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Histological Study of Conjunctiva-associated Lymphoid Tissue in Mouse

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Purpose: To investigate conjunctiva-associated lymphoid tissue (CALT) in the mouse conjunctiva by histological methods.

Methods: The presumed follicular tissue in the conjunctiva of normal mice, age ranging from 4 to 6 weeks, was histologically investigated by the hematoxylin-eosin staining method. Next, we treated the mice with topical instillation of a combined solution of ovalbumin and cholera toxin B to investigate the morphological changes of conjunctival follicles to antigen challenge. The treated mice underwent sequential clinical examinations, and the conjunctival follicular tissue was examined by an immunohistochemical method using anti-CD4 antibody, anti-CD8 antibody, and anti-S-100 antibody.

Results: Follicular tissue was present on the mouse nictitating membrane. Both size and number of follicular tissue areas increased with topical ovalbumin treatment. Immunohistochemical study revealed CD4, CD8, and S-100 positive cells in the follicular tissue. The epithelial layer, corresponding to follicular tissue, demonstrated intra-epithelial pocket and the presence of CD4-positive cells in the intra-epithelial pocket.

Conclusion: Follicular tissue at the nictitating membrane is CALT in the mouse. **Jpn J Ophthalmol 2002;46:364–369**

Key Words: Conjunctiva-associated lymphoid tissue, follicle, immune tissue, mouse, nictitating membrane.

Introduction

In conjunctival tissue, conjunctiva-associated lymphoid tissue (CALT) is present and plays an important role in the local immune system.[1,2] This tissue is composed of lymphoid tissue comprising aggregated lymphatic cells in subconjunctival tissue and has components of a follicular area, para-follicular area, dome area, and lymphatic epithelium, as detected by histological study. This CALT induces an antigen-specific IgA-producing premature B cell against exogenous antigen to conjunctiva.[3,4]

So far, CALT has revealed its presence in humans,[5] monkeys,[6] rabbits, and guinea pigs.[1,2] Because the guinea pig has representative CALT at the palpebral conjunctiva and fornix of the lower lid, it has been investigated by various histological and immunohistochemical methods. In the monkey, high endothelial venule and conjunctival follicles with helix-like structured lymphatic duct were reported by morphological study.[6] In humans, diffuse or follicular CALT tissue was reported by immunohistochemical methods.[5]

In the mouse, the presence of nasopharyngus-associated lymphoid tissue (NALT) and gut-associated lymphoid tissue (GALT) comprising Peyer's patch and the isolated lymphatic nodule in the intestinal duct were reported and their role in the immune system was investigated.[7–10] However, the presence of CALT has not been reported in the mouse to date. In this study, we investigated its presence in the mouse.

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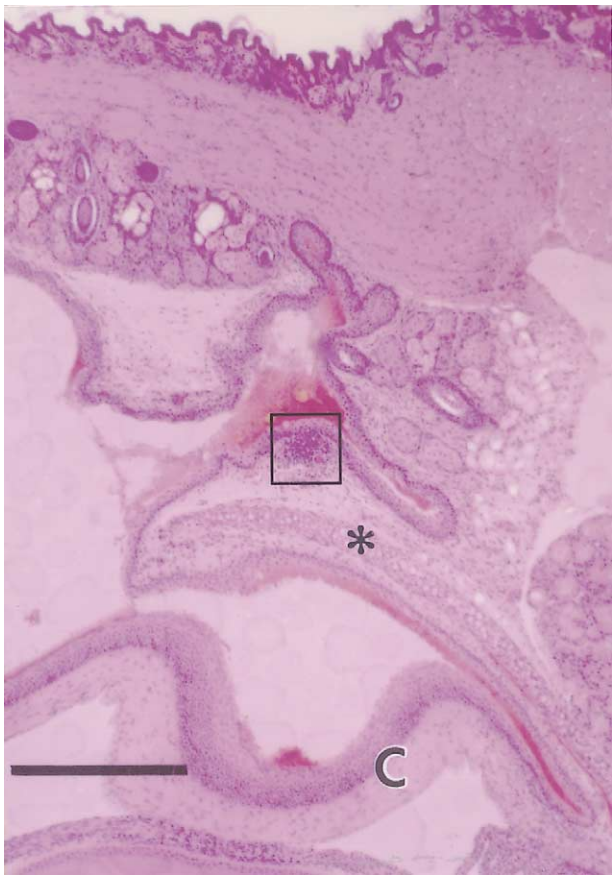

