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# Ocular defense mechanisms with special reference to the demonstration and functional morphology of the conjunctiva-associated lymphoid tissue in Japanese monkeys

Makoto Kageyama[1], Kazuo Nakatsuka[1], Takeshi Yamaguchi[2], Robert L Owen[3], and Tatsuo Shimada[2]

*[1]Department of Ophthalmology and [2]Health Sciences, Faculty of Medicine, Oita University, Oita, Japan; and [3]Department of Medicine, Veterans Affairs Medical Center and University of California, San Francisco, California, USA*

**Summary. In order to define the histological components of ocular defense, the conjunctiva in Japanese monkeys was studied using a whole mount method, light microscopy, and electron microscopy. We investigated the distribution of the conjunctiva-associated lymphoid tissue (CALT) using stereoscopic observations of the conjunctiva immunostained with human leukocyte antigen (HLA)-DR antibody and /or stained with alcian-blue. The outer surface of the conjunctival fornix was lined by sheets of mucus secreting goblet cells, with small epithelial patches without goblet cells, scattered among them. The patches, termed CALT, consisted of flattened epithelial cells, intraepithelial lymphocytes and dendritic cells, and lymphoid follicles with a germinal center. The CALT in Japanese monkeys was fundamentally similar in structure to those found in other animal species. CALT patches ranged in size ranging from 200 $\mu$m to 300 $\mu$m in diameter. The number of patches varied from 20 to 40 in the superior eyelid and 10 to 20 in the inferior eyelid. Latex microspheres administrated as eye drops were selectively taken up first by flattened associated epithelial cells covering the surfaces of CALT patches and then by intraepithelial dendritic cells of the CALT. These morphological findings show that CALT patches in the eyelids of primates are focal sites for particulate uptake and contact with lymphoid constituents, indicating that they are inductive sites for the common mucosal immune system as well as important components in ocular defense.**

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Address for correspondence : Prof. Tatsuo Shimada, MD, Department of Health Sciences, Faculty of Medicine, Oita University, 1-1 Hasama-machi, Yufu, Oita, 879-5593 Japan
Tel & Fax: +81-097-586-5032
E-mail: tshimada@med.oita-u.ac.jp

## Introduction

The conjunctiva in mammals is a transparent mucous membrane which lines the eyelids and is reflected to the front of the eyeball. The conjunctiva is divided into the palpebral conjunctiva, conjunctival fornix, and bulbar conjunctiva. When the eyelids are closed, the conjunctiva forms a closed sac. The ducts of tarsal glands open along the inside free margins of the eyelids, while those of the lacrimal glands open into the lateral recesses of the superior fornix. The entire conjunctiva is composed of stratified columnar epithelium. Superficial cells have a short prismatic form, and mucus secreting goblet cells are scattered between them. The fluid filling the conjunctival sac consists of oily material, tears and mucin, and is spread over the ocular surface by blinking. This mixture of fluid functions to wash away irritating materials, to prevent microbial infection and drying of the cornea and conjunctiva, and to nourish the cornea. In the guinea pig, plasma cells secreting the IgA-specific antibody are distributed in the submucosal layer of the conjunctiva,

which correlates with local immunity on the ocular surface (Shoji *et al.*, 1992).

Lymphoid follicles beneath the epithelium of the conjunctival fornix have been described in most animal species, including humans. Investigators have termed these lymphoid structures conjunctiva-associated lymphoid tissue (CALT), and have identified CALT as a component of the mucosa-associated lymphoid tissue (MALT) (Chandler and Axelrod, 1980; Franklin and Remus, 1984; Ruskell, 1995; Knop and Knop, 2000; Knop E and Knop, 2005).

MALT is prominently represented in the intestines by Peyer's patches, which are composed of a follicular area, parafollicular area, and follicle-associated epithelium (FAE). The FAE includes specially differentiated M cells which take up antigens and transport them from the lumen to the adjacent antigen presenting cells and lymphocytes (Owen and Nemanic, 1978; Kuhn and Kaup, 1996).

In the guinea pig, the CALT is found in the inferior fornix. The follicle-associated epithelium covering the CALT has no goblet cells, and the free surface of the follicle-associated epithelium is composed of flattened cells with uneven microvilli (Latkovic, 1979; Shoji *et al.*, 1989). Injected horseradish peroxidase (HRP) is taken up into the intercellular spaces of the conjunctival epithelium (Stock *et al.*, 1987; Shoji *et al.*, 1989) as was previously observed in Peyer's patches (Owen, 1977). Because the conjunctival membrane is the epithelium most directly exposed to the external environment, it is of great practical importance to elucidate whether the patches of flattened epithelial cells are functional equivalents of M cells in the intestines. Ruskell (1995) found lymph follicles in only the peripheral tarsal and orbital regions of the conjunctiva in the cynomolgus monkey using a whole mount method and light and electron microscopy, and suggested the presence of M cells in the FAE. A detailed distribution of the CALT in primates holds special interest for understanding the possible roles of the CALT in human conjunctival defense against environmental pathogens and antigens.

This study aims to clarify the identification, distribution, and structural organization of the CALT in Japanese monkeys using stereoscopic, light microscopic, and electron microscopic techniques. For stereoscopic studies, we modified the alcian-blue staining method. For transmission and scanning electron microscopy (TEM and SEM), 0.3 $\mu$m latex microspheres were injected into the conjunctival sac of the monkeys. The naked eye alone had not enabled identification of the CALT. In the present study, we developed new approaches to facilitate its macroscopic identification.

## Materials and Methods

Ten adult Macaca fuscatus Japanese monkeys (6 males and 4 females) weighing 6.0-8.0 kg were used for this study. All experimental and animal care procedures were approved by the Institutional Animal Care Committee of Oita University. All animals were deeply anesthetized with ketamine HCI (20mg/kg) followed by sodium pentobarbital (25mg/kg). Under deep anesthesia, two animals were used for the *in vivo* uptake of latex microspheres. All eyelids and eyeballs were taken out, and the superior and inferior eyelids were immediately excised and processed for macro-histochemical, light microscopic, SEM, and TEM examinations according to the procedures described below.

### *Light microscopic examination*

1 mm thick sections were cut sagittally from the eyelids, fixed with 10% formalin, dehydrated, and embedded in paraffin. The palpebral conjunctiva and conjunctival fornix were cut serially to 7 $\mu$m in thickness, stained with alcian-blue (pH 2.5) as well as hematoxylin and eosin (HE), and observed under a light microscope (Nikon, Tokyo).

### *Histochemical examination*

The superior and inferior eyelids were immersion-fixed with 4% paraformaldehyde in a phosphate-buffer (pH 7.4), washed in phosphate buffer saline (PBS), and immunostained by the avidin-biotin-peroxidase complex (ABC) methods using an anti human leukocyte antigen HLA-DR antibody. After immunostaining, they were counterstained according to the alcian-blue staining method (Lev and Spicer, 1964). Other tissue specimens were immersion-fixed with cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde and 2% paraformaldehyde (Karnovsky's fixative), washed with a 0.1M cacodylate buffer (pH 7.4), and then stained only with alcian-blue using a 0.1% alcian-blue solution (pH 2.5). All specimens were observed and photographed using a stereoscope (Leica, Wild M10, Germany). Regions which were macroscopically HLA-DR positive and also alcian-blue negative were dissected under a stereoscope, and paraffin sections were examined by light microscopy.

