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

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Purpose. Secretory IgA (SIgA) 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 SIgA 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.

Methods. Tissues from 18 conjunctivas and 9 LGs of human donor eyes with normal ocular surfaces were analyzed by histology and IHC. Different zones of 12 further conjunctivas and LG tissues were analyzed by RT-PCR for the presence of the respective mRNA.

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 SIgA 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 (SIgA) forms a first line of defense at mucosal surfaces[1,2] 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.[3] The ocular surface and lacrimal drainage system represent a mucosa, similar to that of the intestine and airways,[3] 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[4,5] including lysozyme, lactoferrin,[6] and defensins,[7] components of the specific immune system also occur at the ocular surface. Lymphocytes and plasma cells are known to reside at the ocular surface,[8,9] but their significance is unclear.[10,11] In a historical misconception, they were considered as "inflammatory cells."[8,12–14] 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[15,16] and in other species.[17]

Evidence of a mucosal immune system at the *human* ocular mucosa has increased over the years. Lymphocyte subpopulations[11,18] including mucosa-specific ones,[19,20] along with accessory structures such as specialized high endothelial venules (HEVs)[21,22] for their migration, have been shown. The universal presence of CALT was verified in a large number of normal human conjunctival wholemounts.[21] Later, lymphoid tissue was shown in the lacrimal drainage system,[20,23] which is now termed the lacrimal drainage–associated lymphoid tissue (LDALT).[20] Lymphoid populations at the ocular surface and adnexa are therefore statistically normal and have a physiologic function.[3,11,18,19] Their accepted presence in conjunctiva and lacrimal drainage system along with the LG[24] has led to the concept of the eye-associated lymphoid tissue (EALT)[20,25,26] 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[1] 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 (p)IgA, 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.[2,27] At the luminal surface, pIgA is cleaved together with an extracellular 80-kDa domain of its transporter, termed secretory component (SC),[28] resulting in secretory IgA (SIgA).

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TABLE 1. Tissue Data

	Body Donors	Mean Age (SD)	Sex (F:M)	Tissues Used (*n*)
IHC				
Conjunctiva	16	75.4 y (15.3)	10:6	18
LG	7	77.7 y (20.0)	4:3	9
RT-PCR				
Conjunctiva and LG	6	76.3 y (8.9)	4:2	12

IgA is not restricted to luminal actions that prevent adhesion and invasion of antigens.[1,2] It can also provide intracellular neutralization of virus particles inside the epithelial cells[29] and has an excretory function that "cleans" the tissue via the vectorial transport of IgA-bound antigens.[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.[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 innate and adaptive immunity.[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,[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 consistently verified by immunohistochemistry (IHC).[10,35] Therefore, we compared IgA-positive plasma cells and epithelial SC in the normal human conjunctiva with that in the LGs and verified the IHC findings of these proteins by detection of the respective mRNAs, including J-chain, by RT-PCR.

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 complied 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.

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 355S 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[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 $\times$160; Dako, Hamburg, Germany) and rabbit polyclonal anti-SC against the secretory component from human colostrums (A0187, dilution $\times$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[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 $MgCl_2$, 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

Protein	Primer Sequence	Coded Region (bp)	Intron-Spanning (bp)	PCR Product (bp)
IgA	Forward: 5′-tgc tct tag gtt cag aag cga acc-3′ Reverse: 5′-atg ccc aag tca ggt act tct cgc-3′	654	221	433
SC	Forward: 5′-aat gct gac ctc caa gtg cta aag-3′ Reverse: 5′-atc acc aca ctg aat gag cca tcc-3′	242	—	242
J-chain	Forward: 5′-cac aca cct taa ccc tga ctt ttt-3′ Reverse: 5′-cga gga aca ttt tat tac acc tcc-3′	895	—	895
GAPDH	Forward: 5′-cag aac atc atc cct gca tcc act-3′ Reverse: 5′-gtt gct gtt gaa gtc aca gga gac-3′	258	—	258

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, Jahnsdorf, 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 source of SIgA protein, was used as a positive control. Skeletal muscle tissue with an absence of the secretory proteins was used as a negative control, and additional negative control reactions were performed by 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.