Figure 1. Follicular tissue in normal mouse (hematoxylin-eosin staining). Follicular tissue (□) is present at nictitating membrane (*) with cartilage. In upper part of figure is palpebra and in lower, cornea (C). Bar = 500 μm.

Materials and Methods

We used a specific pathogen-free (SPF) BALB/c strain of mice, age ranging from 4 to 6 weeks. Care and treatment of the animals were undertaken according to the Guideline of the Nihon University School of Medicine on Animal Experiments, as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 36 eyes of 18 female mice were examined in the following study.

Histological Study in Normal Mouse

Three mice were sacrificed by spinal fracture method and both their eyes were removed with palpebral tissue. The removed tissue was fixed with Carnoir solution. After dehydration using an increasing alcohol solution series, the tissue was embedded in Tecnovit 7100® (Heraeus, Wehrheim, Germany) and made into a thin continuous section about 7 μm in thickness, using a microtome. The thin section was stained using hematoxylin-eosin (HE) or Giemsa solution and observed by light microscopy (BH-2, Olympus, Tokyo).

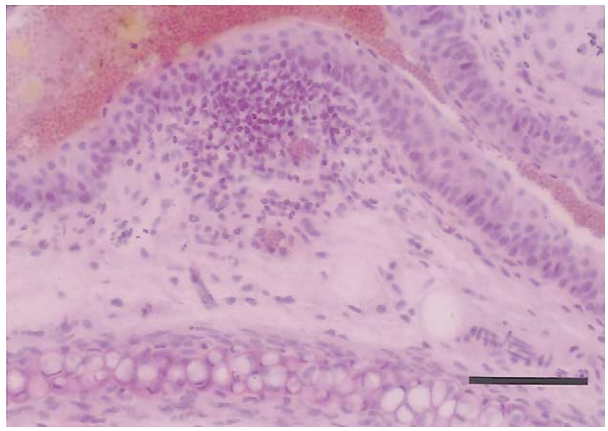

Figure 2. Magnified view of follicular tissue (hematoxylin-eosin staining). Goblet cell is not present in epithelial cell just above follicular tissue. Bar = 100 μm.

Histological Study After Antigen Treatment

Animals were divided into two groups; (A) treated group comprising 9 mice (18 eyes) and (B) nontreated group, consisting of 3 mice (6 eyes). The treated group underwent topical instillation of 0.05 mL of a mixed solution of ovalbumin (OVA), 0.25 μg/mL (Sigma, St. Louis, MO, USA) and cholera toxin B (CT-B),– 0.1 μg/mL (List Biological Laboratories, Campbell, CA, USA) as an adjuvant once a day on days 0, 1, 7, and 8 of the experiment. On days 2, 9, and 14 of the experiment, 6 eyes of 3 mice were studied on each examination day by the methods described above for normal

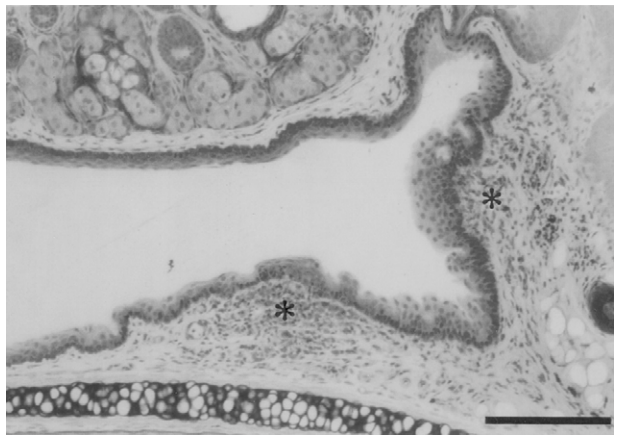

Figure 3. Follicular tissue on day 2 in treated group (Giemsa staining). Follicular tissue (*) is present at both fornix and nictitating membrane. Bar = 200 μm.

