal human specimens investigated by IHC. Until now, this presence has been accepted as being only in the LGs. Moreover, the mRNAs for IgA, SC, and J-chain were shown to be present in both organs for the first time of the secretory in all could have accidentally contaminated the conjunctival specimens. Therefore, tissue of the middle areas of the upper (UB) and lower (LB) and of the UL conjunctiva was also investigated (Figs. 7D, 7E). IgA, SC, and J-chain were also expressed in all eyes in these locations when samples of the LG were compared with conjunctival locations (Fig. 7D) and when tarso-orbital were compared with the UL and LB samples (Fig. 7E). PCR products for IgA, its transporter SC and J-chain were additionally sequenced (MWG Biotech) and the identity confirmed.

In previous reports, the evidence of the presence of plasma cells on the normal human ocular surface is clear in histologic studies because of their unmistakable morphology, but reports for IgA and SC were inconsistent with IHC staining, which were based on relatively few tissues, including pathologic clinical biopsies from ocular surface disease. Other explanations, for example, rabbit, seemed to support the negative human data and led to the opinion that the human conjunctiva does not represent a part of the secretory immune system. However, it is now known that the rat and mouse conjunctiva contain almost no lymphoid cells that differ distinctly from the human and, for example, rabbit, and is therefore not an ideal model. In contrast to IHC, functional evidence for the production of IgA and SC in the human conjunctiva was obtained by an in vitro study.

The lack of consensus on the presence of SC in the conjunctiva may in part be because it was originally described in the acinar epithelium of salivary glands and later mainly in other monolayered epithelia, such as the intestine, that are structurally different from the ocular surface. Still, the absence of SC from goblet cells observed in the present study resembles the results in the intestine. In fact, SC is interestingly even regarded as a marker for intact terminal epithelial differentiation.

In the present study, we have shown that (1) the secretory proteins were consistently found in IHC of numerous normal human tissues, (2) the specificity of the IgA antisera was verified by preadsorption with the protein, (3) the local presence of mRNAs for the secretory proteins was verified by RT-PCR and the PCR products were sequenced, (4) the tissue locations used are clearly different from the typical location of accessory LGs. It can thus be concluded that the normal human conjunctiva is a part of the secretory immune system, with local production of SlgA.

**Functions of SlgA in Ocular Surface Protection**

SlgA is one of the most important effector mechanisms of the mucosal immune system and also represents an important defense mechanism at the ocular surface. Specific IgA antibodies occur naturally against the commensal conjunctival flora and are induced by the presence of pathologic microbes such as Acanthamoeba and Pseudomonas. SlgA-bound microbes are attached via SC to the mucus layer, resulting in their immobilization and discharge with the continuous renewal of the tear film.

In other tissues, it has been shown that SlgA inside the tissue can bind to pathogens, including intracellular viral particles, that have already penetrated the tissue. During the vectorial transport of SlgA toward the lumen, the bound pathogens are cleared from the tissue. These are common events at mucosal surfaces and since the whole ocular surface mucosa is subjected to constant local pathogen exposition including viral infection, this process conceivably also applies here but has not yet been shown. The same is true for the effects of IgA on signaling networks and immune regulation inside the tissue.

Because of these important local actions of IgA inside the tissue, it can be assumed that the luminal action of IgA alone...
(i.e., bathing in an IgA-containing tear film derived from the LG), is not sufficient for ocular surface immune protection. Only local production of IgA inside the tissue of the conjunctiva, as verified in our study by RT-PCR, allows for the necessary clearance from antigens and for the immune modulatory effects of IgA. The local IgA production hence appears as a prerequisite for efficient ocular surface immune protection.

**Multilayered Ocular Surface Epithelia and IgA Transport**

At first glance, it may appear puzzling that SC expression and IgA deposition in the multilayered ocular epithelia is mainly observed in the outermost but not in the basal epithelial layers. However, this staining pattern conceivably results from the fact that, in the multilayered epithelium, only the apical zone of the outermost cell layer is sealed by intercellular tight junctions \(^{(60)}\) (Fig. 6C), which prevent the passive paracellular leakage of molecules. Therefore, an active energy consuming intracellular transport is only necessary through this cell layer where in fact the main expression of SC and IgA was found in the conjunctiva and in excretory lacrimal ducts in the present study. Up to the tight junctions, conjunctival plgA can be passively transported within the interconnected intercellular spaces \(^{(60)}\). A respective model for the transport of plgA through ocular multilayered epithelia and eventually into the tear film \(^{(61)}\) is depicted in Figure 6.

**CONCLUSION**

In the present study, we demonstrated by immunohistochemical staining and by RT-PCR that the conjunctiva has the same components of the secretory immune system as the LG, similar to other mucosal tissues and their associated glands. \(^{(1,2)}\) Together with functional considerations from the literature, our results provide several lines of evidence that the conjunctiva actively participates in the specific immune protection at the ocular surface in concert with the downstream lacrimal drainage system. These tissues together form EALT and function as a component of the mucosal immune system of the body.

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**References**


