Conjunctiva-Associated Lymphoid Tissue in the Human Eye

Nadja Knop and Erich Knop

PURPOSE. Mucosa-associated lymphoid tissue (MALT) represents a part of the immune system located at mucosal surfaces. Its presence in the human eye is the point in question in the current study. Its occurrence, components, topography, and probable functional significance in the human conjunctiva and lacrimal drainage system were investigated.

METHODS. Fifty-three complete conjunctival sacs were obtained from cadaveric eyes, prepared as flat wholemounts, stained, optically cleared, observed in total thickness, and sectioned for light microscopic histology, immunohistochemistry, and electron microscopy. Eight lacrimal sacs and adhering canaliculi were prepared accordingly.

RESULTS. Lymphoid tissue was mainly observed in the palpebral conjunctiva, more pronounced in the upper than in the lower lid. It occurred in different forms: 1) In all specimens, diffuse lymphoid tissue of lymphocytes and plasma cells, most of which were IgA positive, formed a thin layer in the lamina propria. The overlying epithelium produced secretory component. 2) In approximately three fifths of the conjunctival sacs, organized follicular accumulations were embedded in this layer. They had a lenticular shape, were composed of B lymphocytes, and were apically covered by lymphoepithelium. 3) Both types could be associated with the conjunctival crypts. Lymphoid tissue with similar characteristics, including secondary follicles, was also observed inside the lacrimal drainage system. High endothelial venules were present in all types of lymphoid tissue.

CONCLUSIONS. Human conjunctiva and lacrimal drainage system show an associated lymphoid tissue (suggesting the term conjunctiva-associated lymphoid tissue [CALT]) that contains all components necessary for a complete immune response. Expression of immunoglobulins and secretory component indicates that the conjunctiva belongs to the secretory immune system (*Invest Ophthalmol Vis Sci.* 2000;41:1270–1279)

Besides its well-known unspecific defense mechanisms, the conjunctiva is probably provided with a system of specific immune response in the form of mucosa-associated lymphoid tissue (MALT).[1,2] This has only recently received more attention, but still less when compared to other organs.

MALT consists of arrangements of lymphatic cells in different form,[3] situated in and closely underneath the epithelium. This tissue detects antigens and induces an immune response by the direct action of the lymphatic cells or the secretion of soluble antibodies.[4] Induction of tolerance of ubiquitous nonpathogenic antigens is another important function. Specialized vessels allow the recirculation of lymphocytes[5,6] and the communication with the central immune system in which it serves as an outpost. This is described in a number of organs, thus leading to the concept of a common MALT system.[7] Its presence in the eye is unclear, however. It is important to know more about this tissue, because it not only maintains ocular surface homeostasis, but also, lymphatic cells and their soluble immune modulators have been shown to influence pathologic ocular surface processes.[8–10]

In some animal species, components of this tissue were described in the conjunctiva,[11–16] showing species-specific differences.[17] In the human conjunctiva, the existence of lymphatic cells has been known for a long time,[18] but it is still unclear whether they form a functionally active mucosal immune system that could be termed conjunctiva-associated lymphoid tissue, or CALT. Former studies[18–21] were restricted to a histologic description, whereas more recent immunohistologic studies usually have been limited to the investigation of small pieces of tissue obtained by clinical biopsies. The tissue specimens examined in these studies were obtained to investigate lymphocyte subsets in the normal conjunctiva,[22–25] including mucosa-specific lymphocytes,[26] or were examined in a pathologic context.[21,27–30] Because small tissue specimens may not reflect the conjunctiva as a whole,[21] these studies also reported somewhat different results concerning the number and localization of cells.

Therefore, in a combined wholemount approach, we used total conjunctival sacs together with an immunohistochemical and electron microscopic study to investigate the presence, organization, and topographical distribution of CALT in the

Corresponding author: Erich Knop, Department of Cell Biology in Anatomy, Medical School Hannover, D-30625 Hannover, Germany. knop.erich@mh-hannover.de

FIGURE 1. Details of specimen preparation. Topographical anatomy of the conjunctiva (A) with the different zones: T, tarsal; O, orbital; F, fornical; and B, bulbar. (B) Excision and (C) flatmounting of a complete conjunctival sac from the right eye of a human donor was followed by hematoxylin staining and clearing. (D, arrows) Roundish lymphoid accumulations in left eye. Marked area is enlarged in Figure 2A. Bars, 1 cm.
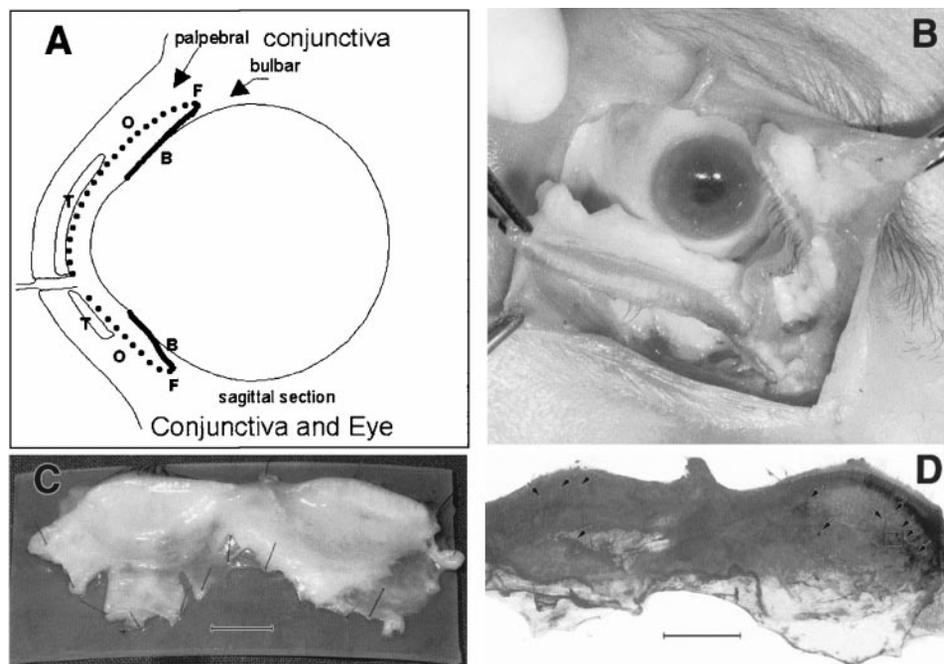
human. This approach could serve as a basis for the evaluation of clinical biopsy specimens in the future. For this purpose, the so-called diffuse type of lymphoid tissue[4] that is mostly disregarded in favor of the more easily detectable organized (follicular) form, received more attention in our study. Furthermore, the presence of specialized vessels (high endothelial venules [HEVs]), indicating the integration of this tissue into the immune system,[31] was analyzed. Another goal was the investigation of plasma cells, as the source of protective immunoglobulins, and the respective transepithelial transporter molecule (secretory component).[32] Because the tears may represent a carrier for soluble immune modulators and for external antigens into the lacrimal drainage system, this tissue was also investigated for the presence of MALT.

