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Immunohistochemical Study on Follicular Dendritic Cell of Conjunctiva-Associated Lymphoid Tissue

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Abstract: We performed an immunohistochemical study of follicular dendritic cells (FDC) in the follicular area of the conjunctiva-associated lymphoid tissue (CALT). Hartley guinea pigs were sensitized with a topical application of an emulsion of ovalbumin and Freund's complete adjuvant in the eye. They were divided into four groups. The control (group A) underwent no sensitization. The sensitized animals were studied at 1 week (group B1) or 2 weeks (group B2) after the sensitization. Additional sensitization at 1 week after the initial sensitization was also performed (group C). Histological methods included methylgreen pyronine staining, alpha-naphthylacetate esterase staining, and enzyme-antibody method against S-100 protein. The uptake of topically applied peroxidase-anti-peroxidase (PAP) in CALT was also examined histologically. In each group, positive reticular patterns by alpha-naphthyl acetate esterase staining and immunoperoxidase staining with anti-S-100 protein antiserum were found in the CALT follicular area. The positively stained cells were found to be dendrite cells by immunoelectronmicroscopy. An uptake of PAP was found in the CALT follicular area, suggesting the function of trapping and retaining antigen-antibody complex by FDC. It was concluded that dendritic cells in the CALT follicular area were identified to be FDC. **Jpn J Ophthalmol 1998;42:1–7**

Key Words: Antigen-presenting cell, conjunctiva-associated lymphoid tissue, follicular dendritic cell, lymphoid follicle, S-100 protein.

Introduction

In the B-cell region of the lymphoid organs, follicular dendritic cell (FDC) is present as an antigen-presenting cell.[1–3] In mucosal tissue, mucosa-associated lymphoid tissue (MALT) is identified as an extranodal lymphoid tissue, and Peyer's patch is one of the components. The presence of FDC is reported in its B-cell region.[4]

FDC is a nomenclature of reticular cell that was known as follicular reticular cell,[2,3] dendritic reticulum cell,[5–7] or antigen-presenting reticular cell,[2,3] which has been the generally accepted terminology since 1982 when Tew[8] identified the cell as one of the dendritic cells. The FDC is classified into B-cell–associated dendritic cells and is differentiated from interdigitating cells or Langerhans cells, which are T-cell–associated dendritic cells. According to the current histological studies, FDC demonstrates a dendritic shape,[1,6] and its cytoplasm is alpha-naphthyl acetate esterase (alpha-NAE) stain[6] and S-100 protein positive[9,10]; both complement[7] and immunoglobulin receptors[1] are expressed on its surface. The FDC also showed an expression of class II major histocompatibility complex as an antigen-presenting cell.[1,5,11,12] However, these histological characteristics are not specific for FDC. Methods to detect trapping and retaining functions of antigen-antibody complex are conventionally used to identify FDC.[5,13]

Conjunctiva-associated lymphoid tissue (CALT) is identified as a MALT compatible system in the conjunctiva.[14–17] That is to say, CALT is an extranodal lymphoid tissue, the surface of which is covered with the lymphoepithelium.[15] Histologically,

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the parafollicular area and dome area are observed around the follicular area in CALT.[16] Clinically, it is thought to be a tissue corresponding to conjunctival follicle. So far as we know, however, the presence of dendritic cell has not been reported in CALT. We investigated the FDC in the follicular area in CALT by histochemical and immunohistochemical methods.

Materials and Methods

Identification of Cellular Marker in FDC

Sensitization and sample process. Twenty-four eyes of 12 female Hartley strain guinea pigs (350–600 g) were used. Provoked antigen was an emulsion solution of an equivalent amount of ovalbumin, 5 mg/mL (Sigma Chemical Co., St. Louis, MO, USA) and Freund's complete adjuvant. The animals were divided into four groups. Each group included six eyes of three guinea pigs. The control group (group A) did not undergo sensitization. The basic sensitization was a 50-μL topical application of antigen solution once a day for 3 days. Group B was divided into two subgroups, depending on the examined period after the sensitization. That is, the basic sensitized group was examined at 1 week (group B1) and 2 weeks (group B2) after the sensitization. Group C underwent booster antigen challenge once at 2 weeks after the initial treatment and was examined at 1 week after the second treatment. At the examination, animals were sacrificed by intra-abdominal injection of sodium pentobarbital, and the eyelid and eyeball were sampled. The specimens were fixed immediately in periodate-lysin-paraformaldehyde (PLP) solution or Zamboni solution for 1 hour and were frozen rapidly with embedding using OCT compound (Tissue-Tak®: Miles Inc., USA). Then, about an 8-μm frozen section was processed using cryostat.