For scanning electron microscopy, the eyelids were incubated with the HLA-DR antibody using the ABC method and were placed in 1% osmium tetroxide for 2 h at room temperature, dehydrated in ethanol of ascending concentrations and then dried by the t-butylalcohol freeze-

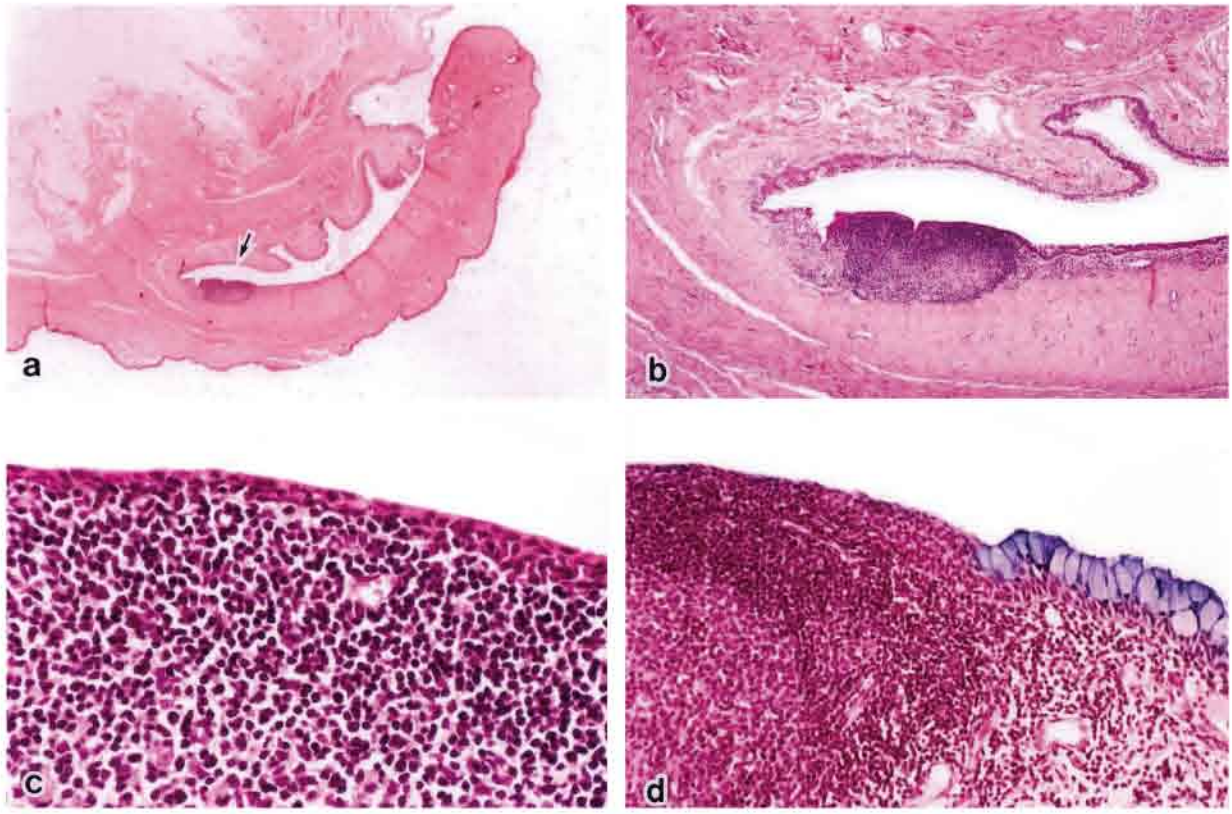


**Fig. 1.** Light micrographs of the superior eyelid in the monkeys. **a:** In a low power micrograph, there is a lymphoid follicle (arrow) at the conjunctival fornix. ×8. **b:** The lymphoid follicle includes a germinal center. ×30. **c:** The follicle-associated epithelium consists of flattened cells and no goblet cells. ×300. **d:** Goblet cells stained blue with alcian-blue are absent over CALT patches. ×150

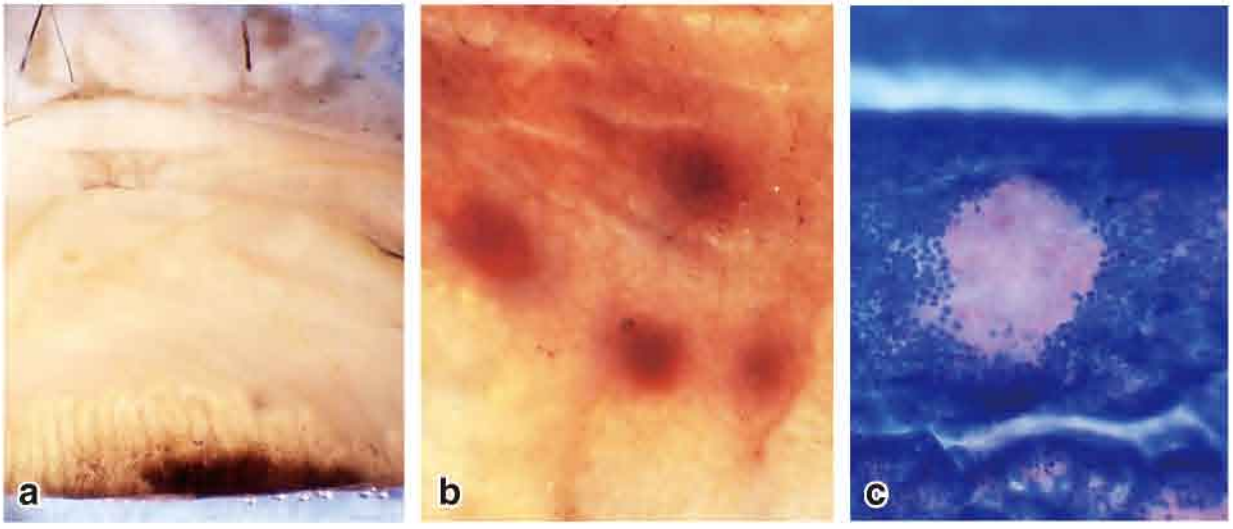


**Fig. 2.** Stereoscopic images of the conjunctival epithelium in the monkey. **a:** The CALT is not visible in the unstained specimen. ×8. **b:** The CALT is identified in a specimen immunostained with the HLA-DR antibody. ×30. **c:** In a specimen after immuno-reaction followed by alcian-blue reaction, the CALT is negative for alcian-blue reaction. ×34