MATERIALS AND METHODS

Specimen Preparation

Conjunctival sacs ($n = 53$) and lacrimal sacs ($n = 8$) with adherent canaliculi were obtained from human cadavers ($n = 27$) from 1994 through 1998 at the Department of Anatomy, Medical School Hannover. The average age of the donors was 76.1 ± 12.3 years, and the sex distribution was 16:11 (female to male). The average postmortem time before fixation was 2.3 ± 2.2 days. Only macroscopically normal conjunctivas were used. These specimens were taken from donors who had given informed consent to donate their bodies for education and science, and the study was in compliance with the Declaration of Helsinki.

The complete conjunctival sac was excised from the lid margin toward the corneal limbus (Fig. 1A), remaining connected at the nasal canthus, whereas the lateral canthus was divided (Fig. 1B). Specimens were then placed on plastic board and gently flattened without touching the conjunctival surface, and the margins of the wholemounts were secured with metal

pins (Fig. 1C). They were immediately fixed by immersion in a 4% paraformaldehyde solution (for light microscopy) or in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde diluted in 0.1 M cacodylate buffer (pH 7.4) for transmission electron microscopy (TEM).

Clearing Procedure

For identification of lymphoid tissue, 36 of the flattened wholemounts were stained en bloc in undiluted Mayer's hematoxylin (Merck, Darmstadt, Germany) for 8 minutes and consecutively cleared by different procedures. In one procedure, clearing in anise embedding oil[20] produced clear wholemounts but later necessitated re-embedding in another material (such as paraffin, Vogel Histo-Comp, Giessen, Germany) to allow sectioning. Embedding of osmium tetroxide–immersed wholemounts in Epon[15] resulted in too dark specimens in our case. We observed that optical clearing could also be achieved by embedding the tissue in 2-hydroxy-methacrylate resin (Kulzer, Hanau, Germany).[33]

Morphometric Analysis of Follicular Spots

In 36 cleared conjunctival sacs of 21 individuals, dense follicular spots were counted using a stereo magnifier (Wild-Leitz, Wetzlar, Germany) with a zoom optic for enlargements between 60- and 500-fold. Size was measured with a calibrated eyepiece. Only dense spots that resembled those previously identified in combined observation and sectioning of the wholemounts were counted, fulfilling the following criteria: roundish shape with relatively distinct outline, location directly underneath the epithelium, and prominence at the conjunctival surface. The totals, mean values, and SDs and Student's t-test were calculated with commercial software (Excel; Microsoft, Redmond, WA).

TABLE 1. Immunochemicals Used for Immunohistochemistry

Immunochemical	Source	Type	Dilution	Pretreatment
CD3	Sigma* C7930	Rabbit, polyclonal	×800	0.4% Pepsin
CD20	Dako† M0755	Mouse, monoclonal	×200	Microwave
IgA	Dako M0728	Mouse, monoclonal	×160	0.1% Trypsin
IgM	Dako M0702	Mouse, monoclonal	×80	Microwave
Secretory component	Dako A0187	Mouse, monoclonal	×1000	0.1% Trypsin
IgA Protein	Sigma I1010	Human colostrum	—	—

* Munich, Germany.
† Hamburg, Germany.

Paraffin Immunohistochemistry

Seven conjunctival and six lacrimal sacs were embedded in paraffin, sectioned at 5- to 10-μm thickness and stained with hematoxylin-eosin. Later, primary antibodies were used according to the data in Table 1 and incubated with deparaffinized sections overnight in a refrigerator at 4°C. Biotinylated secondary antibodies from the goat (Jackson/Dianova, Hamburg, Germany) were used to detect binding of the primary antibodies. This complex was marked by streptavidin-conjugated peroxidase (Dianova) and visualized by the chromogen diaminobenzidine (DAB), developed to a brown reaction product. For negative controls, primary antibodies were replaced by normal serum, and anti-IgA antiserum was additionally preadsorbed with the respective protein (Sigma, Munich, Germany) to verify the identity of staining. Accessory lacrimal glands were used as a positive control. Counterstaining was performed with hematoxylin.

Electron Microscopy

Six conjunctival and two lacrimal sacs were fixed as described, dehydrated in a graded alcohol series, and embedded in Epon. Semithin sections (1 μm) from areas of interest were stained with toluidine blue. Thin sections (70 nm) were stained with lead citrate and uranyl acetate and examined on a transmission electron microscope (model EM 10; Carl Zeiss, Oberkochen, Germany).

RESULTS

The excised conjunctival material consisted of the epithelium and its loose connective tissue layer (lamina propria). In the palpebral region, the tarsal plate was completely preserved, together with the conjunctiva (Fig. 1C); sometimes an additional layer of adipose connective tissue remained underneath (Fig. 1C). The method of embedding hematoxylin-stained tissue in methacrylate resin produced translucent flat conjunctival wholemounts (Figs. 1D, 2A). The light microscope displayed structural details in a better resolution, whereas observation with a stereo magnifier revealed the three-dimensional organization. Elevations were produced by accumulations of lymphocytes causing bulges at the surface; the conjunctival crypts appeared as grooves (Figs. 2A, 2B). After identification, areas of special interest could be readily sectioned.[33]
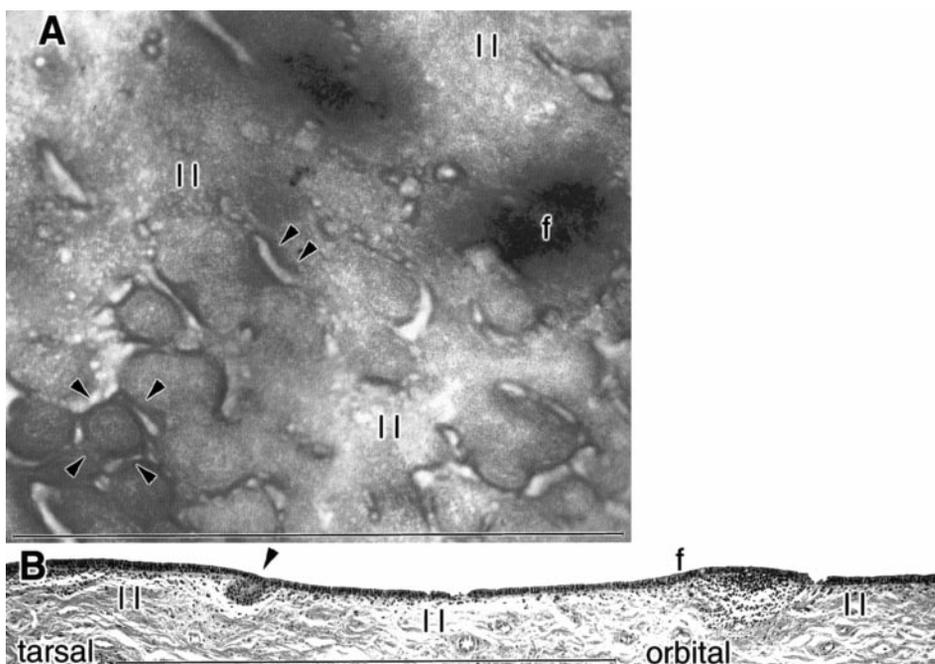

FIGURE 2. Combined wholemount and section approach. Magnification of the tarso-orbital zone (**A**) in a translucent conjunctival sac shows different forms of lymphoid tissue: a lymphoid layer (ll) with embedded dense follicular accumulations (f) and conjunctival crypts with associated lymphoid tissue (*arrowheads*). Perpendicular section (**B**) through a similar specimen also shows these forms of lymphoid tissue and reveals the lenticular shape of the follicle (f). Bars, 1 mm.

