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Ultrastructural and immunoelectron microscopic studies on infiltrating mononuclear cells in lymphocytic submandibulitis in NOD mice

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Summary. The ultrastructural relations of the infiltrating mononuclear cells to the parenchymal tissues were studied in the submandibular gland of the female non-obese diabetic (NOD) mouse. In addition, the phenotype of mononuclear cells infiltrating the submandibular gland has been determined by light and electron microscopy by using monoclonal antibodies against T-cell subsets (Thy1.2, Lyt1, Lyt2).

Ultrastructurally, lymphoid cells were frequently observed around and in the acini and ducts. Some of the lymphoid cells observed in the acini and ducts were irregular in shape and sometimes sent spike-like projections into acinar and ductal cells. Immunohistochemical study demonstrated that Thy1.2$^+$ cells were predominant among the infiltrating cells, and the majority of these infiltrating T-cells were composed of Lyt1$^+$ cells with a small proportion of Lyt2$^+$ cells. By immunoelectron microscopy, lymphocytes carrying Thy1.2, Lyt1 or Lyt2 antigen were identified, as is demonstrated by an electron-dense reaction product on the entire cell surface, and these immunopositive cells were frequently observed around and in the acini and ducts. Some of the Thy1.2$^+$, Lyt1$^+$ or Lyt2$^+$ cells observed in the acini and ducts demonstrated a close contact with acinar and ductal cells and both Lyt1$^+$ and Lyt2$^+$ cells sent spike-like projections into them.

Occasionally, a partial degeneration of acinar cell adjacent to the invading lymphocytes was observed. These observations suggest that T-lymphocytes are involved in the direct destruction of acinar and ductal cells in the NOD mouse submandibular glands.

Key words: NOD mouse, Submandibular gland, Ultrastructure, Immunoelectron microscopy, T-lymphocyte

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Introduction

The non-obese diabetic (NOD) mouse, has been recently established as an useful animal model for type I diabetes mellitus (Makino et al., 1980). This diabetic condition is caused by the destruction of pancreatic ß-cells by lymphocyte infiltration. In addition to this lymphoproliferative lesion in the pancreatic islet, the NOD mouse also develops the same lymphoproliferative lesion spontaneously and frequently in the submandibular gland (Asamoto et al., 1984; Miyagawa et al., 1986). Although the pathogenesis of «lymphocytic submandibulitis» observed in NOD mice has been suggested to be an autoimmune process (Asamoto et al., 1984; Miyagawa et al., 1986), little is known of its precise mechanism.

In the pancreas of the NOD mouse, it is suggested that T-lymphocytes come in close contact with pancreatic ß-cells and destroy them (Shimizu et al., 1987). Similarly, in the submandibular gland, it has been suggested that T-lymphocytes may play an important role in the direct acinar and ductal cell destruction (Miyagawa et al., 1986). However, there seem to be no publications which show, by immunoelectron microscopy, that the invading lymphocytes in the acini and ducts are T-lymphocytes. Further, it has not been clarified which phenotype of T-lymphocytes invades the acini and ducts and what relationship, exists between the invading lymphocytes and acinar or ductal cells.

To explore this question, we investigated the distribution of Thy1.2$^+$, Lyt1$^+$ and Lyt2$^+$ lymphocytes in the submandibular gland lesion, and studied the ultrastructural features of the interaction between infiltrating lymphocytes and the submandibular gland parenchyma in NOD mice.

Materials and methods

Twenty female NOD mice of 19-37 weeks of age, 26-32g weight, were studied. The practical procedures of

this experiment were as follows: all mice were anesthetized with pentobarbital sodium given intraperitoneally. Vascular perfusion through the left ventricle was begun with physiological saline solution for 1 min, and followed by fixative solution of 0.1M cacodylate-buffered 2% paraformaldehyde, pH 7.2-7.4. After perfusion for 5 min, the submandibular glands were immediately removed and each gland was divided into two halves and one half was used for light and electron microscopic studies, while the other half was used for immunohistochemical and immunoelectron microscopic studies.