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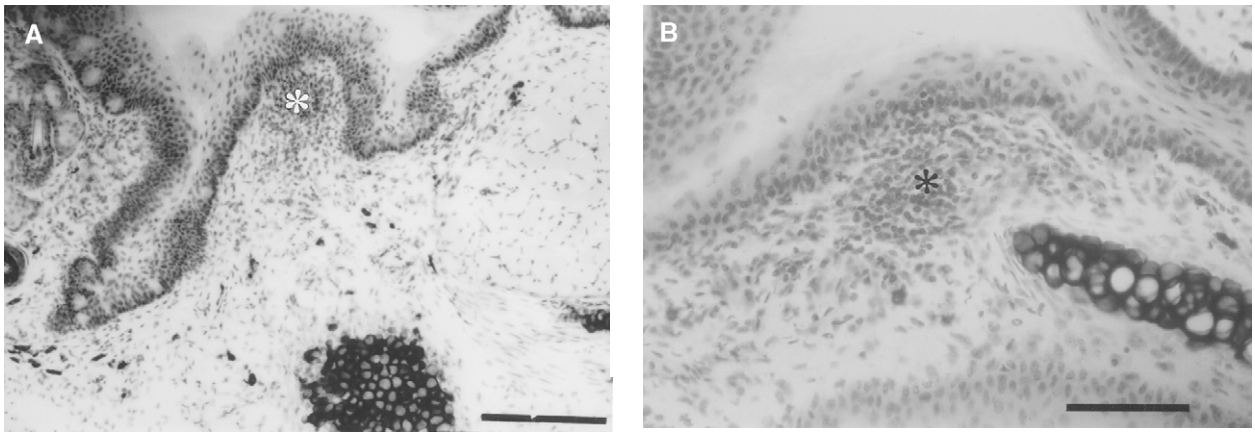

Figure 4. Follicular tissue on day 9 in treated group. (Giemsa staining). Follicular tissues (*) are present at several places (same eye). (**A**) Fornix area (Bar = 100 μm). (**B**) Center of nictitating membrane (Bar = 200 μm).

mice. The nontreated group, which did not undergo OVA instillation, was examined at day 14 by the same methods as used in the treated group.

Immunohistochemical Study of Follicular Tissue

The remaining 6 eyes of 3 mice underwent topical instillation of OVA solution as described above on days 0, 1, 7, and 8 of the experiment and were sacrificed on day 9. Eyeballs with palpebral conjunctiva were removed and fixed using 2% periodate lysin paraformaldehyde solution for 1 hour and embedded in Tissue-Tek® (Sakura Seiki, Tokyo) by the dry ice-isopentane freezing method. Frozen tissues were sectioned, about 7 μm in thickness, using a cryostat. The thin sections were stained by anti-mouse CD4 monoclonal antibody (Beckman Coulter, Tokyo), anti-mouse CD8 monoclonal antibody (Beckman Coulter) and anti-bovine brain S-100 protein rabbit antibody (Serotec, Oxford, UK) as a first antibody; then stained by indirect enzyme-antibody method (avidin-biotin complex method: ABC method). In the ABC method, Vectastain ABC kit® (Vector, Burlingame, CA, USA) was used and Karnovsky solution with 3,3′-diaminobenzidine tetra hydrochloride (Wako Pure Chemical, Osaka) was used. Nuclear staining was performed with methyl green staining and observed by light microscopy.