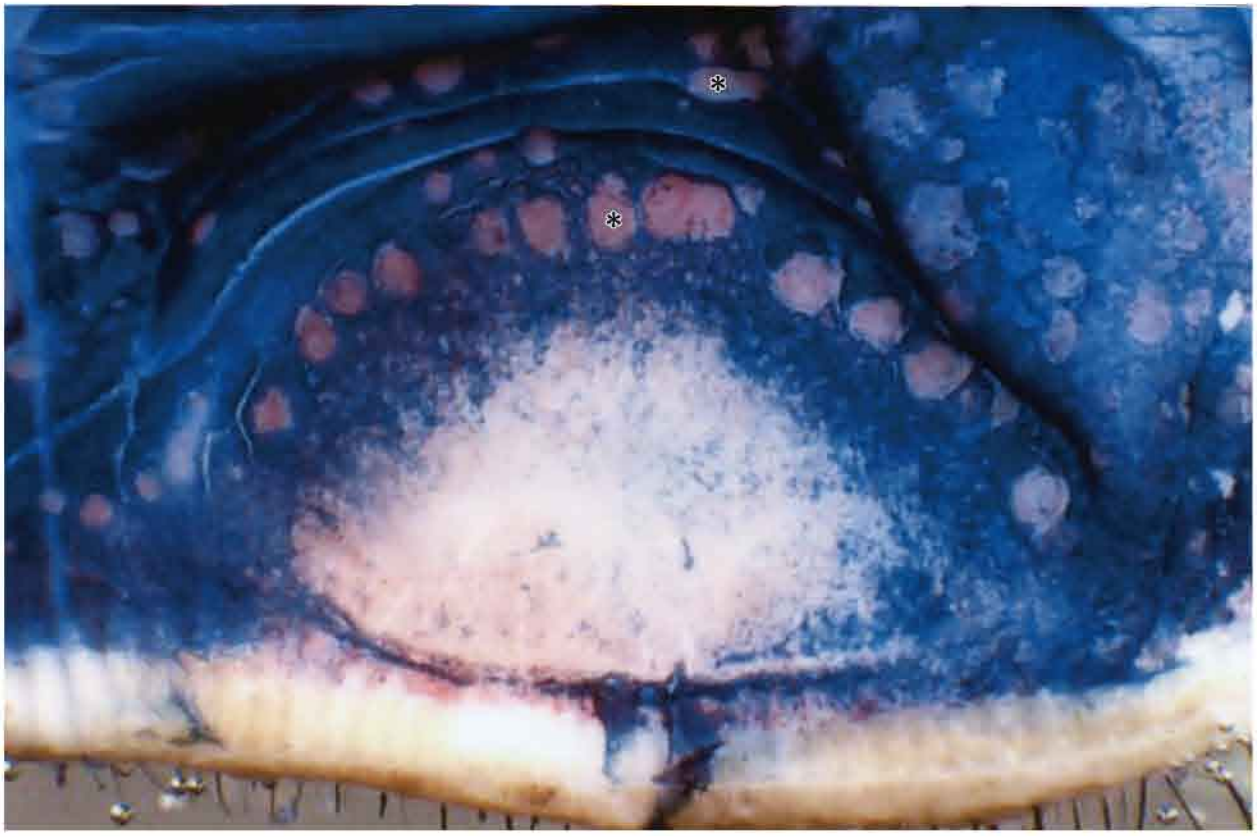

**Fig. 3.** Stereoscopic image showing the conjunctival surface of the superior eyelid in the monkey. The tissue was immunostained with the HLA-DR antibody, and then counterstained with alcian-blue. The CALT patches (*), which were positive for HLA-DR and negative for alcian-blue, show a round shape and vary in size. They are distributed at or near the conjunctival fornix (upper). ×10

drying method. The specimens were coated with carbon and examined at 25 kV in a Hitachi S-800 scanning electron microscope equipped with a GW 30 back-scattered electron detector (Hitachi, Tokyo).

### *Uptake of latex microspheres*

In two animals, 0.5 m*l* of a 5% aqueous solution of 0.3 $\mu$m latex microspheres (Sigma, St. Louis, USA) was instilled into the conjunctival sac. After 15 min or 30 min, the conjunctival fornix was removed and immersed in Karnovsky's fixative. Tissue specimens were processed for both SEM and TEM examination.

For SEM, the conjunctival fornices were washed thoroughly in physiologic saline and again fixed in Karnovsky's fixative. They were placed in 1% aqueous tannic acid for 1 h, postfixed in cacodylate buffered 1% osmium tetroxide for 2 h, dehydrated as above, freeze-dried, and coated with gold. They were then examined at 15 kV in a scanning electron microscope.

For TEM, small blocks of the conjunctival fornix were immersion-fixed in Karnovsky's fixative for 2 h at 4℃, postfixed in 0.1 M cacodylate buffered (pH 7.4) 2% osmium tetroxide-0.5% potassium ferrocyanide for 2 h at 4℃, dehydrated in ascending grades of ethanol, and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100C-X transmission electron microscope (JEOL, Tokyo).