Preparation of specimens for light and electron microscopy

For light microscopy, the excised submandibular glands were immersed in 10% formalin for several hours, and were dehydrated through a graded series of ethanol and embedded in paraffin. They were sliced at 3-5 μm and stained with hematoxyline-eosin.

For ultrastructural study, the excised submandibular glands were fixed in 0.1M cacodylate-buffered 2.5% glutaraldehyde and 2% paraformaldehyde at 4° C for about 1 hour. After being rinsed overnight in the 0.1M cacodylate-buffer solution containing 3% sucrose at 4° C, pH 7.2-7.4, the specimes were postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer at 4°C, pH7.4, dehydrated through a graded series of ethanol and embedded in Spurr's resin. Ultrathin sections were cut, doubly stained with uranyl acetate and lead citrate, and examined under a Hitachi HS-9 or a JEM 1200-EX electron microscope.

Immunoperoxidase techniques

Avidin-biotin-peroxidase complex (ABC) method was performed following the procedure described by Hsu et al. (1981). Briefly, the excised submandibular glands were fixed with periodate-lysine-paraformaldehyde (PLP) solution. After washing with PBS, they were soaked in 10% DMSO in PBS (used as a cryoprotectant) for 1 hr at 4° C, embedded in OCT compound, frozen by rapid immersion in acetone chilled by dry ice, and stored at -80° C in a freezer.

The frozen block was cut into sections 10-30 μm in thickness in a cryostat at -20° C. For light microscopic examination, the sections were placed on glass slides, and for electron microscopic examination, free floating sections in PBS were used. To inactivate endogenous peroxidases, sections were treated in 0.3% H_2O_2 in methanol for 30 min. After washing with PBS, the sections were treated with biotin-conjugated anti-Thy1.2, Lyt1 or Lyt2 monoclonal antibody (5 μg/ml; Becton-Dickinson, Inc., CA.) at 4° C, overnight and washed with PBS. Next, the sections were treated with avidin-biotinylated horseradish peroxidase complex (Dako corporation, USA) at room temperature for about 40 min and washed with PBS.

For light microscopic examination, the sections were exposed to DAB-H_2O_2 for about 10 min and mounted in glycerin jelly.

For electron microscopic examination, the sections, treated with avidin-biotinylated horseradish peroxidase complex, were washed with PBS and further fixed with 0.1M cacodylate-buffered 1% glutaraldehyde, pH 7.4 for 1 hour. After washing with PBS, they were exposed to DAB-H_2O_2 for about 10 min. The sections were then postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hour, dehydrated through a graded series of ethanol, treated with propylene oxide and embedded in Spurr's resin. The ultrathin sections were stained with uranyl acetate only or doubly stained with uranyl acetate and lead citrate and examined under a Hitachi HS-9 or a JEM 1200-EX electron microscope.

Results

Light and electron microscopic findings

All mice, except one (19 weeks old), had one to several foci of lymphoproliferative lesions at the periductal and perivascular region in the submandibular gland (Fig. 1a). Infiltrating cells mainly consisted of small or medium-sized lymphocytes and a small amount of plasma cells (Figs. 1b, c).

Some of the acini or ducts which lay close to the lesion showed retrogressive changes as they involuted and reduced in size (Fig. 1b). In the fibrous connective tissue stroma near the acini and ducts, lymphoid cells were frequently observed. Furthermore, lymphoid cells were frequently observed in the interepithelial space of acinar or ductal cells. These interepithelial lymphoid cells were usually seen to be located in basal regions of the acini and ducts, with irregular cell outlines, 4 to 12 μm in apico-basal diameter. Intracellular organelles were poorly developed in these interepithelial lymphoid cells and only a small number of mitochondria, one or two small elements of rough endoplasmic reticulum and a small Golgi apparatus could be recognized in the cytoplasm. Some of the interepithelial lymphoid cells sent spike-like projections into acinar and ductal cells (Fig. 1d).