Staining of the cellular marker. The following staining was performed to detect FDC in CALT.

1. *Methylgreen pyronine staining:* the cryostat section was stained with methylgreen pyronine solution (Muto Chemical, Japan) for 10 minutes then differentiated and dehydrated in n-butanol. The specimen was rinsed in pure xylene and then examined by light microscopy.
2. *Alpha-NAE staining:* 10 mL of ethyleneglycol monomethylether solution with 93 mg of alpha-naphthylacetate was prepared as a 50-mM basic solution. Then, a reagent solution was prepared with 0.2 mL of basic solution, 9.6 mL of 1/15-M phosphate buffer solution (pH = 7.6), and 0.2 mL of hexazotized pararosanilin. The cryostat section was incubated in the reagent solution for 30 minutes at room temperature and examined by light microscopy.
3. *Immunohistochemical methods (avidin biotin complex [ABC] methods):* the cryostat section was stained by indirect enzyme-antibody method using a Vecstain® ABC kit (Vector Labs, Burlingame, CA, USA) with the following first polyclonal antibody: rabbit anti-S-100 protein antibody (Serotec Ltd., Oxford, England). The cryostat section was incubated in normal serum to block nonspecific reaction for 30 minutes, and then it was incubated in the first antibody for 60 minutes. Indirect enzyme-antibody reaction (ABC method) was performed based on the directions accompanying the kit. The specimen was placed in a solution of 3,3′-diaminobe ntidine tetra-hydrochloride (DAB) (20 mg/100 mL) dissolved in 0.05-M tris-HCl buffer (pH = 7.6) containing 0.005% H_2O_2 for 5 minutes and examined by light microscopy. Counterstaining was performed with methylgreen. The specimen, which was processed by enzyme-antibody method for S-100 protein, was postfixed by 1% osmium tetroxide for 60 minutes. After postfixation with OsO_4, it was then embedded in epoxy resin (Epok 812®, Ouken Shouji, Japan) after dehydration using an increasing concentration gradient of ethanol. An ultra-thin section was made and examined either without staining or with lead citrate staining using electron microscopy.

Trapping and Retaining Functions of Antigen-Antibody Complex

Six eyes of three guinea pigs underwent the same treatment as in group C. Furthermore, they underwent topical application of rabbit peroxidase-anti-peroxidase (PAP) antibody soluble complex (Dako, Japan) as an antigen-antibody complex. The animals' eyelids and eyeballs were taken at 24 hours after the treatment. The tissues were fixed immediately using PAP solution for 1 hour and processed by the above-mentioned methods. The frozen section was treated with biotinated anti-rabbit IgG antibody for 60 minutes and was then treated with avidin-biotinylated peroxidase complex for 30 minutes and incubated in DAB containing 0.005% H_2O_2 for 5 minutes. The localization of PAP in the specimen was examined by light microscopy.

Results

Methylgreen Pyronine Staining

The follicular and parafollicular areas were identified in CALT in groups A, B, and C. Dome area was

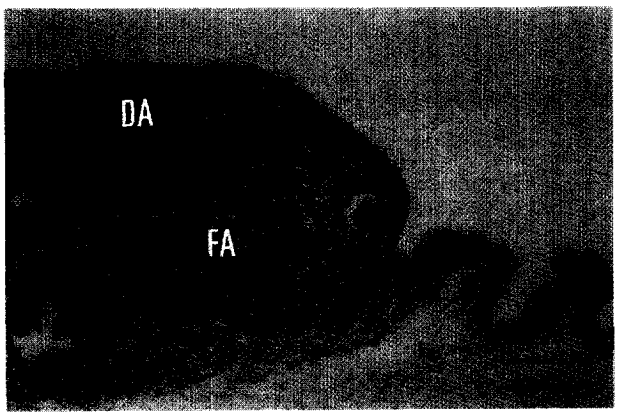

Figure 1. Light micrograph of conjunctiva-associated lymphoid tissue (CALT) in group B1. Follicular area (FA), parafollicular area, and dome area (DA) are observed in CALT (methylgreen pyronin staining; bar = 50 μm).

present between the parafollicular area and the epithelium. The follicular area was composed of medium-size cells with pyronine positive staining in its cytoplasm and pyronine positive large cells (Figure 1).