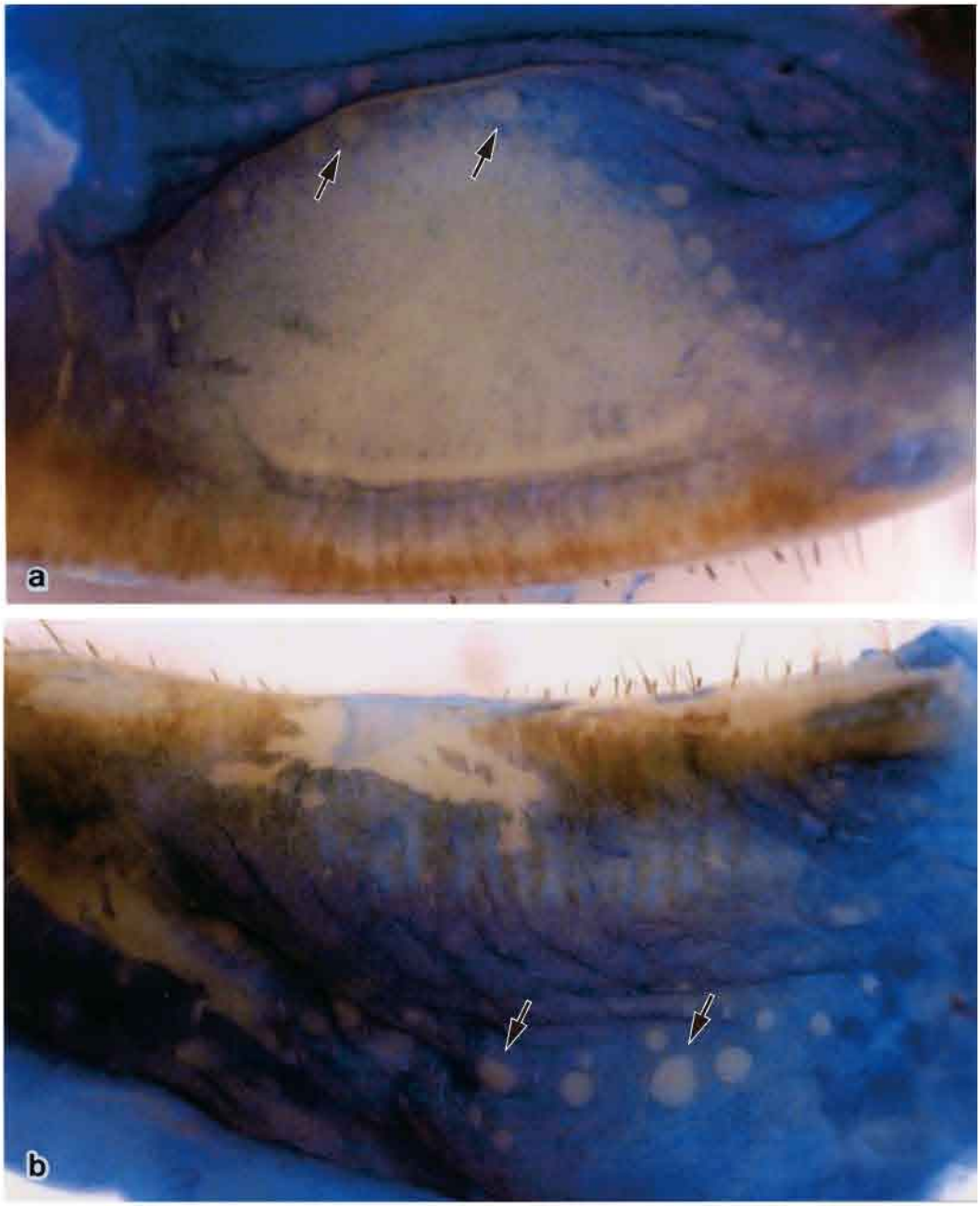


**Fig. 4.** Stereoscopic images showing the conjunctival surfaces of the superior (**a**) and inferior (**b**) eyelids stained with alcian-blue. Non-reactive areas (arrows), the so-called CALT, are more abundant in the superior eyelid than in the inferior one. ×8

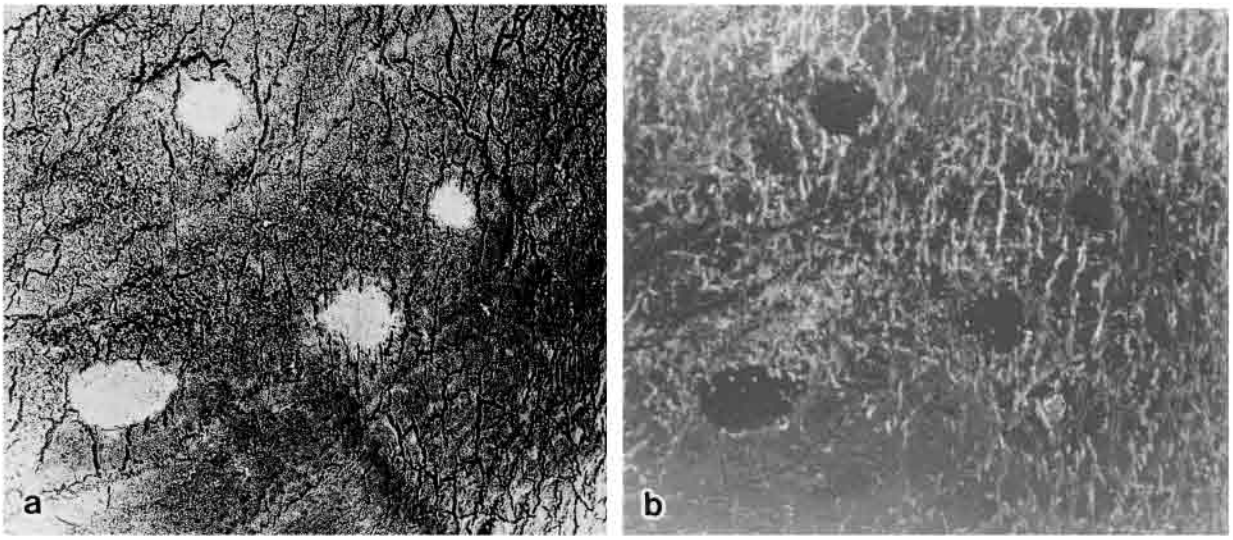


**Fig. 5.** Backscatter (**a**) and secondary (**b**) electron microscopic images of the conjunctiva immunostained with HLA-DR. The CALT reacts positively and appears as bright, round areas in the former image, while it is dark in the latter image. ×40

## Results

### *Light microscopic findings of the conjunctiva*

We found no histological differences between the conjunctiva of male and female monkeys. Although the epithelium near the margins of the palpebral conjunctiva was stratified squamous, the preponderant structure of the conjunctiva of other regions was a stratified columnar epithelium (Fig. 1a). Near or at the conjunctival fornix, epithelial cells were taller and there was a great number of goblet cells. Light microscopic observations of serial sections stained with HE revealed lymphoid follicles distributed beneath the epithelium in the conjunctival fornix (Fig. 1a–c). These follicles showed the appearance of secondary follicles with germinal centers (Fig. 1b, c). Furthermore, the follicle-associated epithelium consisted of flattened cells, and contained lymphocytes but no goblet cells (Fig. 1c). In sections stained with alcian-blue (pH 2.5) for identification of acid glycocarbohydrate, blue stained goblet cells, which were prominent in other parts of the conjunctiva, were absent over CALT patches (Fig. 1d). This morphological feature in monkeys was practically identical to that of the conjunctiva-associated lymphoid tissue (CALT) demonstrated in rabbits (Franklin and Remus, 1984) and guinea pigs (Shoji *et al.*, 1989, 1992). We detected CALT in monkeys in both the superior and inferior conjunctival fornices.

### *Stereoscopic analyses of the CALT*

It was not possible to identify the CALT from the posterior surface of the unstained superior and inferior eyelids under a stereoscope (Fig. 2a). Accordingly, the present study attempted to demonstrate the CALT by utilizing histochemical techniques. In both upper and lower eyelids immunostained with the anti HLA-DR antibody, small brown oval regions showing a positive reaction were scattered near or at the conjunctival fornix (Fig. 2b). Tissue blocks containing the HLA-DR positive regions were microdissected under a stereoscope, and then paraffin sections were made for light microscopy. The HLA-DR positive region had no goblet cells, and a lymphoid follicle was situated beneath the conjunctival epithelium. The HLA-DR positive region was clearly identical with the CALT. When the eyelids were immunostained and then counter-stained with alcian-blue, HLA-DR positive areas were negative for alcian-blue stained goblet cells found in the surrounding epithelium (Fig. 2c).