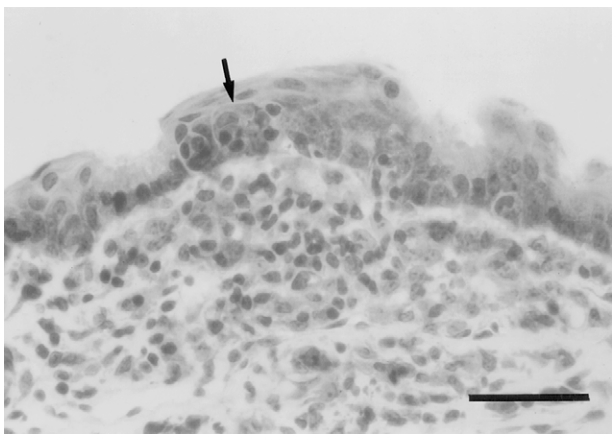

Figure 5. Histological finding of intra-epithelial pocket on day 9 in treated group (Giemsa staining). Arrow indicates formation of intra-epithelial pocket. Bar = 50 μm.

Results

Histological Study in Normal Mouse

Nictitating membrane with cartilage was present at the inner canthus of the mouse eyes. Nictitating membrane has conjunctival epithelium and its subconjunctival tissue demonstrated one follicular tissue area comprising an aggregation of lymphatic cells (Figure 1). Although goblet cells were present in the surrounding epithelium, they were not present in the epithelium covering the follicular tissue (Figure 2).

Histological Study After Antigen Treatment

In the nontreated group, only one follicular tissue area was observed and it was limited to the nictitating membrane. However, in the treated group, on day 2, follicular tissue was observed not only at the nictitating membrane but also at the fornix area (Figure 3). On day 9, more than one follicular tissue area was observed at the nictitating membrane (Figure 4). Furthermore, on day 9, an intra-epithelial pocket was formed in the epithelium covering follicular tissue (Figure 5). On day 14, several follicular tissue areas were present at the nictitating membrane, but the size of each tissue area was smaller than that on day 9 (Figure 6).

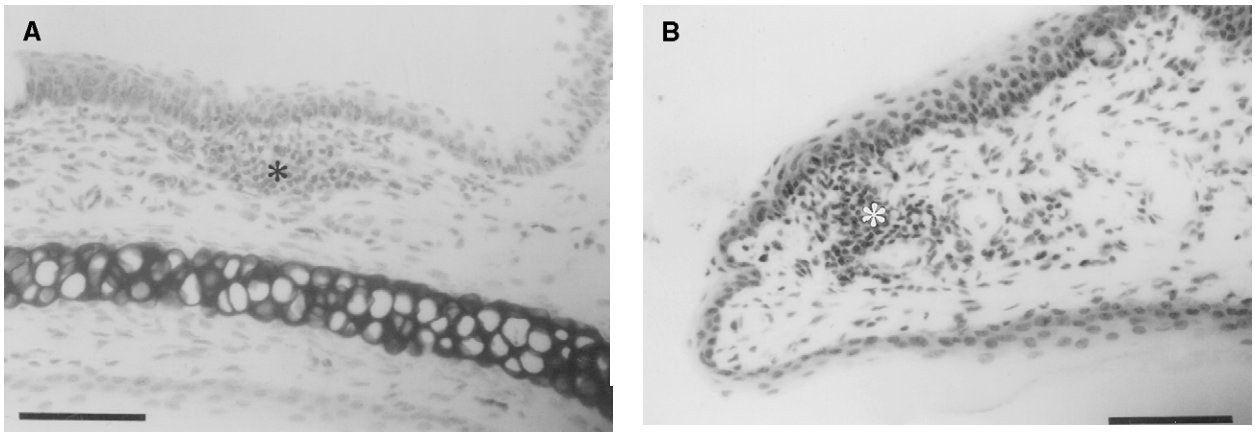

Figure 6. Follicular tissue on day 14 in treated group. (Giemsa staining). Several follicular tissues (*) are present, but their size tends to be decreased (same eye). (**A**) Fornix side of nictitating membrane. Bar = 100 μm. (**B**) Head of nictitating membrane. Bar = 100 μm.

Immunohistochemical Study of Follicular Tissue

CD4- and CD8-positive cells were present on the follicular tissue and its surrounding area (Figures 7 and 8), and their distribution did not show any characteristic feature. CD4-positive cells were present in the epithelial pocket (Figure 9). S-100-positive cells were demonstrated in the meshwork pattern in the center of the follicular area (Figure 10).