Alpha-NAE Staining

In groups A, B, and C, macrophage was detected as large and round brown cells stained positively by alpha-NAE in the follicular area, and the intra-lymphocyte space was also stained brown in reticular form (Figures 2 and 6).

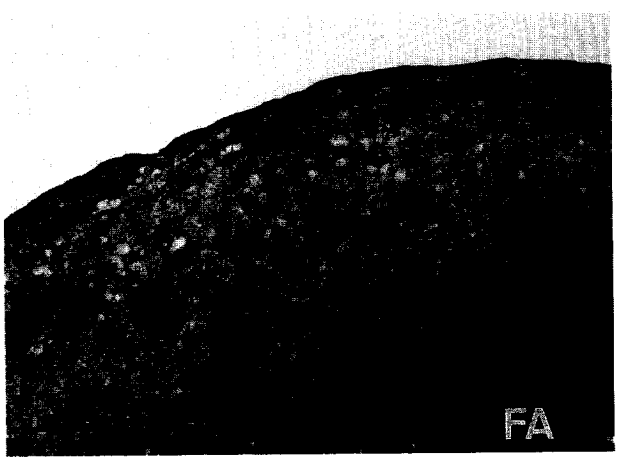

Figure 2. Light micrograph of conjunctiva-associated lymphoid tissue in group B2. There are enzyme-positive reticular formation and round cells in the follicular area (FA) (alpha-naphthyl acetate esterase staining; bar = 50 μm).

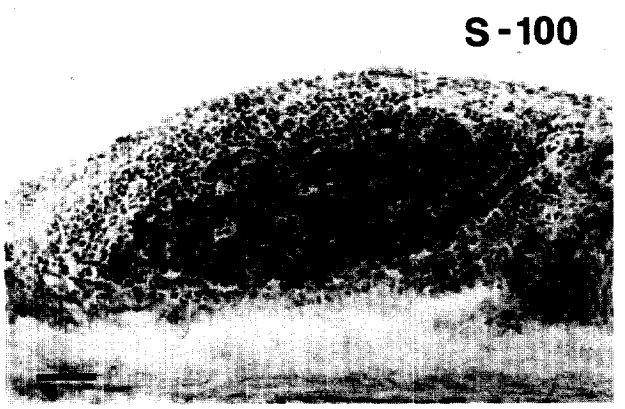

Figure 3. Light micrograph of conjunctiva-associated lymphoid tissue of guinea pigs weighing 350 g in group A. The reticular pattern formed by S-100 protein positive cells is clearly shown in the follicular area (immunoperoxidase staining with the anti-S-100 protein antiserum; bar = 25 μm).

Immunohistochemical Methods

In terms of immunoperoxidase staining with anti-S-100 protein antiserum, a brown reticular form similar to S-100 protein positive findings was localized in the follicular area in group A (Figures 3 and 4). In group B, a primary follicular area without a germinal center and a secondary follicular area were present concomitantly. The primary follicular area in group B showed uniform brown staining in reticular form, but its density was low (Figure 5). A reticular staining pattern with alpha-NAE was also identified in the same pat-