FIGURE 3.   Characteristics of the diffuse CALT: (**A**) orbital zone. Plasma cells (p) and lymphocytes (l) form a diffuse layer covered by an epithelium with intraepithelial lymphocytes (*arrowhead*) and an HEV (hev) underneath. Typical roundish endothelial cells (e) are seen with lymphocytes within and around the wall (*arrows*). TEM shows an intraepithelial lymphocyte (**B**, l) lying between epithelial cells (e) and deformed probably by passage (*arrow*) through the basement membrane (*arrowheads*). Immunostaining shows T cells (**C**) inside the epithelium (*arrowhead*), in the lymphoid layer (l), and around or in the wall (*arrows*) of an HEV (*∗*). Ultrastructurally, an HEV in the conjunctiva (**D**) is composed of large, bright endothelial cells (e) and a pericyte layer (pe) and shows adjacent (l) or intramural (*arrow*) lymphocytes. Most plasma cells in the lymphoid layer are IgA positive (**E**), as are the deposits in the epithelium (*arrowheads*), whereas there are fewer IgM-positive plasma cells (**F**). The overlying epithelium is strongly positive for the transporter secretory component (**G**), which shows homogeneous staining. A plasma cell (**H**) in the loose collagenous tissue (c) has extended cisternae of rough endoplasmic reticulum (rer), numerous mitochondria (m), a typical large nucleolus (n) and radially arranged heterochromatin masses. Bars, (**B**, **D**, and **H**) 1 μm; (**A**, **C**, and **E** through **G**) 10 μm. Staining, (**A**) hematoxylin and eosin; (**C**) anti CD3; (**E**) anti IgA; (**F**) anti IgM; (**G**) anti SC; (**E** through **G**) counterstained with hematoxylin; (**B**,**D**,**H**) TEM.
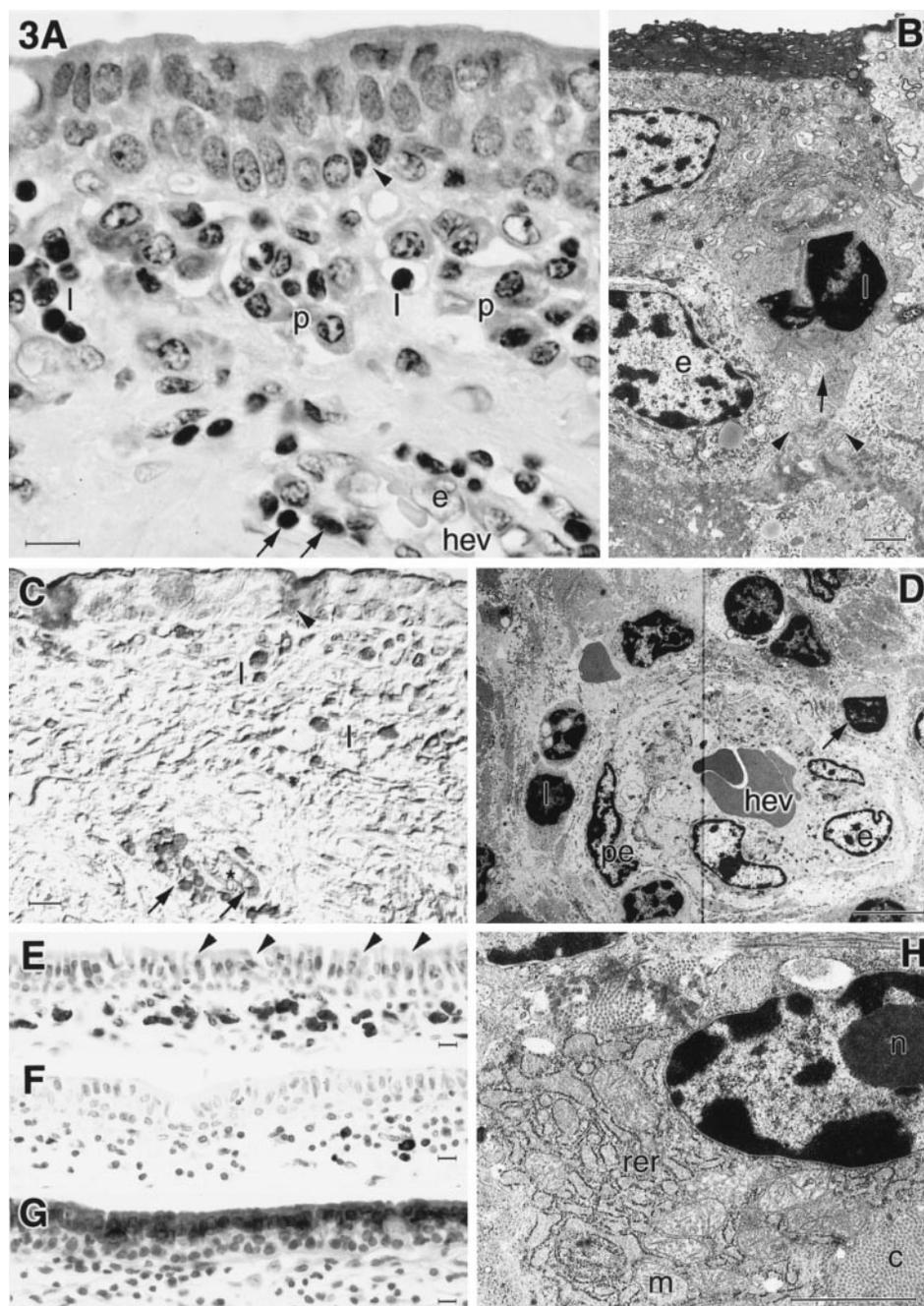
Different Forms of Lymphoid Tissue

In the stained and cleared tissue, the lymphocytes appeared, depending on their amount, as masses of varying darkness (Figs. 1D, 2A, 2B), because they had a relatively small cytoplasmic rim around the compact nucleus. They were organized in different forms as shown in the tarso-orbital zone of a translucent wholemount and in a similar section (Figs. 2A, 2B). The conjunctiva showed a layer of lymphatic cells that was continuous but of inhomogeneous density that seemed to extend like a carpet along the conjunctiva and showed a certain regional distribution. Embedded into this lymphoid layer were occasional dark, roundish spots resembling solitary lymphoid follicles. Lymphatic cells were also observed in the conjunctival crypts.