The goblet cells were scanty at the margin of the palpebral conjunctiva but were richly distributed near and in the conjunctival fornix. Within these sheets of goblet cells in the fornix, we found small islands of epithelium without goblet cells scattered near and at the conjunctival fornix, clearly demarcated from the surrounding mucous epithelium. These regions were oval in shape, variable in

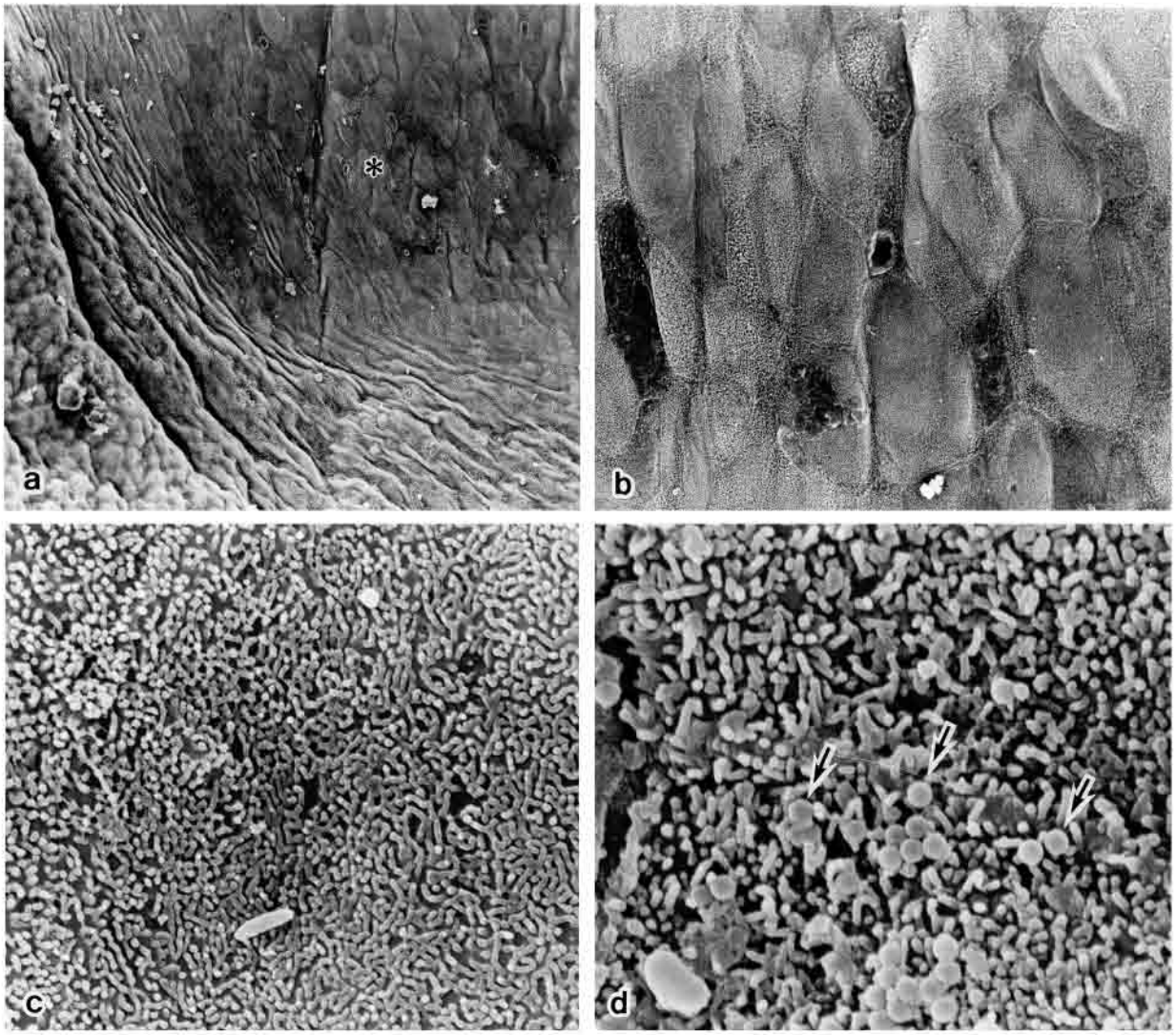


**Fig. 6.** SEM images showing the surface of the CALT and its surroundings in the superior conjunctival fornix. **a:** The surface of the CALT (*) is relatively flat, while its surroundings form small folds. ×270. **b:** The superficial cells are polygonal in shape and vary in size. ×1,100. **c:** In a higher magnification, the cell surface is densely packed with short microvilli and microridges. ×8,300. **d:** In tissue specimens 15 min after administration of a latex solution, latex microspheres (arrows) adhere to a surface cell. ×14,000

size ranging from 200 μm to 300 μm, and arranged in concentric lamellae (Fig. 3).

The alcian-blue staining method thus enabled us to identify easily the CALT, which showed a starkly negative reaction for alcian-blue in the fornix (Fig. 4a, b). The number of the CALT patches in both the superior and inferior eyelids varied according to the individual. The number of CALT patches was approximately 20 to 40 in the superior (Fig. 4a), and approximately 10 to 20 in the inferior eyelid (Fig. 4b). Conjunctiva of the superior eyelid was bigger in area than that of the inferior lid (Fig. 4a, b).

### *Scanning electron microscopy (SEM) of the CALT*

The free surface of the epithelium in the conjunctival

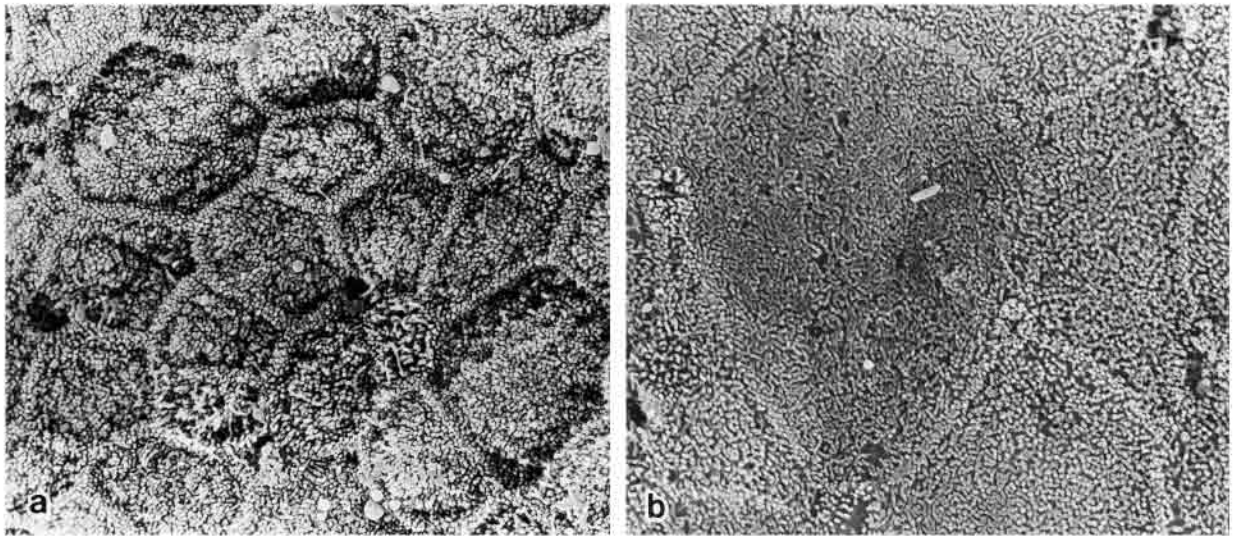


**Fig. 7.** SEM images of goblet cells (**a**) and microfold cells (**b**) in the superior conjunctival fornix. Both types of cells have a great number of microvilli, but the goblet cells are smaller in surface area than the microfold cells and have prominent, raised junctions with adjacent cells. ×4,800