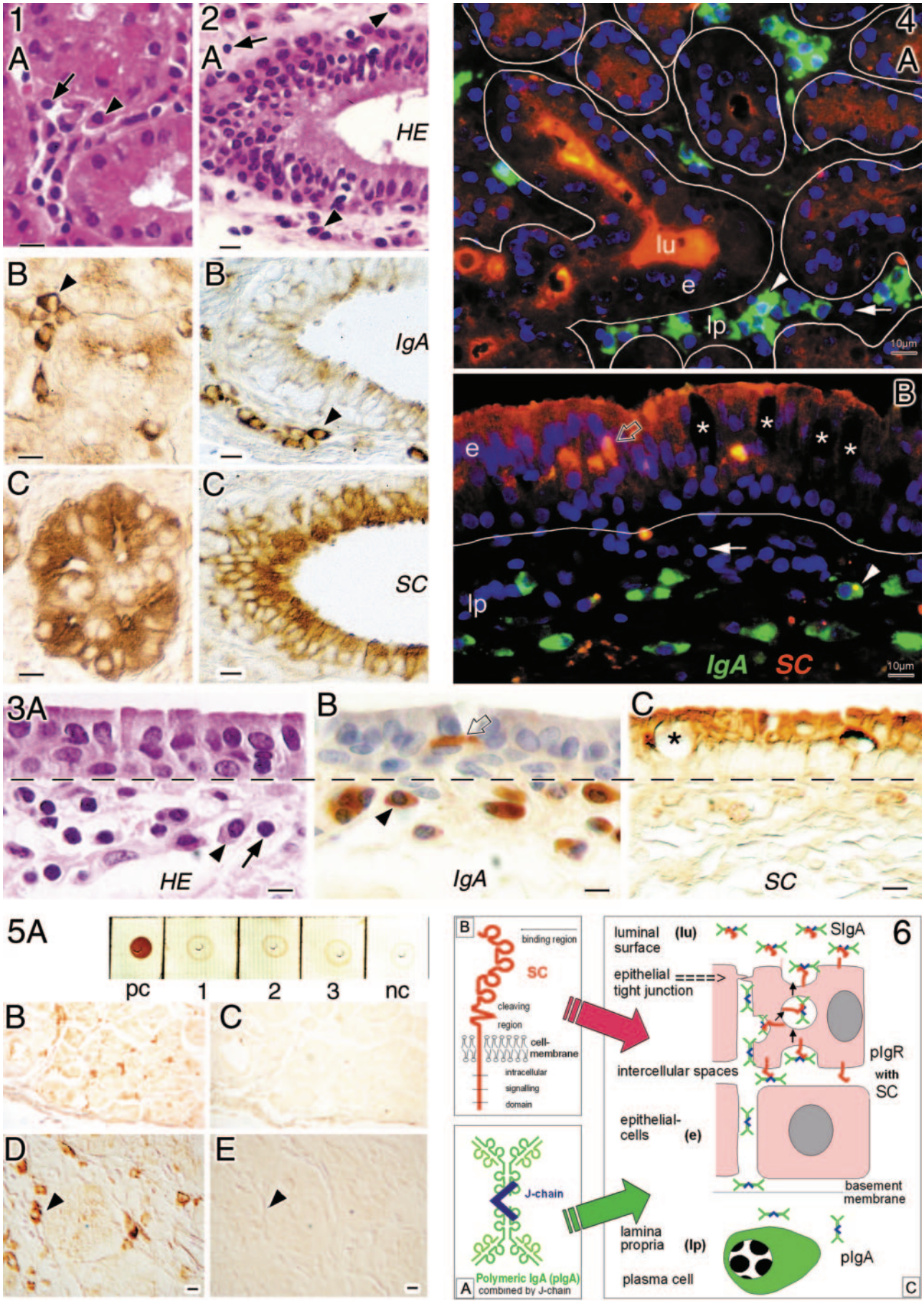
Figures 1 and 2. Components of the secretory immune system in the LG and excretory duct. (**1A–C**) LG lymphocytes (*arrow*) and plasma cells (*arrowhead*) between the roundish LG acini. (**1B**) IgA was strongly found in the plasma cells and as weaker, patchy staining in the cytoplasm of some acinar epithelial cells, which more strongly expressed SC (**1C**). The excretory lacrimal ducts (**2A–C**) that connected the LG to the conjunctiva had similar characteristics, but in the epithelium, IgA (**2B**) and SC (**2C**) were mainly expressed in the luminal epithelial layer. Scale bars in Figures 1 to 5, 10 μm.