Lymphoid Layer

The lymphoid layer had a varying thickness in the range of the conjunctival epithelium (Fig. 3A) and was composed of small, dark lymphocytes and larger plasma cells. Deeper within the lamina propria, these cells were at times accompanied by granular mast cells. The epithelium contained intraepithelial lymphocytes (Figs. 3A, 3B, 3C). Occasionally, single granulocytes were seen inside the epithelium or underneath. The lymphoid layer was associated with a rich network of small vessels among which venules with roundish, bright endothelial cells characteristic of HEVs (Figs. 3A, 3C, 3D) were observed. The number of HEVs correlated with the thickness of the lymphoid layer. In a thin layer, they were rare, whereas in a thick layer numerous HEVs might be seen. Lymphocytes were
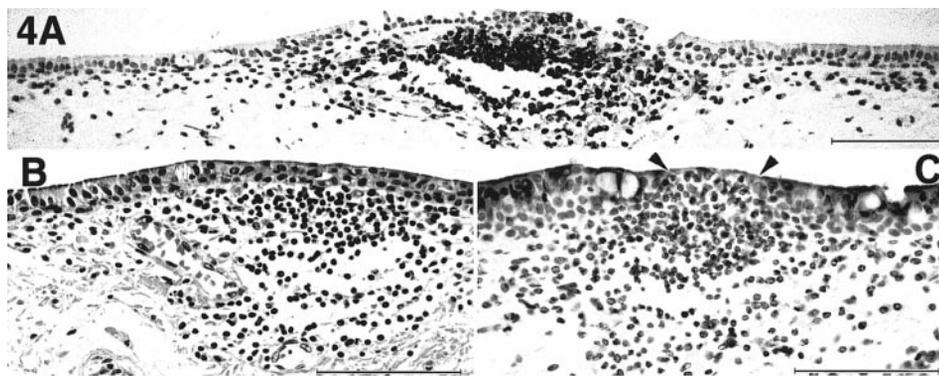
FIGURE 4.   Characteristics of follicular CALT. Even smaller lenticular lymphocyte accumulations are primarily composed of B cells (**A**) in immunostaining. Over the apex, the overlying epithelium becomes flatter (**B**), goblet cells are absent, immunostaining for secretory component is weaker (**C**, *arrowheads*), and numerous intraepithelial lymphocytes are present (**A** and **B** represent sections of the same follicle as shown in Fig. 2B). Bar, 100 μm. Staining, (**A**) anti CD20; (**B**) hematoxylin and eosin; (**C**) anti SC; (**A** and **C**) counterstained with hematoxylin.

located in and around these vessels and occasionally also around the usual vessels.

Immunostaining revealed that the lymphocytes in the lymphoid layer and the overlying epithelium primarily belonged to the T subtype (Fig. 3C). Positive anti-IgA and anti-IgM staining of the plasma cells and of scattered deposits inside the epithelium showed that they produced immunoglobulin. Plasma cells positive for IgA were more frequent and dominated the lymphoid layer (Fig. 3E), whereas those positive for IgM were relatively few (Fig. 3F). Secretory component was strongly and homogeneously expressed inside the overlying epithelium (Fig. 3G) with the exception of the intraepithelial goblet cells (Fig. 4C). Immunostaining was absent when a nonimmune serum was used instead of the primary antibody, and staining for IgA was completely inhibited after preadsorption of the antiserum with IgA protein.

In TEM, intraepithelial lymphocytes (Fig. 3B) were seen lying in the basal layers or close to the basement membrane and were detected by the density of chromatin and absence of intermediate filament bundles. Plasma cells (Fig. 3H) had an abundance of mitochondria and rough endoplasmic reticulum with distended cisternae, indicating secretory activity and presumably representing the immunoglobulin identified by immunohistochemistry. The endothelial cells of the HEVs (Fig. 3D) showed a large, roundish cytoplasm with cell organelles, but of characteristic low electron density. The nucleus was mostly roundish and contained a prominent nucleolus, altogether accounting for an active cell type. The vessel wall around the endothelium was composed of one or more layers of pericytes and was frequently accompanied by a cuff of lymphocytes.

Dense Follicular Spots

The dense accumulations of lymphocytes had a lenticular shape in sections and produced a mild elevation of the conjunctival surface in small (Fig. 4) and also in larger accumulations (Fig. 6C). Bright areas of germinal centers were not observed, but other features indicating them to be solitary lymphoid follicles were found. Even small spots consisted predominantly of CD20-positive B lymphocytes, which were rare in the embedding lymphoid layer (Fig. 4A). The overlying epithelium became flatter toward the apex, goblet cells disappeared (Figs. 4B, 4C), and immunostaining for the secretory

component was reduced or absent (Fig. 4C). The number of intraepithelial lymphocytes was increased, and they were sometimes arranged in groups (Figs. 4C, 6A). Staining for B and T lymphocytes in parallel sections confirmed that B lymphocytes formed central accumulations, whereas T lymphocytes were arranged complementarily to the B cells in the periphery and around the HEVs (Figs. 5B, 5C). All follicular lymphocyte accumulations had an abundance of HEVs (Figs. 5A, 5B, 5C, 6B).
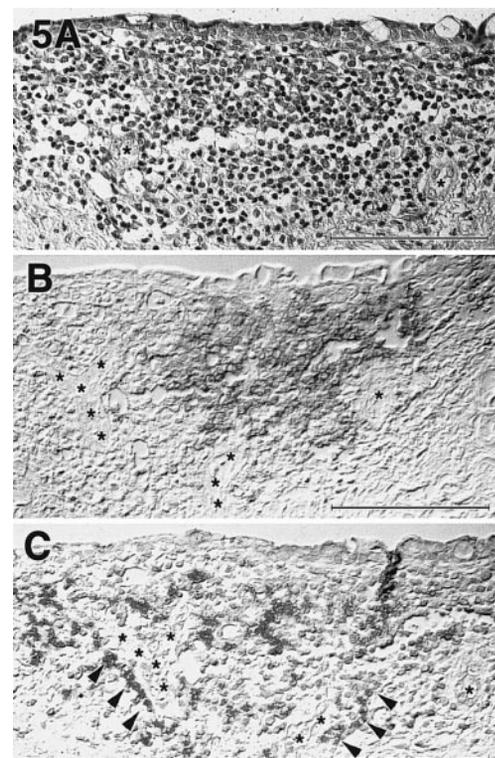