fornix was further characterized by SEM. The backscatter electron microscopic observations of the tissues immunostained with HLA-DR enabled us to identify the CALT (Fig. 5a), which formed small islands conforming to the islands of goblet cell free epithelium observed under a stereoscope. In secondary electron microscopic images, the CALT showed a dark appearance (Fig. 5b). The CALT was flat, oval, or round in shape and ranged from 150 to 500 $\mu$m. The cells covering the surface of the CALT were polygonal in shape (Fig. 6a, b), ranging from 15 to 25 $\mu$m in diameter, and their surfaces were densely packed with numerous short microvilli and microridges (Fig. 6c, d, 7b). The surface morphology of these cells was similar to that of microfold cells as illustrated in the duct-associated lymphoid tissue (DALT) of monkey parotid glands (Matsuda *et al.*, 1997). A few bacteria were seen adhering to the epithelial surface.

To confirm morphologically whether the CALT in monkeys was zones of particulate uptake as is characteristic in other locations of mucosa associated lymphoid tissue (MALT), an eye drop solution of 0.3 $\mu$m latex particles was put into monkey's eyes. SEM observation revealed that, 15 min after the administration of eye drops, latex particles were picked up only by the large polygonal epithelial cells over the surfaces of CALT patches, (Fig. 6d), and not by cells in the surrounding goblet cell rich epithelium.

The surfaces of the conjunctival fornix were mostly covered with goblet cells, which have a diameter of about 7 $\mu$m, bulged slightly, and showed an irregular surface (Fig. 7a). Thus, goblet cells were different in size and surface appearance from microfold cells over CALT patches (Fig. 7a, b). Only a few goblet cells were detected at the periphery of the CALT patches.

### *Transmission electron microscopy (TEM) of the CALT*

When a latex solution in eye drops was instilled into the conjunctival sacs, the CALT appeared as small plaques under a stereoscope. These small regions including the CALT were selected for investigation by TEM. Epithelial cells overlying the lymphoid follicle took up small numbers of latex particles into their cytoplasm 15 min after the instillation of eye drops (Fig. 8a). After 30 min epithelial cells over the surfaces of CALT patches phagocytosed particles at the microvillus surface and incorporated single or groups of latex particles in vesicles (Fig 8b). The epithelial cells overlying the lymphoid follicles showed a characteristic morphology in contrast to that of goblet cells. The superficial cells were often flattened, had short irregular microvilli, and were tightly connected to each other by junctional complexes containing desmosomes. Their cytoplasm possessed many vesicles and lysosomes, numerous mitochondria, a moderately developed Golgi apparatus,

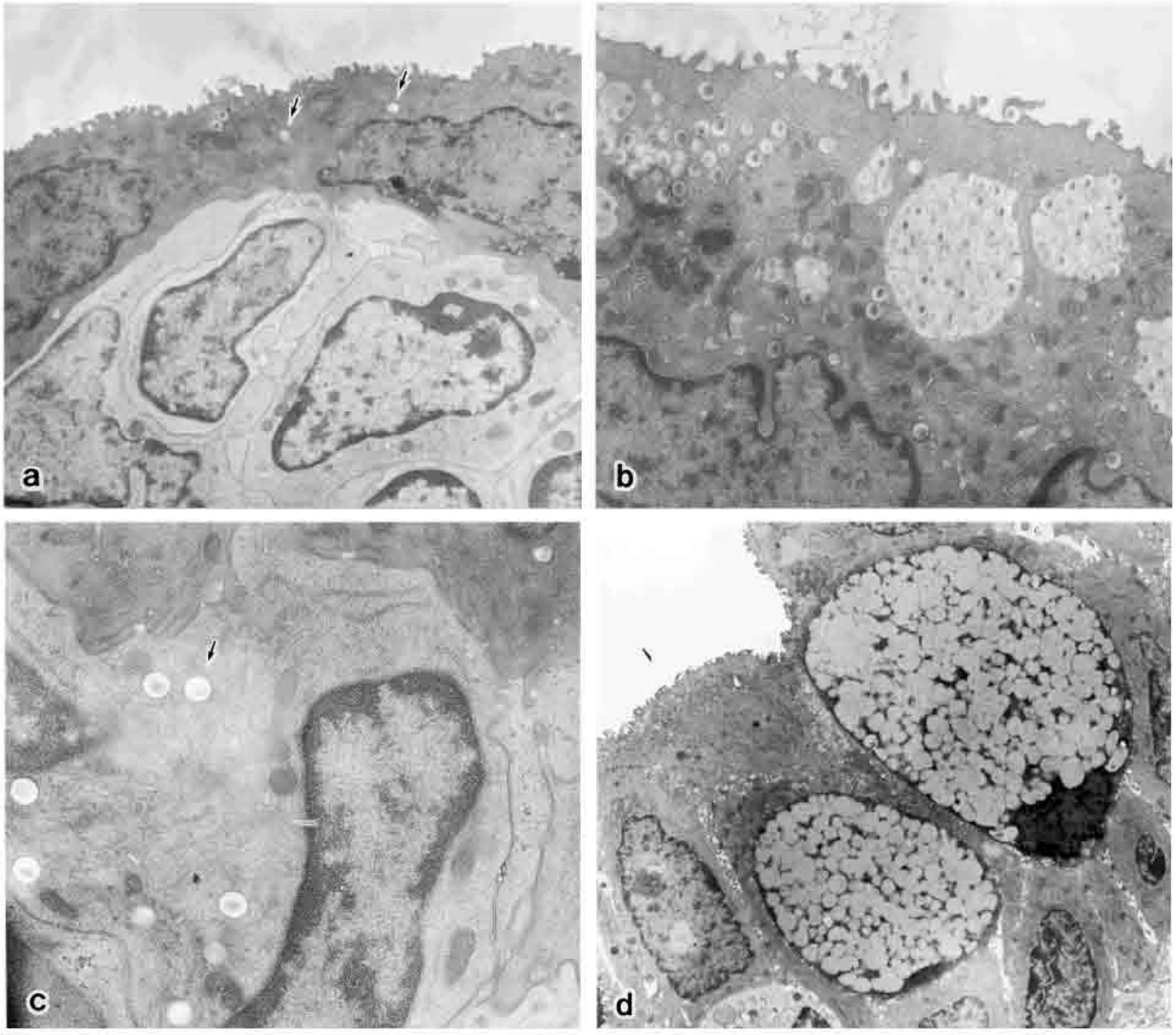


**Fig. 8.** TEM images of the conjunctival epithelium overlying the lymphoid follicle after administration of a latex solution. **a:** The surface epithelial cells take up a few latex microspheres (arrows) 15 min after administration. ×3,800. **b:** They take up many latex microspheres 30 min after administration. ×6,700. **c:** Latex microspheres are detected between the cytoplasmic processes of intraepithelial dendritic cells as well as in their cytoplasm (arrow) 30 min after administration. ×10,100. **d:** There are no latex microspheres in goblet cells near the CALT. ×1,700