Discussion

An investigation method for CALT is comprised of three principle methods, though there have been minor variations. (1) Detection of CALT component tissue such as follicular area, para-follicular area, and lymphoid tissue.[1,3,5,6,11] (2) Detection of lymphatic cells at the follicular area.[5,6,12] (3) Detection of the intra-epithelial pocket, which plays a role as antigen presentation site of the lympho-epithelium.[3]

Detection of CALT Component Tissue

In intestinal tissue, peripheral lymphatic tissue such as Peyer's patch was found anatomically before establishment of its functional role as GALT. In the neonatal stage or in SPF environment, immaturity of lymphatic tissue, especially in the germinal center of the follicular area or para-follicular area, has been reported.[13] These lymphatic tissues are activated by antigen stimulation.[14] In NALT of the neonatal mouse, it is reported that a B-lymphatic cell area with I region-associated antigen (Ia antigen) positive

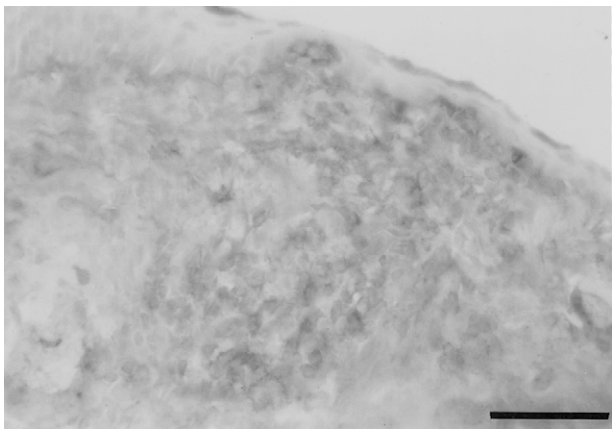

Figure 7. Indirect enzyme antibody method with anti-CD4 antibody. CD4-positive finding is observed mainly at surrounding cells and partly cells in intra-epithelial pocket. Bar = 100 μm.

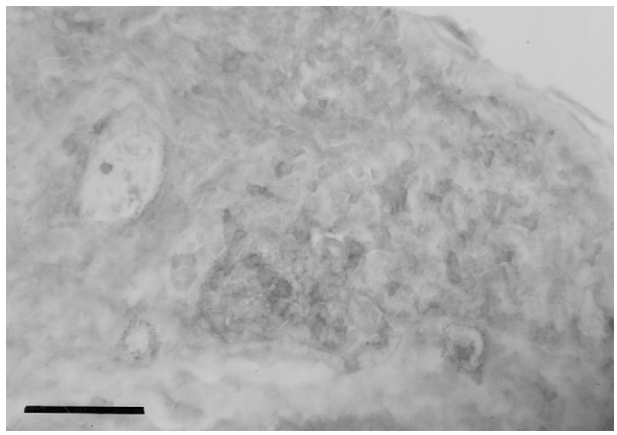

Figure 8. Indirect enzyme antibody method with anti-CD8 antibody. CD8-positive finding is observed in surrounding area. Bar = 100 μm.

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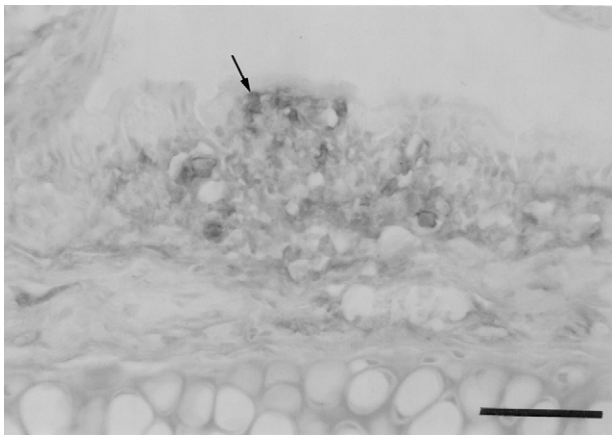