Figure 3. Secretory immune system in the conjunctiva. The conjunctiva (here tarsal zone) also showed a subepithelial diffuse lymphoid tissue (**3A**), with IgA strongly expressed in the plasma cells and patchy, weak expression (*open arrow*) in the epithelium (**3B**, counterstained with hematoxylin). Immunostaining for SC was stronger and predominated in the superficial layer of the two- to three-layered conjunctival epithelium (**3C**). The basal cells were SC negative, similar to the goblet cells (*asterisk*). The basement membrane level is indicated by a *dasbed line* in Figure 3 and *solid lines* in Figure 4.

Figure 4. Comparison of IgA and SC distribution in conjunctiva and LG. Double-labeling fluorescent IHC with DAPI counterstain (triple fluorescence) for IgA (*green*), SC (*red*), and cell nuclei (*blue*, DAPI) showed in overlays that the components of the secretory immune system were similarly arranged in the LG (**4A**) and the conjunctiva (**4B**, orbital zone). IgA-positive plasma cells were diffusely interspersed in the lamina propria (lp) of both tissues—in the LG, frequently in groups. The epithelium (e) showed strong staining for SC, restricted in the conjunctiva (**4A**) to the superficial epithelial layer with occasional bright deposits (*open arrow*). Goblet cells (✶) were negative for SC. A mixed color indicating both proteins (SIgA) was seen in the tubulo-acinar lumina (lu in **4A**) of the LG and frequently delineated the luminal cell surface.

Figure 5. Preadsorption of IgA antisera abolished specific staining. In a dot blot experiment (**5A**), a positive control (pc) serum, preadsorbed to increasing IgA concentrations (1–3), resulted in an almost complete block of staining compared with a negative control (nc) without IgA antiserum. Staining (**5B**, **5D**) was also inhibited in consecutive tissue sections treated with preadsorbed antiserum (**5C**, **5E**). Overview (**5B**, **5C**) and magnification (**5D**, **5E**) of a lacrimal gland; *arrowheads*: corresponding locations of IgA-positive plasma cells.

Figure 6. Molecular components of the conjunctival secretory immune system are depicted in a model for IgA transport derived from observation of the immunostaining in the multilayered conjunctival epithelium: pIgA joined by the peptide J-chain (**6A**) and excreted by plasma cells reaches passively through the basement membrane and the interconnected open intercellular epithelial spaces until it reaches the luminal epithelial tight junction (**6C**). At the basolateral membrane of the superficial epithelial layer, pIgA gets in contact with the polymeric Ig receptor (pIgR^{3,2}) including SC (**6B**), and is internalized and transcytosed. At the luminal membrane, pIgR is cleaved and SC shed together with IgA as SIgA (**6C**, SIgA).


1
A
2
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HE
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IgA
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4
A
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B
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3A
HE
B
IgA
C
SC
5A
pc
1
2
3
nc
B
C
D
E
B
binding region
SC
cleaving region
cell-membrane
intracellular signalling domain
J-chain
A
Polymeric IgA (pIgA) combined by J-chain
6
luminal surface (lu)
SIgA
epithelial tight junction
pIgR with SC
intercellular spaces
epithelial-cells (e)
basement membrane
lamina propria (lp)
pIgA
plasma cell
C

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 tarsoorbital 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