and rough endoplasmic reticulum (Fig. 8a, b). The CALT was also characterized by the presence of two types of intraepithelial immunoreactive cells: lymphocytes and dendritic cells (Fig. 8a, c). The dendritic cells were identified by their lobulated nuclei and cytoplasmic processes. Their cytoplasm was less dense than epithelial cells having mitochondria, a Golgi apparatus, and rough endoplasmic reticulum. Birbeck granules were not found. The dendritic cells were in contact with intraepithelial lymphocytes, epithelial cells, and each other without forming junctional complexes. Latex particles were found not only between intraepithelial lymphocytes but also in the cytoplasm of intraepithelial dendritic cells (Fig. 8c). The follicular and parafollicular compartments of the lymphoid follicles in the CALT showed fundamental ultrastructural similarities to Peyer's patch lymphoid

follicles (Owen, 1977). The follicular area contained numerous small and large lymphocytes, follicular dendritic cells, and macrophages. In the parafollicular area, plasma cells and high-endothelial venules were observed (data not shown). The conjunctival epithelium surrounding the CALT patches was composed of goblet cells packed with mucous granules. There were no latex microspheres within the cytoplasm of goblet cells (Fig. 8d).

## Discussion

The present study illustrates that the epithelial surface of the conjunctival fornix in the superior and inferior eyelids of monkeys is predominantly composed of mucus secreting goblet cells. Anatomically, the ducts of the lacrimal glands opened onto the lateral part of the superior fornix. It is likely that tears and mucus from the many goblet cells play an important role in ocular defense by trapping particles and bacteria in mucus and washing them away with tears. The conjunctival fornix in most animal species has been shown to contain lymphoid follicles with an epithelium covering the lymphoid follicles histologically different from the surrounding epithelium (Latkovic, 1979). This organized lymphoid tissue was first referred to as the conjunctiva-associated lymphoid tissue (CALT) by Chandler and Axelrod (1980). Thereafter, the presence of the CALT was reported in many animal species, including the turkey (Fix and Arp, 1989), chicken (Fix and Arp, 1991), mouse (Sakimoto *et al.*, 2002), Lewis rat (Dua *et al.*, 1996), guinea pig (Dwyer *et al.*, 1983; Shoji *et al.*, 1989, 1992), rabbit (Knop N and Knop, 2005), angora goat (Asti *et al.*, 2000), dog (Giuliano *et al.*, 2002), and cynomolgus monkey (Ruskell, 1995; Ruskell and VanderWerf, 1997). Human conjunctival fornices have also been shown to possess CALT (Knop and Knop, 2000), but the functional significance of CALT in humans or other primates has not been demonstrated previously. In Japanese monkeys we found focal areas, which consisted of lymphoid follicles with reactive centers and specialized epithelium in the conjunctival fornix. Thus, it is further confirmed that the CALT is present in not only lower mammals but also humans and other primates.

Although the CALT in the guinea pig was localized to the inferior fornix (Shoji *et al.*, 1989,1992), in monkey it was present in the fornices of both the superior and inferior eyelids. Previously, morphological studies of the CALT were largely limited to the light microscopy of serial sections and transmission electron microscopy (TEM). Accordingly, the distribution of the CALT remained unclear. It had been impossible to identify the CALT in unstained tissue with only the naked eye under a stereoscope. Our immuno-staining method using the HLA-DR antibody and alcian-blue counterstaining enabled us to identify clearly the distribution of CALT under a stereoscope. The latter method was especially simple and useful. By this method, CALT patches appeared spherical or oval in shape, being about 300 $\mu$m in size and distributed at or near the fornix. The number of patches ranged from 10 to 40 and was larger in the superior fornix than the inferior one. This may be due to the fact that the superior eyelid is larger in area than the inferior one.

From many light microscopic and TEM examinations of the CALT, the follicle-associated epithelium was seen to consist of flattened cells with irregular microvilli and had no goblet cells. The cytoplasm of the superficial epithelial cells had numerous mitochondria, small lysosomes, a moderately developed Golgi apparatus, many small vesicles, and rough endoplasmic reticulum. Consequently, the superficial epithelial cells of the CALT showed fundamentally the same morphological features as M cells in Peyer's patches (Owen and Jones, 1974). Even so, the term "M cell" was used as a diminutive of the microfold cell in human Pyer's patches by Owen and Jones (1974). M cells in the conjunctival epithelium of the guinea pig (Latkovic,1979) and the cynomolgus monkey (Ruskell, 1995) were covered with stunted, irregular microvilli rather than microfolds. In the present SEM and TEM observations, the superficial cells over lymphoid follicles were larger in surface area compared with the goblet cells, and had more numerous and irregular microvilli.

The ultrastructural features of superficial cells of the monkey CALT were fundamentally similar to those of M cells in GALT (Owen and Jones, 1974), NALT (Claeys *et al.*, 1996) and DALT (Matsuda *et al.*, 1997). However, it was not previously clear whether these cells were identical with M cells functionally. Morphological studies have shown that particles such as latex microspheres and microorganisms are readily taken up by M cells of Peyer's patches (Lefevre *et al.*, 1978; Sass *et al.*, 1990) and that M cells are positive for the anti HLA-DR antibody (Nagura *et al.*, 1991). In our study, follicle-associated epithelial cells were positive for the anti HLA-DR antibody and took up injected latex microspheres administered as eye drops. These findings strongly indicate that specialized conjunctival cells in the CALT are functionally equivalent to M cells in GALT. Lymphoid follicles in the CALT are composed of T cells and B cells (Franklin and Remus, 1984). Follicular dendritic cells, which function as antigen-presenting cells in the germinal center of lymph nodes (Yoshida and Takaya, 1989) and Peyer's patches (Yamakawa *et al.*, 1991), were previously identified in the CALT follicular area of the guinea pig. In this study,

dendritic cells with processes were also found within the follicle-associated epithelium of primates as demonstrated in the monkey DALT (Matsuda *et al.*, 1997), and injected latex microspheres were taken by them after passing through epithelial cells. This demonstrates the histological components and functional attributes of the CALT that are necessary for the initiation of an immune response to particulate antigens to supplement the mechanical defenses provided by mucus and tears. Our observation of the uptake of particles from eye drops may be useful in laying a foundation for exploiting this route for the administration of insoluble particulate therapeutic as well as immunizing agents.