FIGURE 5.   A small, almost flat follicular accumulation that appears homogeneous in hematoxylin-eosin staining (**A**) shows that there is a central accumulation of B cells (**B**) around which T cells (**C**) are arranged complementarily in the periphery and around (*arrowheads*) the HEVs (∗). Bar, 100 μm. Staining, (**A**) hematoxylin and eosin; (**B**) anti CD20; (**C**) anti CD3.
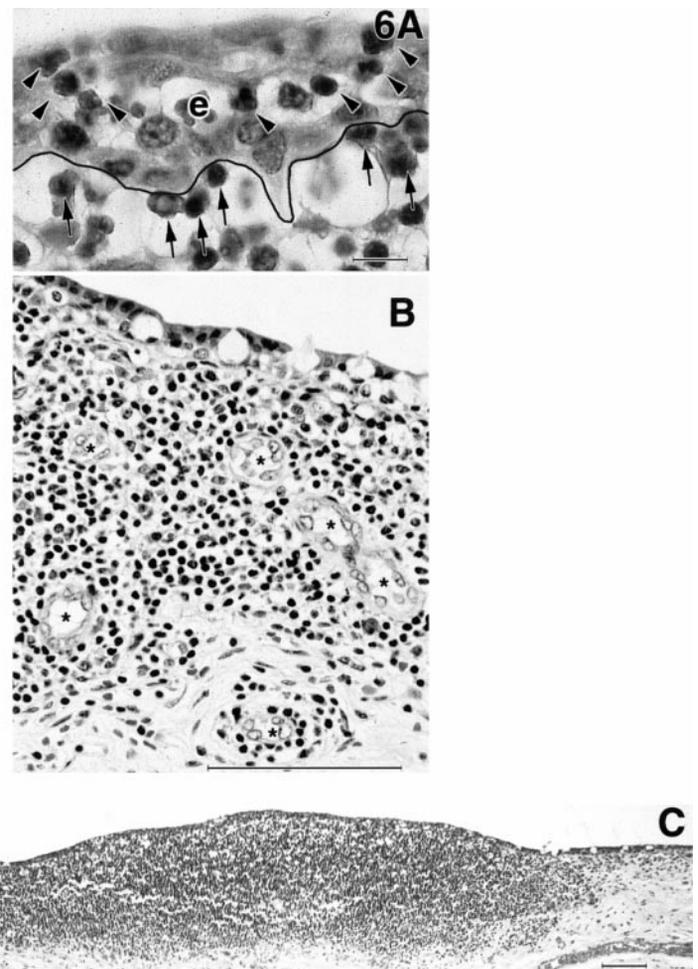
FIGURE 6. A follicle-associated epithelium (**A**) is seen to contain groups of lymphocytes (*arrowheads*); others are underneath (*arrows*) the basement membrane (indicated by a *solid line*). Well developed HEVs (**B**, *) are in the periphery of the same follicle. Even large follicular accumulations as this can still have a lenticular shape (**C**). Bar, (**A**) 10 μm; (**B,C**) 100 μm. Staining, (**A**) through (**C**), hematoxylin and eosin.

Follicular spots were present in a majority (21 of 36) of conjunctival sacs, more frequent in the upper (20 sacs) than in the lower (8 sacs) conjuctiva (Table 2). The diameter of the spots reached from 0.1 mm to approximately 1.25 mm and was usually below 0.5 mm (approximately 0.25 mm, on average). One of the conjunctivas that contained approximately seven times more follicular spots than the average was later excluded as probably abnormal. In those conjunctival sacs where follicular spots were present, the average number of spots was 10.25 (8 in the upper and 2.25 in the lower sac, $P = 0.00052$). In 15 pairs of donor eyes, 4 pairs with no follicles in one eye also had no follicles in the fellow eye and 6 with follicles in one eye also had them in the fellow eye. If minor differences (up to three follicles in one eye with none in the fellow eye) are accepted, altogether 13 of the 15 pairs had relatively homogeneous occurrence of follicular spots in both eyes, indicating a right–left symmetry.

Crypt-Associated Lymphoid Tissue

Accumulations of lymphoid cells were also associated with the conjunctival crypts. In regions with isolated crypts they were aggregated along the crypt wall creating a dense lining (pericryptal arrangement). In other locations with more frequent and interwoven crypt furrows, these sometimes encircled dense roundish spots of lymphoid tissue (intercryptal arrangement), similar to solitary follicles. Sections of the crypt-associated dense spots revealed follicular characteristics as described (not shown).

Lacrimal Drainage System

Mucosa-associated lymphoid tissue was also observed in the lacrimal sac with characteristics similar to those in the conjunctiva. The circumference was encircled by a layer of diffuse lymphoid tissue containing IgA-positive plasma cells in all specimens (Fig. 7A). The overlying epithelium at times contained deposits of IgA and was strongly positive for the secretory component with the exception of the numerous intraepithelial goblet cells. In the periphery, glandular tissue resembling accessory lacrimal glands occurred with immunoglobulin-positive plasma cells around, and secretory component inside the acinar cells (Fig. 7A). In four lacrimal sacs, follicular lymphocyte accumulations were embedded in the diffuse lymphoid tissue. In two sacs, these contained a bright germinal center characteristic of secondary follicles (Fig. 7B). No follicles and fewer lymphoid cells were observed at the lacrimal canaliculi.

Topographic Anatomy of Lymphoid Tissue

The topography of the different forms of lymphoid tissue (Fig. 8) was relatively homogeneous and more pronounced in the upper than in the lower conjunctiva. It was preferably ob-

TABLE 2. Morphometric Analysis of Follicular Accumulations in 35 Normal Human Conjunctival Sacs

		Number in Different Zones										Number per Diameter (in mm)							
	Total Sacs	Upper Conjunctiva	T	O	F	B	Lower Conjunctiva	T	O	F	B	0.1	0.2	0.3	0.4	0.5	0.75	1.0	1.25
Total	205	160	76	61	17	6	45	18	8	14	5	54	77	51	14	5	2	1	1
Mean	10.25	8.00	3.80	3.05	0.85	0.30	2.25	0.90	0.40	0.70	0.25	2.70	3.85	2.55	0.70	0.25	0.10	0.05	0.05
± SD	± 7.27	± 5.51	± 5.18	± 3.00	± 1.66	± 0.98	± 3.89	± 2.13	± 0.88	± 1.72	± 0.91	± 4.03	± 3.30	± 2.54	± 0.92	± 0.44	± 0.31	± 0.22	± 0.22

The number of follicles (mean ± SD) is shown for the topographical distribution (in the different zones, the upper and lower conjunctiva, and the total sacs) and for the size distribution, respectively. T, tarsal; O, orbital; F, fornical; B, bulbar.

served in the tarsal and orbital zones of the palpebral conjunctiva, broadening nasally and temporally and decreasing toward the bulbar zone. In the upper lid a local minimum occurred in the midtarsal zone with the exception of the marginal area: Marginal and tarso-orbital lymphoid tissue were sometimes continuous, both nasally and temporally. The lacrimal punctum in both eyelids (more pronounced in the lower) was encircled by a meshwork of crypt-associated lymphoid tissue. In the bulbar zone there was less lymphoid tissue in general, and usually its density sharply declined toward the limbal zone. Lymphoid tissue also continued inside the lacrimal drainage system.

DISCUSSION

Method

By applying a method using flat, cleared, and stained wholemounts of the conjunctiva, combined with section morphology and immunohistochemistry, we avoided the problem of restriction to only limited parts of the conjunctiva, as occurs with small clinical biopsy specimens.[21–27,29,34] We were able to identify MALT of the organized (follicular) and diffuse type that should be considered part of the mucosal immune system and can be referred to as CALT.