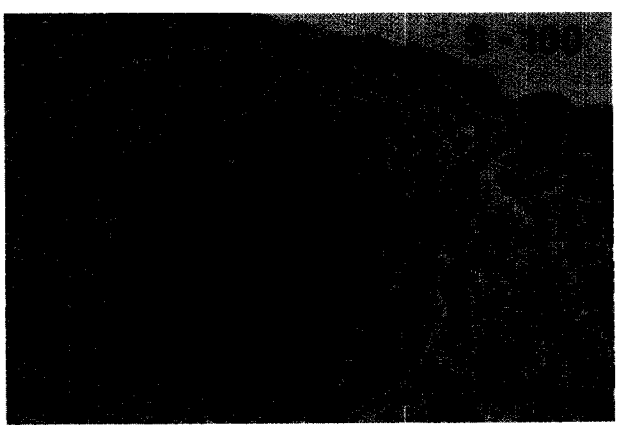

Figure 4. Light micrograph of conjunctiva-associated lymphoid tissue of guinea pigs weighing 500 g in group A. A differentiation of follicular area (FA), parafollicular area, and dome area is demonstrated. The S-100 protein positive reticular form is observed in FA (immunoperoxidase staining with anti-S-100 protein antiserum; bar = 25 μm).

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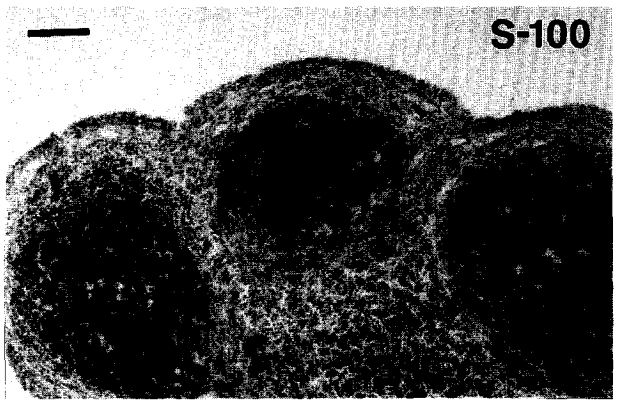

Figure 5. Light micrograph of conjunctiva-associated lymphoid tissue (CALT) in group B1. The primary follicles in CALT indicate the dendritic network revealed with anti-S-100 protein antibody (immunoperoxidase staining with anti-S-100 protein antiserum; bar = 50 μm).

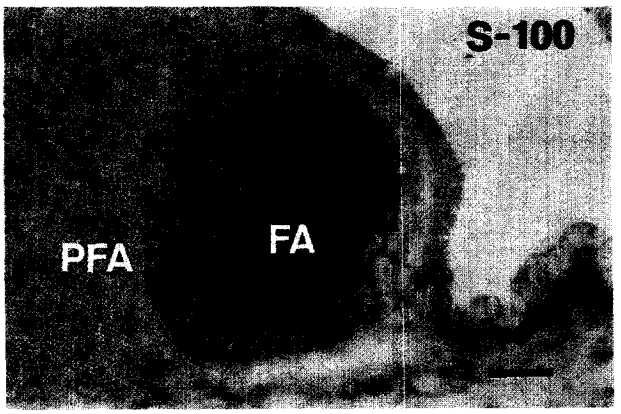

Figure 7. Light micrograph of the follicular area (FA) of conjunctiva-associated lymphoid tissue in group C. Secondary follicle is observed in the FA. The central network pattern of S-100 protein positive cells is visible in the secondary follicle. PFA = parafollicular area (immunoperoxidase staining with anti-S-100 protein antiserum; bar = 50 μm).

tern with immunoperoxidase staining for S-100 protein by a study with serial specimen sections (Figure 6). The increase of the secondary follicular area with a germinal center was demonstrated in group C. The center of the follicular area was diffusely stained by immunoperoxidase staining for S-100 protein (Figure 7). The staining pattern demonstrated a complex dendritic pattern in the intra-lymphocyte spaces with a higher magnification (Figure 8).

An electron microscopic examination with enzyme-antibody method using anti-S-100 protein antibody detected cells with a diffuse high electron dense