Immunohistochemical and immunoelectron microscopic findings

At the light microscopic level, the Thy1.2$^+$ (pan T) cells were predominant among infiltrating cells (Fig. 2a) and showed a tendency of forming groups at the center of the infiltrate. The majority of infiltrating cells were Lyt1$^+$ cells (Fig. 2b) with a small proportion of Lyt2$^+$ cells (Fig. 2c).

At the electron microscopic level, Thy1.2$^+$, Lyt1$^+$ and Lyt2$^+$ cells were identified, as was demonstrated by an electron-dense reaction product on the entire cell surface, and these immunopositive T-cells were frequently observed in the fibrous connective tissue stroma near the acini (Figs. 3a,c) and ducts (Fig. 3b). Furthermore, we could often observe Thy1.2$^+$, Lyt2$^+$ and Lyt1$^+$ lymphocytes invading the involuted acini and ducts (Figs. 3c-e, 4,5). The cytoplasm of these T-lymphocytes was sparse, containing free ribosomes, a few mitochondria

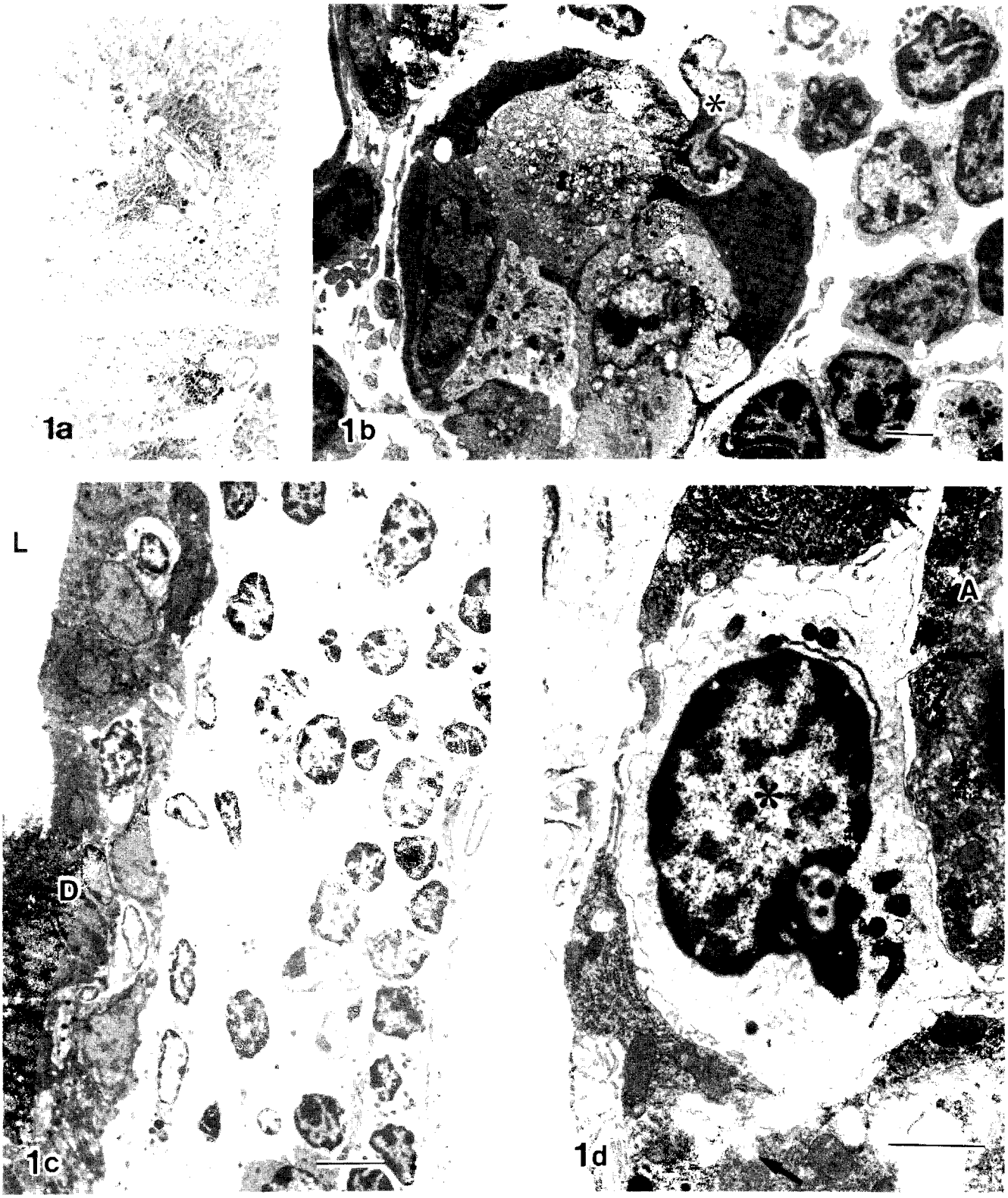

Fig. 1. Lesion of submandibular gland in the female NOD mouse. **a.** Periductal and perivascular mononuclear cell infiltration is seen. = 50. **b.** Involuted acinus- or duct-like structure is surrounded by lymphocytes. A lymphoid cell (*) is just invading this acinus-or duct-like structure. × 4,000. Bar = 2 μm. **c.** Infiltrating cells mainly consisted of small- or medium-sized lymphocytes and a small amount of plasma cells. Lymphoid cells (*) are seen in the duct. × 2,400. Bar = 5 μm. L: lumen, D: duct. **d.** A lymphoid cell (*) is seen in the acinus (A). This lymphoid cell has irregular surface and sends spike-like projections (arrows) into the acinar cells (A). × 17,000. Bar = 1 μm.