Types and Location of Lymphoid Tissue in the Human Conjunctiva

We confirmed results in bioptical studies[22-25,34] of the predominance of T cells, but were able to extend these data by revealing the topographical distribution of the lymphoid tissue. If biopsies are performed without this knowledge, the specimens may not be representative and could be misleading, as happens for example in the midtarsal area of the upper lid where a lower amount of lymphoid tissue was described than in the bulbar zone.[25] We found a local minimum here, whereas the tarsus in total showed distinctly more lymphatic tissue than the bulbar conjunctiva. We also found more follicles in the tarso-orbital zones than previously described in the fornix,[35] because even a larger preselected region such as the fornix may still not provide information on the conjunctiva as a whole and, in our study, did not represent the preferred location of CALT. Furthermore, by investigation of the whole organ, we could identify the distribution of the nonfollicular lymphocytes and plasma cells as a separate entity,[36] representing so-called diffuse lymphoid tissue.[4]

Lymphoid Layer

This represented the main expression of CALT. It has occasionally been mentioned before[18,19,37-39] but was often given the misleading names adenoid layer[39] or inflammatory cells.[21] Its composition of lymphocytes and plasma cells was correctly described earlier but was assumed to be an inflammatory infiltration even by investigators who found it in approximately 85% of their specimens.[19] Plasma cells were called inflammatory cells, although it was stated that they occur in every normal conjunctiva in high amounts.[21]

In the present study we showed that it is not a diffuse inflammatory infiltrate as indicated by its wide expression and consistent composition of cells. This is supported by the identification of dominating suppressor–cytotoxic T cells[23–26] that
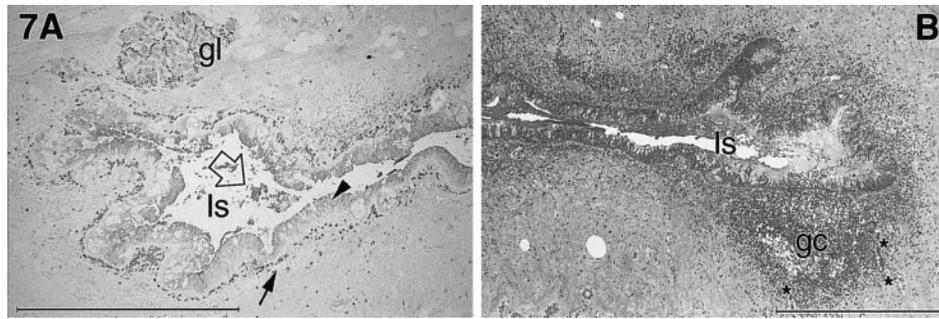
FIGURE 7. Aspects of lymphoid tissue in the lacrimal sac. Cross section (**A**) shows a layer of IgA-positive plasma cells (*arrow*) around the circumference of a lacrimal sac (ls) with IgA deposits inside the epithelium (*arrowhead*) and inside the lumen (*open arrow*). A neighboring gland (gl) also contains IgA-positive plasma cells in a periacinar position. In another lacrimal sac (**B**, ls), a secondary follicle with a bright germinal center (gc) is seen underneath an epithelium with intraepithelial lymphocytes. In its periphery are high endothelial venules (∗). (**A**) Anti-IgA; (**B**) semithin section, toluidine blue. Bars, 1 mm.

are believed to play a heavy immunoregulatory[24] rather than an inflammatory role. The relative absence of IgM, representing an early answer to immunologic stimulation, may further support the macroscopic diagnosis of a normal uninflamed tissue.

This lymphoid layer shows important criteria for identification as so-called diffuse lymphoid tissue.[4] It represents the efferent limb of mucosal immunity, namely to host plasma cells for antibody production, which was supported in our study by the detection of immunoglobulin-positive plasma cells with expanded cisternae of rough endoplasmic reticulum and the respective transporter molecule in the overlying epithelium. The plasma cells are densely filled with immunoglobulin compared with levels in spot-like deposits in the epithelium, contradicting a probable uptake from the luminal tear fluid. The identity of IgA was verified by a preadsorption control. The expression of secretory component is not limited to simple columnar epithelia as could be assumed by its preferable expression in the intestine but also is observed in the pseudostratified epithelium of the upper airways[40] and the stratified epithelium of the vagina.[41] Therefore, the lymphoid layer of the conjunctiva is able to perform a humoral immune response and appears to be a part of the secretory immune system.[32] Thus, the immune protection of the ocular surface may not solely depend on the lacrimal tissues[42–44] but can be

maintained, at least in part, by the conjunctiva itself. The presence of plasma cells in the normal human conjunctiva is controversial[18,19,21,23,24,42,45] but our findings indicate that they not only represent a normal component of a healthy conjunctiva but may be necessary to protect it against pathologic invasion.

Follicular CALT

Follicular CALT was present inconstantly but in the majority of the human conjunctival sacs and showed a right–left symmetry. The follicular lymphocyte accumulations had a relatively flat lenticular shape and therefore differed from the prominent roundish ones observed in the rabbit by us and others[11,17,46–48] or in the monkey.[15] Those found here still showed follicular characteristics as central B-cell accumulations covered by a follicle-associated epithelium lacking, for example, goblet cells and secretory component.[49] The diameter of follicles observed here was in the range reported in other studies in human[19] and animal tissues.[11,15,17,46–48]

Therefore, the human conjunctiva may also be provided with an afferent limb of mucosal immunity to sample antigens, as shown in the rabbit[12] where B-lymphocytes from conjunctival follicles could be stimulated to become immunoglobulin-producing plasma cells. Therefore the conjunctiva itself is able
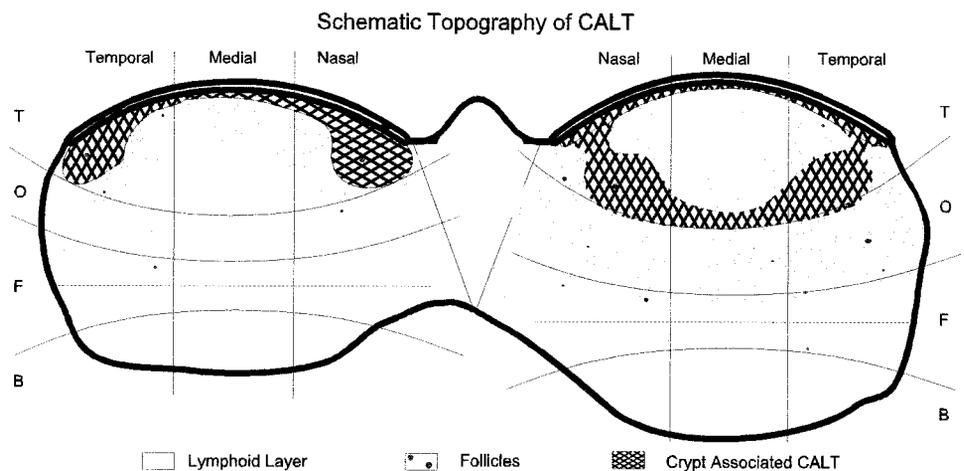

FIGURE 8. Synopsis of the topographical distribution of CALT in the zones of the human conjunctiva. The different types of CALT are indicated by different *symbols.* Increasing grade of *shading* refers to an increasing amount of the diffuse lymphoid layer (showing local inhomogeneities), whereas the number and size of follicles is indicated by dark *dots* of different sizes. Crypt-associated CALT is represented by *hatched lines.*

to locally produce plasma cells specific for ocular surface antigens. An ability to induce tolerance after topical instillation of antigens is also shown.[50] The differences in comparison with animals could be species specific, but also, and probably more likely, they are due to age differences. Whereas our human donors were of old age, the investigated animals[11,15,46,47] were usually young adults. An age-related reduction of follicles in the human conjunctiva is confirmed by Østerlind[19] who found that their amount increased in early youth and later decreased.