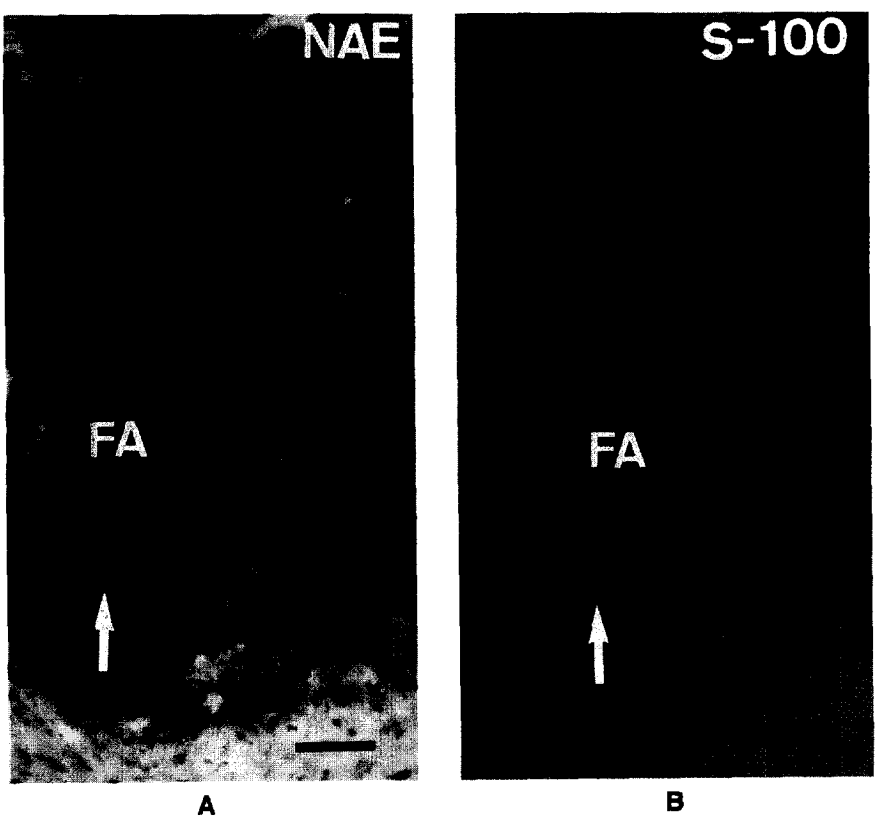

Figure 6. Light micrograph of the follicular area (FA) of conjunctiva-associated lymphoid tissue in group B1. Reticular staining pattern (arrow) in the FA stained with alpha-naphthyl acetate esterase (alpha-NAE) is basically similar to that seen with S-100 protein immunoperoxidase staining (alpha-NAE staining [**A**] and immunoperoxidase staining with anti-S-100 protein antiserum [**B**]; bar = 50 μm).

Figure 8. High magnification of the light micrograph of the follicular area (FA) in group C. In the central portion of the secondary follicle, positive cell immunostained by S-100 protein shows a well-delineated network pattern composed of spreaded processes in the intra-lymphocyte spaces in the FA (Immunoperoxidase staining with anti-S-100 protein antiserum; bar = 12.5 μm).

cytoplasm. Their nucleus possessed well-defined nucleoli and showed a euchromatin pattern with a thin rim of heterochromatin along the inner nuclear membrane (Figure 9). The cytoplasmic processes that developed a dendritic form were extended among the surrounding lymphocytes (Figure 10).

Trapping and Retaining of Antigen-Antibody

Peroxidase-anti-peroxidase staining revealed a brown granular or a lineal positive staining pattern in the follicular area, but dendritic cells with a uniform positive staining were not detected (Figure 11).

Discussion

Mucosa-associated lymphoid tissue plays a role in the local immune system, which recognizes exogenous antigen and induces B-cell lymphocyte producing antigen-specific IgA antibody. To reveal this function, however, FDC as an antigen-presenting cell to B-lymphocytes has to be detected in the follicular area where the B-cell region is located. So far, Langerhans cells, which are known as T-cell–associated dendritic cells, were reported as being antigen presenting in the ocular tissue,[18] but the presence of antigen-presenting cells in CALT was not detected. Therefore, we set out to identify FDC in CALT by immunohistochemical methods.