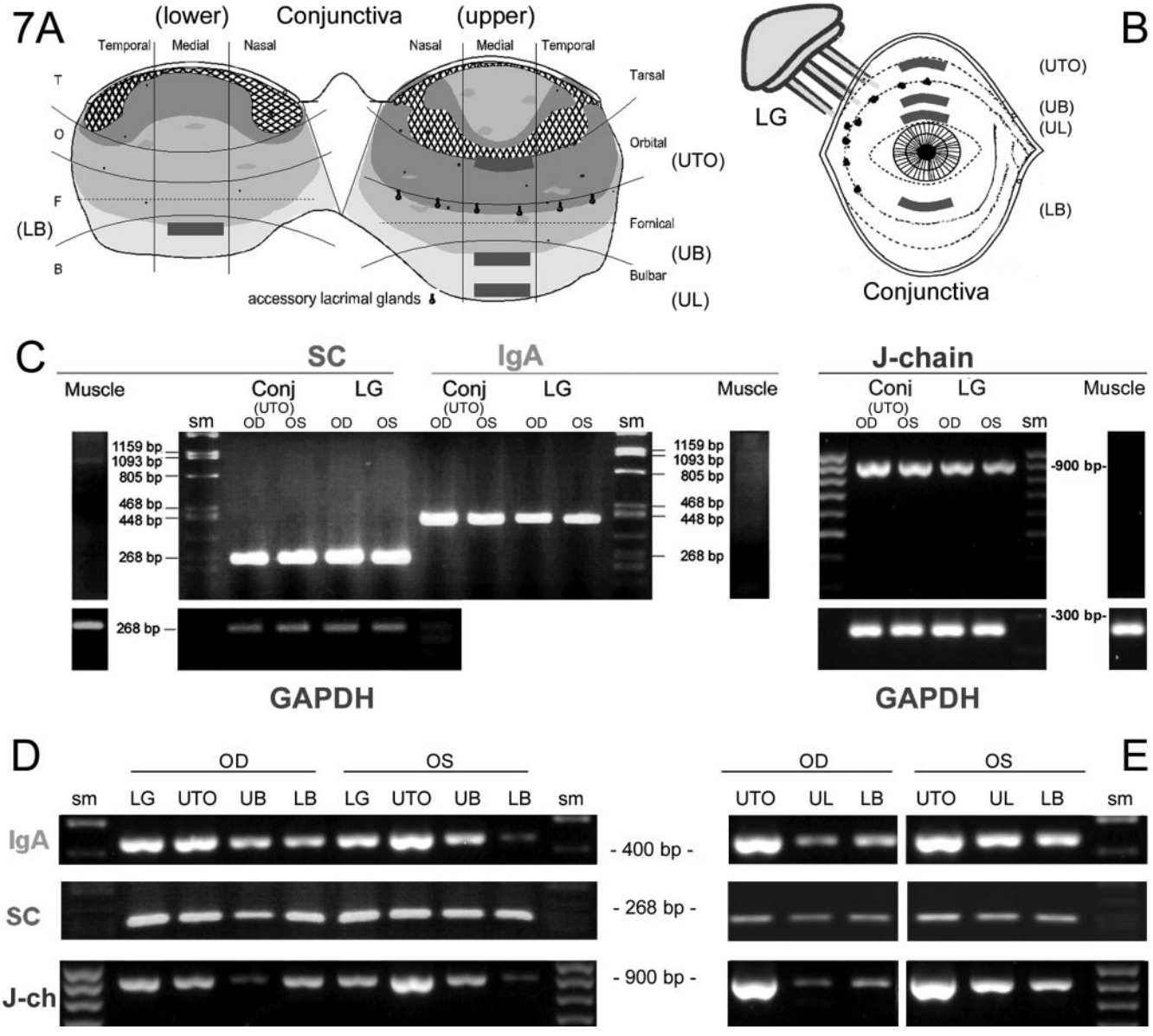

FIGURE 7. Local presence of mRNAs for the secretory proteins was verified by RT-PCR. The locations of tissue samples for RT-PCR are shown in the drawings. A flat wholemount of the conjunctival sac with topography of lymphoid tissue (**A**) and a frontal view with opened lids (adopted from Ref. 9), as in the clinical setting (**B**); accessory LGs show as large dark spots (small spots in **A** represent lymphoid follicles). Gel electrophoresis of PCR products from tissues of both eyes of a representative donor (**C**) verified that mRNAs of SC, IgA, and J-chain were expressed in the LG and UTO conjunctiva and had bilateral symmetry (**C**, OD, OS). The housekeeping mRNA (GAPDH) was also present in the negative control tissue (skeletal muscle) but not the mRNAs for SC, IgA, and J-chain. Comparison of LG and UTO conjunctiva in fellow eyes (OD, OS) with UB and LB conjunctiva (**D**) showed that the mRNA for the secretory proteins was also present here. Comparison of UTO with UL and LB conjunctiva (**E**) verified that the mRNA for IgA, SC, and J-chain were also present in perilimbal conjunctiva. sm, size marker (in **C**–**E**).