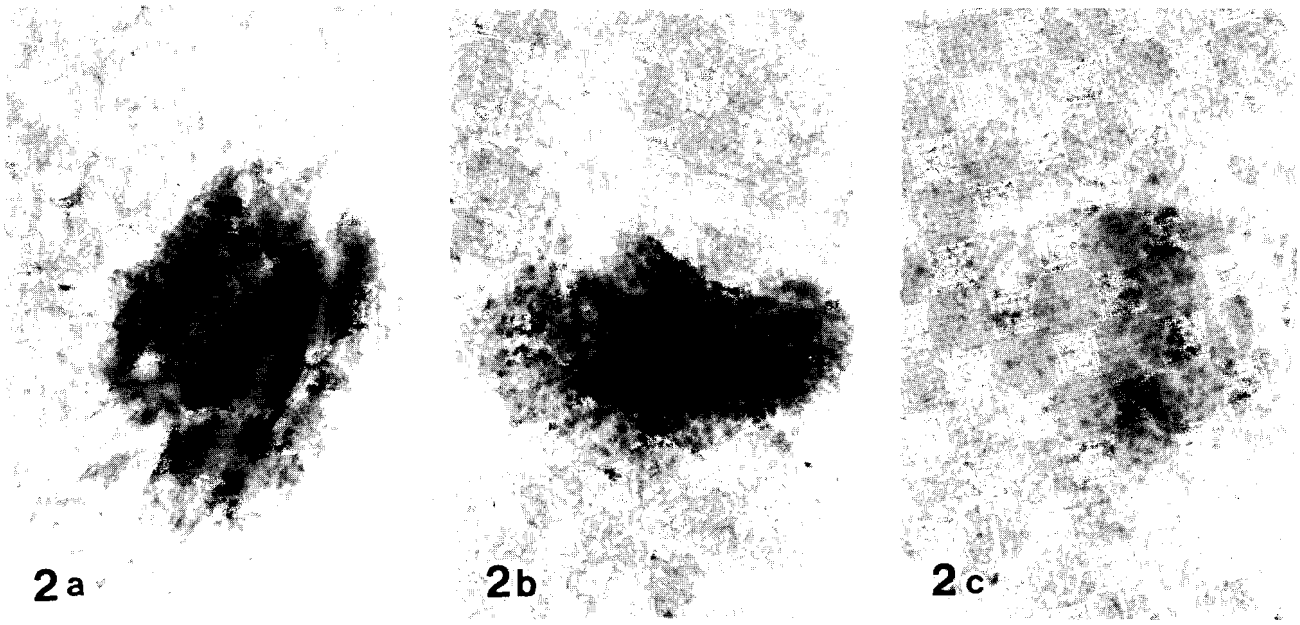

Fig. 2. ABC immunoperoxidase stain of the infiltrating mononuclear cells with Thy1.2 (2a), Lyt1 (2b) and Lyt2 (2c) monoclonal antibodies. The majority of lymphocytes are recognized as Thy1.2$^+$ cells (2a) which were composed chiefly of Lyt1$^+$ 2b) and a lesser number of Lyt2$^+$ cells (2c). (2a-c, ABC jmmunoperoxidase staining, × 100).

and endoplasmic reticulum. Differences in shape between these Thy1.2$^+$, Lyt1$^+$ or Lyt2$^+$ T-cells could not be made out. These invading T-lymphocytes were usually located in the interepithelial space of acinar or ductal cells and were frequently in contact with acinar and ductal cell membrane via cytoplasmic projections showing broad or pointed contacts (Figs. 3d, e, 4b, c, 5b, c). Occasionally, a partial degeneration of acinar cells adjacent to the invading lymphocytes was observed (Fig. 5a).

Discussion

In the present study, we have confirmed the previous findings (Asamoto et al., 1984; Miyagawa et al., 1986) that the female NOD mouse frequently and spontaneously develops «lymphocytic submandibulitis». It is of clinical interest that the lesion of the submandibular gland of NOD mice closely resembles that of the salivary gland in patients with Sjögren's syndrome (SS) (Miyagawa et al., 1986). In SS patients' salivary glands, the inflammatory round cells in the periductal infiltrates are predominantly T-cells (Fox et al., 1982; Kilpi et al., 1983), and a similar immunohistochemical staining pattern, with T-lymphocytes tending to occupy the center of the infiltrate, has also been observed in the salivary glands of NOD mice (Miyagawa et al., 1986). We have also shown, using ABC immunohistochemical method, that most of the infiltrates are T-lymphocytes.