Plasma cells are representative cells that stain pyronine positive by methylgreen pyronine staining. Centroblasts and FDC also stain pyronine positive. It is possible that the large cells observed in the follicular area of CALT this time that stained positive for methylgreen pyronine are FDC and that the medium-sized cells are centroblasts. Histochemical and immunohistochemical studies revealed the alpha-

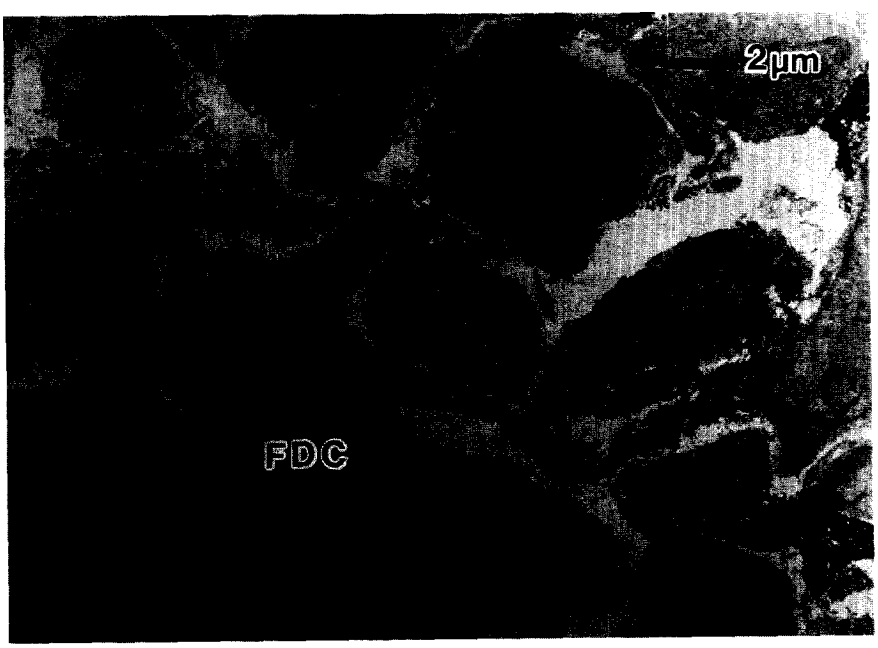

Figure 9. Electron micrograph of a follicular dendritic cell (FDC).[1] The follicular dendritic cell shows an euchromatic nucleus and an electron dense cytoplasm (immunoperoxidase staining with anti-S-100 protein antiserum; bar = 2 μm).

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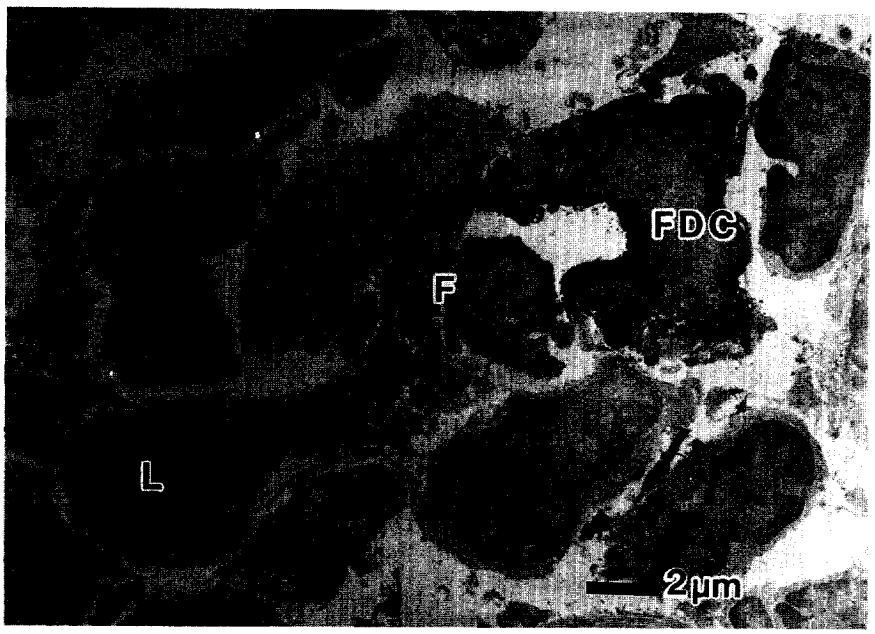

Figure 10. Electron micrograph of a follicular dendritic cell (FDC).[2] Cytoplasmic processes of the FDC include closely related collagen fibers (F) spread among surrounding lymphocytes (L) (immunoperoxidase staining with anti-S-100 protein antiserum; bar = 2 μm).