In 1984, Chandler and Axelrod advanced the concept that the CALT is a probable component of the mucosa-associated lymphoid tissue (MALT). Subsequent morphological investigations, including the present study, strongly support this concept and indicate that the CALT provides a focal route for the uptake and transport of potential antigenic particles and microorganisms for the initiation of protective mucosal immune responses. In the submucosal tissue beneath the conjunctiva and in the lacrimal glands, a considerable number of plasma cells are present. These cells are active in the synthesis of immunoglobulins, particularly IgA (Cohen and Allansmith, 1981). The outer eye is thus provided with a system of local secretory immunoglobulins. In conclusion, CALT patches are uniquely positioned and histologically composed to participate as inductive sites for the common mucosal immune system.

## References


Aşti RN, Kurtdede N, Altunay H, Özen A: Electron microscopic studies on conjunctiva associated lymphoid tissue (CALT) in Angora goats. *Dtsch Tierarztl Wochenschr* 107: 196-198 (2000).

Chandler JW, Axelrod AJ. Conjunctiva-associated lymphoid tissue: a probable component of the mucosa-associated lymphoid system. In: *Immunologic diseases of the mucous membranes; pathology, diagnosis and treatment* (O'Connor GR, ed), New York, Masson Publishing USA, 1980 (p. 63-70)

Claeys S, Cuvelier C, Quatacker J, Cauwenberge PV: Ultrastructural investigation of M-cells and lymphoepithelial contacts in naso-pharyngeal associated lymphoid tissue (NALT). *Acta Otolaryngol (Stokh) Suppl* 523: 40-42 (1996).

Cohen EJ, Allansmith MR: Fixation techniques for secretory component in human lacrimal gland and conjunctiva. *Am J Ophthalmol* 91: 789-793 (1981).

Dua HS, Jindal VK, Gomes JAP, Amoaku WA, Donoso LA, Laibson PR, Mahlberg K: The effect of topical cyclosporin on conjunctiva-associated lymphoid tissue (CALT). *Eye* 10: 433-438 (1996).

Dwyer RSTC, Darougar S, Monnickendam MA: Unusual features in the conjunctiva and cornea of the normal guinea-pig: Clinical and histological studies. *Br J Ophthalmol* 67: 737-741 (1983).

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

Fix AS, Arp LH: Morphologic characterization of conjunctiva-associated lymphoid tissue in chickens. *Am J Vet Res* 52: 1852-1859 (1991).

Franklin RM, Remus LE: Conjunctival-associated lymphoid tissue: Evidence for a role in the secretory immune system. *Invest Ophthalmol Vis Sci* 25: 181-187 (1984).

Giuliano EA, Moore CP, Phillips TE: Morphological evidence of M cells in healthy canine conjunctiva-associated lymphoid tissue. *Graefe's Arch Clin Exp Ophthalmol* 240: 220-226 (2002).

Knop E, Knop N: The role of eye-associated lymphoid tissue in corneal immune protection. *J Anat* 206: 271-285 (2005).

Knop N, Knop E: Conjunctiva-associated lymphoid tissue in the human eye. *Invest Ophthalmol Vis Sci* 41: 1270-1279 (2000).

Knop N, Knop E: Ultrastructural anatomy of CALT follicles in the rabbit reveals characteristics of M-cells, germinal centers and high endothelial venules. *J Anat* 207: 409-426 (2005).

Kuhn EM, Kaup FJ: Morphological characteristics of the ileal Peyer's patches in the Rhesus macaque: a histological and ultrastructural study. *Anat Histol Embryol* 25: 65-69 (1996).

Latkovic S: The ultrastructure of the normal conjunctival epithelium of the guinea pig. Ⅲ. The bulbar zone, the zone of the fornix and the supranodular zone. *Acta Ophthalmol* 57: 305-320 (1979).

Lefevre ME, Olivo R, Vanderhoff JW, Joel DD: Accumulation of latex in Peyer's patches and its subsequent appearance in villi and mesenteric lymph nodes. *Proc Soc Exp Biol Med* 159: 298-302 (1978).

Lev R, Spicer SS: Specific staining of sulfate groups with alcian blue at low pH. *J Histochem Cytochem* 12: 309 (1964).

Matsuda M, Ina K, Kitamura H, Fujikura Y, Shimada T: Demonstration and organization of duct-associated lymphoid tissue (DALT) of the main excretory duct in the monkey parotid gland. *Arch Histol Cytol* 60: 493-502 (1997).

Nagura H, Ohtani H, Masuda T, Kimura M, Nakamura S: HLA-DR expression on M cells overlying Peyer's patches is a common feature of human small intestine. *Acta Pathol Jap* 41: 818-823 (1991).

Owen RL: Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology* 72: 440-451 (1977).

Owen RL, Jones AL: Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* 66: 189-203 (1974).

Owen RL, Nemanic P: Antigen processing structures of the mammalian intestinal tract: An SEM study of lymphoepithelial organs. *Scanning Electron Microsc 1978*/ Ⅱ: 367-378 (1978).

Ruskell GL: Organization and cytology of lymphoid tissue in the cynomolgus monkey conjunctiva. *Anat Rec* 243: 153-164 (1995).

Ruskell GL, VanderWerf F: Sensory innervation of conjunctival lymph follicles in cynomolgus monkeys. *Invest Ophthalmol Vis Sci* 38: 884-892 (1997).

Sakimoto T, Shoji J, Inada N, Saito K, Iwasaki Y, Sawa M: Histological study of conjunctiva-associated lymphoid tissue in mouse. *Jpn J Ophthalmol* 46: 364-369 (2002).

Sass W, Dreyer HP, Seifert J: Rapid insorption of small particles in the gut. *Amer J Gastroenterol* 85: 255-260 (1990).

Shoji J, Fujisawa A, Ebato B, Sakimoto T, Kitano S. Ishii Y: The lymphoepithelium of the conjunctival associated lymphoid tissue (In Japanese). *Journal of the Eye* 6: 1181-1185 (1989).

Shoji J, Inada N, Kasai H, Ishii Y, Kitano S: Immuno-responses to the external antigen in conjunctival-associated lymphoid tissue (In Japanese). *Acta Soc Ophthalmol Jpn* 96: 432-439 (1992).

Stock EL, Sobut RA, Roth SI: The uptake of horseradish peroxidase by the conjunctival epithelium of the guinea-pig. *Exp Eye Res* 45: 327-337 (1987).

Yamakawa M, Kudo S, Ohe S, Imai Y. Morphological and functional studies of dendritic cells in the Peyer's patch. In: *Frontiers of mucosal immunology* (Tsuchiya *et al.*, eds), Excerpta Medica, Amsterdam, 1991 (p. 649-652)

Yoshida T, Takaya K: Follicular dendritic reticular cells in the germinal center of the rat lymph node as studied by immuno-electron microscopy. *Arch Histol Cytol* 52: 327-335 (1989).