Figure 9. Indirect enzyme antibody method with anti-CD4 antibody. CD4-positive finding is observed at cell in intra-epithelial pocket (arrow). Bar = 100 μm.

dendritic cells appears at first, then a T-lymphatic cell area with high endothelial venule develops with growth on days 7 and 14.[15] Although the formation of NALT is completed in the 4-week-old mouse, the presence of definitive follicular and para-follicular areas have not been reported. In our present study, the normal mouse demonstrated a follicular area at its nictitating membrane, but its structure did not show a marked differentiation such as the follicular, para-follicular, or dome area observed in CALT in the guinea pig. Size and number of follicular areas increased in the mouse, but the differentiation between follicular and para-follicular areas was not marked. Furthermore, an enlarged follicular area showed shrinkage with the elapse of time. CALT in a mouse is similar to NALT in morphological study.

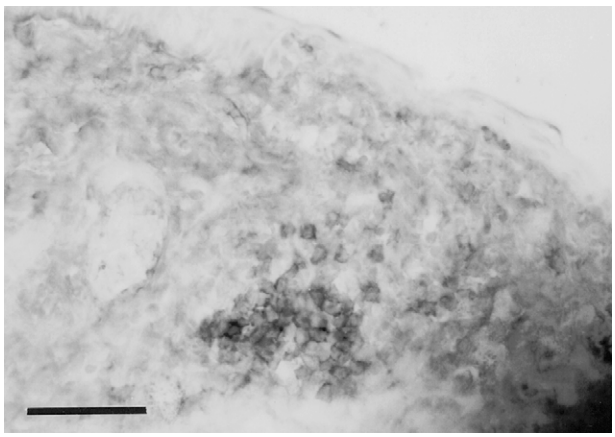

Figure 10. Indirect enzyme antibody method with anti-S-100 protein antibody. Meshwork pattern of S-100 protein-positive finding is observed in center of follicular cell. Bar = 100 μm.

Immunohistochemical study with S-100 protein antibody revealed meshwork-like positive staining. In the follicular area, follicular dendritic cells having antigen-presenting activity to B-lymphatic cells form a meshwork structure, and B-lymphatic cells are present in this region.[16] Takaura et al reported the presence of follicular dendritic cells by detecting S-100 protein cells in the germinal center of CALT in an experimental allergic model in the guinea pig.[17] Therefore, the presence of an S-100 protein cell in the present study is compatible with the demonstration of follicular dendritic cells in previous reports and revealed the presence of a follicular area in the mouse.

Detection of Lymphatic Cells at the Follicular Area

In the Pyell's plate in intestinal tissue, T-cells such as CD4- and CD8-positive T-cells are present dominantly in the para-follicular and dome areas. NALT and GALT in the mouse also have CD4- and CD8-positive cells in the T-cell area, but their ratio is reported to be 4:1.[18] In our present study, anti-CD4 antibody and anti-CD8 antibody were positive in the follicular area; thus we could conclude that the T-cell area was formed in the follicular area.

Detection of Intra-epithelial Pocket in Lympho-epithelium

The lympho-epithelial cell is characterized by the formation of a cytoplasmic facet called intra-epithelial pocket. Shoji et al reported histological findings of an intra-epithelial pocket in CALT in the guinea pig.[3] In the present study, the specimen on day 9 showed the histological findings of intra-epithelial pocket and CD4-positive cells in that tissue by immunohistochemistry. These findings indicate that antigen trapped by epithelial cells forms an intra-epithelial pocket and causes migration of lymphocytes. Therefore, the epithelial cell has the character of lympho-epithelium.

In this study, follicular tissue showed enlargement and increase in number due to antigen stimulation. It was an aggregation of lymphatic cells and had a germinal area showing an S-100 protein-positive meshwork structure. Furthermore, both the T-cell area and lympho-epithelial cells with intra-epithelial pockets could be demonstrated. Therefore, it could be concluded that follicular tissue at the nictitating membrane in the mouse is CALT.

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