Crypt-Associated CALT

The reason for the close association of lymphoid tissue with the conjunctival crypts is unknown. It may provide an arrangement in which foreign antigenic materials are accumulated by the mechanical forces of lid movement in such a way that CALT comes into contact with a source of enriched antigens.

Lacrimal Drainage System

This may also be true for the presence of MALT in the lacrimal drainage system, because antigens from the ocular surface are presumably transported here by the tear flow.[48] When the flow rate slows down in the lacrimal sac due to its wider lumen, it may represent a suitable location to sample antigens as indicated by the observed secondary follicles to assist the afferent immune function of CALT in the conjunctiva. This also requires the presence of a protective IgA shield, however, as found in this study, to avoid infection.

HEVs

The presence of HEVs in the normal human conjunctiva and lacrimal sac indicates that they require a more effective and regulated emigration[6] of lymphoid cells from the bloodstream into lymphatic tissue[5] than that provided by the rich network of ordinary vessels. That the number of HEVs correlated with the amount of lymphatic cells in the layer may be the result of a respective influx of lymphocytes into the conjunctival and also lacrimal lamina propria. The HEVs were not so densely packed with lymphocytes here as is occasionally seen in other secondary lymphoid organs, probably because of a lower rate of migration in the normal state. Their light and electron microscopic morphology[51] showed strong similarities to respective vessels in other tissues.[52] They were also reported in follicular CALT of the monkey[15] but not detected in the human by immunohistochemistry.[53] Therefore, they were assumed to appear only in a pathologic context in the human—for example, they may be upregulated in inflammatory diseases. Although this possibility seems reasonable, the present study clearly shows on a morphologic basis that HEVs are a regular component of the normal conjunctiva and lacrimal sac.[54]

CONCLUSION

The question of whether CALT is a normal feature of the conjunctiva is usually based on the proportion of lymphatic tissue observed. Therefore, investigators finding it in a minority of specimens[35] concluded it must be abnormal, whereas others reporting it present in the majority consequently considered it normal.[19] However, the proportion of lymphatic tissue detected may also depend on the applied technique and knowl-

edge of topographic distribution as indicated earlier. The occurrence of CALT seems to be related to the presence of antigenic stimuli. This is supported by the findings of a rapid increase of conjunctival lymphoid tissue in early youth,[19] a reduced amount under germ-free conditions,[55] and the general presence of diffuse lymphoid tissue and a relative right–left symmetry of follicles as observed in the present study. Because antigen contact is an inevitable component of normal life and lymphoid tissue is designed to manage this threat, we conclude that it represents a normal feature of a disease-free conjunctiva, even though the actual amount of it and the differential composition of the lymphoid components may be different in individuals and may vary depending on environmental conditions and age.

References

1. Collins FM. Cellular antimicrobial immunity. *CRC Crit Rev Microbiol.* 1978;7:27–91.
2. Chandler JW, Gillette TE. Immunologic defense mechanisms of the ocular surface. *Ophthalmology.* 1983;90:585–591.
3. Hein WR. Organization of Mucosal Lymphoid Tissue. In: Kraehenbuhl JR, Neutra MR, eds. *Defense of Mucosal Surfaces: Pathogenesis, Immunity and Vaccines*. Berlin: Springer–Verlag, 1999:1–15.
4. Kraehenbuhl JP, Neutra MR. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol Rev.* 1992;72:853–879.
5. Gowans JL. The recirculation of lymphocytes from blood to lymph in the rat. *J Physiol.* 1959;146:54–54.
6. Pabst R, Westermann J. Which steps in lymphocyte recirculation are regulated by interferon-gamma? *Res Immunol.* 1994;145:289–294.
7. Mestecky J, McGhee JR, Michalek SM, Arnold RR, Crago SS, Babb JL. Concept of the local and common mucosal immune response. *Adv Exp Med Biol.* 1978;107:185–192.
8. Jones DT, Monroy D, Ji Z, Atherton SS, Pflugfelder SC. Sjogren's syndrome: cytokine and Epstein–Barr viral gene expression within the conjunctival epithelium. *Invest Ophthalmol Vis Sci.* 1994;35:3493–3504.
9. Sacks EH, Jakobiec FA, Wieczorek R, Donnenfeld E, Perry H, Knowles DMJ. Immunophenotypic analysis of the inflammatory infiltrate in ocular cicatricial pemphigoid: further evidence for a T cell- mediated disease. *Ophthalmology.* 1989;96:236–243.
10. Baudouin C, Haouat N, Brignole F, Bayle J, Gastaud P. Immunopathological findings in conjunctival cells using immunofluorescence staining of impression cytology specimens. *Br J Ophthalmol.* 1992;76:545–549.
11. Axelrod AJ, Chandler JW. Morphologic characteristics of conjunctival lymphoid tissue in the rabbit. In: Silverstein AM, O'Connor GR, eds. *Proceedings of the Second International Symposium on the Immunology and Immunopathology of the Eye*. New York: Masson Publishing, 1979:292–301.
12. Franklin RM, Remus LE. Conjunctival-associated lymphoid tissue: evidence for a role in the secretory immune system. *Invest Ophthalmol Vis Sci.* 1984;25:181–187.
13. Latkovic S. Ultrastructure of M cells in the conjunctival epithelium of the guinea pig. *Curr Eye Res* 1989;8:751–755.

14. Fix AS, Arp LH. Conjunctiva-associated lymphoid tissue (CALT) in normal and *Bordetella* avium-infected turkeys. *Vet Pathol.* 1989; 26:222–230.

15. Ruskell GL. Organization and cytology of lymphoid tissue in the cynomolgus monkey conjunctiva. *Anat Rec.* 1995;243:153–164.

16. Knop E, Knop N. MALT tissue of the conjunctiva and nasolacrimal system in the rabbit and human. *Vision Res.* 1996;36:60.

17. Chodosh J, Nordquist RE, Kennedy RC. Comparative anatomy of mammalian conjunctival lymphoid tissue: a putative mucosal immune site (in process citation). *Dev Comp Immunol.* 1998;22: 621–630.

18. Virchow H. Mikroskopische Anatomie der äusseren Augenhaut und des Lidapparates. In: Saemisch T, ed. *Graefe-Saemisch Handbuch der gesamten Augenheilkunde.* Band 1, 1. Abteilung, Kapitel II. 2nd ed. Leipzig: Verlag W. Engelmann; 1910:431.

19. Osterlind G. An investigation into the presence of lymphatic tissue in the human conjunctiva, and its biological and clinical importance. *Acta OphthalmolCopenh.* 1944;(suppl):23:1–79.