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[9] and as observed by us in stained translucent wholemounts,[21] we intended in further experiments to exclude the theoretical possibility that accessory LG tissue with a known presence of the secretory proteins 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.

Discussion

IgA-positive plasma cells were consistently found in the connective 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 and also occurred in all examined specimens.

IgA and SC had been observed in the human LG by IHC,[6,24,38,39] and the same is true for J-chain.[40] On this basis, the LG was accepted as the source of specific immune defense via antibodies that are distributed over the ocular surface by the tear flow. However, this had never been verified by RT-PCR and similar IHC evidence in the conjunctiva[21] was not unequivocally accepted. In the present study, we intended to show the identity and local production of the mRNAs by RT-PCR combined with the exact location of the produced protein in the tissue and its identity by IHC. The present results verify that the whole conjunctiva performs as a local production source of SIgA.

Conjunctival production of SIgA appears to be an important component of the ocular immune defense, which is functionally in line with the observation that monkeys after extirpation of the LG did not exhibit increased ocular surface infection.[41] The conjunctiva has a considerable surface area with a plasma cell content calculated to be in the range of the LG.[35] Of interest, during eye closure at night, the concentration of IgA in the preocular tear film rises to very high levels, although the secretion by the LG almost ceases.[5,42] These observations can reasonably be explained only by local production in the conjunctiva, as verified in the current study by the presence of the mRNAs for IgA, SC, and J-chain. This local conjunctival production appears to represent an important mechanism for the maintenance of ocular surface immune protection and not only at night. Evidence of a considerable contribution of the human conjunctiva to ocular secretory immunity is derived from observations that the tears from patients with atopic dermatitis contain less SIgA.[43] Atopic dermatitis is a disease that primarily affects the conjunctiva and not the LG. In Sjögren's disease, LG secretory function is impaired, but the tear film IgA concentration at the ocular surface is still normal.[44]

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,[8,9,35] but reports for IgA[10] and SC[33] were inconsistent by IHC staining, which were based on relatively few tissues, including pathologic clinical biopsies from ocular surface disease. Other explanations for previous inconsistent results may that the investigators in the early studies[10,33,45] found the results of IHC highly dependent on methodological influences. Later findings of absent IgA and SC staining in the conjunctiva of a common laboratory animal (rat[46]) 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[17,47] which differs distinctly from the human and, for example, rabbit[16,17,48] 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.[49]

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[28] and later mainly in other monolayered epithelia, such as the intestine,[45,50] 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.[45,50] In fact, SC is present in numerous epithelial tissues early in development,[51] even in the liver, and therefore is a basic epithelial transport mechanism. It also occurs in the superficial cells of the stratified vaginal epithelium[52] similar to the findings in the conjunctiva in our study. In the rabbit conjunctiva, which closely resembles the human,[16,17,48] SC is interestingly even regarded as a marker for intact terminal epithelial differentiation.[53]

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 antiserum 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 SIgA.

Functions of SIgA in Ocular Surface Protection

SIgA is one of the best-defined 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[54,55] and are induced by the presence of pathologic microbes such as *Acanthamoeba*[56] and *Pseudomonas.*[57] SIgA-bound microbes are attached via SC to the mucus layer,[58] resulting in their immobilization and discharge with the continuous renewal of the tear film.[59]

In other tissues, it has been shown that pIgA inside the tissue can bind to pathogens, including intracellular viral particles,[29] that have already penetrated the tissue. During the vectorial transport of pIgA toward the lumen, the bound pathogens are cleared from the tissue. These are common events at mucosal surfaces[30] 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.[31,32]

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 pIgA can be passively transported within the interconnected intercellular spaces.[60] A respective model for the transport of pIgA 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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