NAE and S-100 protein positive reticular findings in the follicular area. Heusermann et al[6] reported the presence of alpha-NAE positive dendritic reticulum cells in the follicles of rabbit spleen. Carbone et al[9] investigated human lymphoid organs such as lymph node, pharynges, tonsilla, appendix, and thymus by immunohistochemical methods using anti-S-100 protein antibody and human FDC specific DCR-1 antibody. They revealed a reticular staining pattern in the follicular area by the two antibodies. They concluded that FDC is an S-100 protein positive cell.

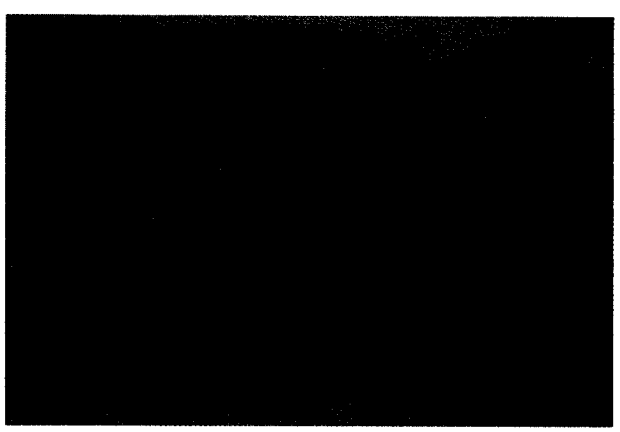

Figure 11. Trapping and retaining of antigen-antibody complex in conjunctiva-associated lymphoid tissue. Note the brown, positive granular and linear staining patterns indicating retained peroxidase-anti-peroxidase (PAP). FA = follicular area (immunoperoxidase staining; bar = 50 μm).

Follicular dendritic cells in the rat lymph node and spleen[10] or Peyer's patch[17] are reported to be S-100 protein positive. The reticular staining pattern by alpha-NAE and S-100 protein in this study agreed with these previous results. Furthermore, nuclear and cytoplasmic findings of S-100 protein positive cells in the follicular area in CALT by electron microscopy agreed with those of FDC reported by Imai et al[5] and Cocchia et al.[10]

The method to demonstrate a trapping of PAP, which is an antigen-antibody complex, was used to detect a function of FDC, which plays a role in trapping and retaining antigen-antibody complexes.[5,13] The antigen-antibody complex was demonstrated on the cell surface, especially at the process of its dendrite, and this finding showed nonspecies specificity of used antigen-antibody complex in this method. The result of PAP-positive findings in the follicular area in this study could reveal that FDC and CALT trapped antigen-antibody complex. Peroxidase-anti-peroxidase positive staining also indicated granular or linear findings corresponding to the trapping of antigen-antibody complex on the dendritic process of the cell surface. This made a difference in the staining pattern from S-100 protein, which stained cytoplasm. Therefore, we believe that cells in the follicular area in CALT may be identified as FDC.

In this study, guinea pigs weighing more than 500 g in the control group (group A) demonstrated a differentiation of follicular area, parafollicular area, and dome area in CALT. Follicular area in CALT

develops from primary to secondary follicles with a germinal center by an exposure to antigen. In accordance with this development of follicles, S-100 protein staining changed from a weak reticular pattern in the primary follicle to a dense reticular pattern in the secondary follicle. In terms of the difference in morphology of FDC between primary and secondary follicles, Imai et al[5] classified FDC into two types based on the labyrinth-like structure of its process. They concluded that FDC in the secondary follicle showed more developed labyrinth-like structure than that in the primary one. In the present study, follicles in all the groups demonstrated S-100 protein positive staining, meaning that FDC localized in the follicles without regard for the primary or secondary follicles. Denser S-100 protein staining in the secondary follicle than in the primary one indicates the increase of FDC and development of its labyrinth-like structure in the secondary follicle.

Finally, we concluded that we could identify FDC, which is a B-cell–associated dendritic cell in the follicular area in CALT. It could be said that conjunctiva also has B-cell–associated dendritic cells apart from Langerhans cells, which are T-cell–associated cells. The morphological change of FDC by antigen treatment indicates that FDC plays a role in local immune response in conjunctiva.

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