20. Kessing SV. Mucous gland system of the conjunctiva: a quantitative normal anatomical study. *Acta Ophthalmol Copenh.* 1968; (suppl)95:1–133.

21. Allansmith MR, Greiner JV, Baird RS. Number of inflammatory cells in the normal conjunctiva. *Am J Ophthalmol.* 1978;86:250–259.

22. Belfort R Jr, Mendes NF. T- and B-lymphocytes in the human conjunctiva and lacrimal gland. In: Silverstein AM,O'Connor GR, ed. *Proceedings of the Second International Symposium on the Immunology and Immunpathology of the Eye.* 1979:287–291.

23. Bhan AK, Fujikawa LS, Foster CS. T-cell subsets and Langerhans cells in normal and diseased conjunctiva. *Am J Ophthalmol.* 1982; 94:205–212.

24. Sacks EH, Wieczorek R, Jakobiec FA, Knowles DM. Lymphocytic subpopulations in the normal human conjunctiva: a monoclonal antibody study. *Ophthalmology.* 1986;93:1276–1283.

25. Hingorani M, Metz D, Lightman SL. Characterisation of the normal conjunctival leukocyte population. *Exp Eye Res.* 1997;64:905–912.

26. Dua HS, Gomes JA, Jindal VK, et al. Mucosa specific lymphocytes in the human conjunctiva, corneoscleral limbus and lacrimal gland. *Curr Eye Res.* 1994;13:87–93.

27. Belfort R Jr, Mendes NF. Identification of T and B lymphocytes in the human conjunctiva and lacrimal gland in ocular diseases. *Br J Ophthalmol.* 1980;64:217–219.

28. Jakobiec FA, Lefkowitch J, Knowles DM. B- and T-lymphocytes in ocular disease. *Ophthalmology.* 1984;91:635–654.

29. Medeiros LJ, Harris NL. Lymphoid infiltrates of the orbit and conjunctiva: a morphologic and immunophenotypic study of 99 cases. *Am J Surg Pathol.* 1989;13:459–471.

30. Wotherspoon AC, Diss TC, Pan LX, et al. Primary low-grade B-cell lymphoma of the conjunctiva: a mucosa-associated lymphoid tissue type lymphoma. *Histopathology.* 1993;23:417–424.

31. Westermann J, Pabst R. How organ-specific is the migration of "naive" and "memory" T cells? *Immunol Today.* 1996;17:278–282.

32. Brandtzaeg P, Berstad AE, Farstad IN, et al. Mucosal immunity: a major adaptive defence mechanism. *Behring Inst Mitt.* 1997;98: 1–23.

33. Knop E, Knop N. Flachpräparation des Konjunktivalsackes: eine Methode zur topografischen und mikroskopischen Untersuchung seiner Bestandteile. *Verh Anat Anz.* 1996;(suppl 179):262.

34. Dua HS, Gomes JA, Donoso LA, Laibson PR. The ocular surface as part of the mucosal immune system: conjunctival mucosa-specific lymphocytes in ocular surface pathology. *Eye.* 1995;9:261–267.

35. Wotherspoon AC, Hardman Lea S, Isaacson PG. Mucosa-associated lymphoid tissue (MALT) in the human conjunctiva. *J Pathol.* 1994; 174:33–37.

36. Knop E, Knop N. The mucosa associated lymphoid tissue of the human conjunctiva consists of three components: solitary follicles, crypt associated MALT, and a lymphoid layer. [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1997;38(4):**S**125. Abstract nr 612.

37. Stöhr P. Über den Bau der Conjunctiva palpebrarum. *Sitzungsb-.der physik.-med.Ges.in Würzburg* 1885.

38. Duke-Elder S, Wybar KC. The anatomy of the visual system. In: Duke-Elder S, ed. *System of Ophthalmology.* London: Henry Kimpton, 1961:541–558.

39. Raehlmann E. Pathologisch-anatomische Untersuchungen über die follikuläre Entzündung der Bindehaut des Auges oder das Trachom. *Arch Ophthalmol.* 1883;29:73–86.

40. Brandtzaeg P, Jahnsen FL, Farstad IN. Immune functions and immunopathology of the mucosa of the upper respiratory pathways. *Acta Otolaryngol.* 1996;116:149–159.

41. Kaushic C, Frauendorf E, Wira CR. Polymeric immunoglobulin A receptor in the rodent female reproductive tract: influence of estradiol in the vagina and differential expression of messenger ribonucleic acid during estrous cycle. *Biol Reprod.* 1997;57:958–966.

42. Sullivan DA. Ocular mucosal immunity. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee J, Bienenstock J, eds. *Handbook of Mucosal Immunology.* 2nd ed. New York: Academic Press, 1999: 1241–1281.

43. Allansmith MR. Defense of the ocular surface. *Int Ophthalmol Clin.* 1979;19:93–109.

44. Wieczorek R, Jakobiec FA, Sacks EH, Knowles DM. The immuno-architecture of the normal human lacrimal gland: relevancy for understanding pathologic conditions. *Ophthalmology.* 1988;95: 100–109.

45. Allansmith MR, Kajiyama G, Abelson MB, Simon MA. Plasma cell content of main and accessory lacrimal glands and conjunctiva. *Am J Ophthalmol.* 1976;82:819–826.

46. Krause W. *Die Anatomie des Kaninchens.* 2nd ed. Leipzig; 1884.

47. Niimi Y. Studien über den Lymphfollikel in der Conjunctiva, I: Mitt. Über den Lymphfollikel in der Conjunctiva bei normalen Kaninchen. *Acta Soc Ophthalmol Jpn.* 1935;39:808–823.

48. Knop N, Knop E. The MALT tissue of the ocular surface is continued inside the lacrimal sac in the rabbit and human [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1997;38(4):**S**126. Abstract nr 613.

49. Gehrke I, Pabst R. The epithelium overlying rabbit bronchus-associated lymphoid tissue does not express the secretory component of immunoglobulin. *Cell Tissue Res.* 1990;259:397–399.

50. Dua HS, Donoso LA, Laibson PR. Conjunctival instillation of retinal antigens induces tolerance. *Ocul Immunol Inflamm.* 1994;2:29–36.

51. Knop E, Knop N. Fine structure of high endothelial venules in the human conjunctiva. *Ophthalmic Res.* 1998;30(suppl):169.

52. Anderson ND, Anderson AO, Wyllie RG. Specialized structure and metabolic activities of high endothelial venules in rat lymphatic tissues. *Immunology.* 1976;31:455–473.

53. Zierhut M, Mihalic AM, Duijvestijn AM, Thiel HJ. High endothelial venules in the eye. *Invest Ophthalmol Vis Sci.* 1992;33(4):**S**1245. Abstract nr 2761.

54. Knop E, Knop N. High endothelial venules are a normal component of lymphoid tissue in the human conjunctiva and lacrimal sac. [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1998;39(4):**S**548. Abstract nr 2525.

55. McMaster PR, Aronson SB, Bedford MJ. Mechanisms of the host response in the eye, IV: the anterior eye in germ-free animals. *Arch Ophthalmol.* 1967;77:392–399.