By light microscopy, the infiltrating lymphocytes were composed primarily of Thy1.2$^+$, Lyt1$^+$ cells with occasional Lyt2$^+$ T-cells. The composition of lymphocytes subsets in the lesion is comparable to that reported in the pancreatic islet in NOD mice (Koike et al., 1987; Shimizu et al., 1987). Some investigators have suggested that the disease process in the NOD mouse is helper T-lymphocyte (L3T4$^+$ cell) dependent, and T-lymphocytic cell-mediated cell cytotoxicity might not play an essential role in the onset of insulitis of NOD mice (Koike et al., 1987; Wang et al., 1987). However, other investigators have suggested, using electron microscopy that T-lymphocytes come into close contact with pancreatic ß-cells and send projections into them, and cell-mediated cytotoxicity may play an essential role in the development of autoimmunity in NOD mice (Shimizu et al., 1987; Maruyama et al., 1988).

In the present study, we have shown by immunoelectron microscopy that Thy1.2$^+$, Lyt1$^+$ and Lyt2$^+$ lymphocytes were located in the interepithelial space of acinar or ductal cells. In regard to the interepithelial lymphocytes, attention must be drawn to the fact that they may appear not only in pathological condition but also in normal condition. Takeda and Fujimura (1985) described the occurrence of interepithelial lymphocytes in the normal rat submandibular gland. Although the origin, significance and function of these interepithelial lymphocytes occurring in normal condition are still known only in fragments, it is thought that one of the important roles of interepithelial lymphocytes is to form a first immunological barrier (Takeda and Fujimura, 1985). It is therefore probable that not all of the interepithelial lymphocytes present in the submandibular gland of NOD mice are involved in the acinar or ductal cell destruction. However, in the present study, we have observed that lymphocytes invaded the acini and ducts, and Lyt2$^+$ cells with known potential cytotoxic function were located in the acini and ducts and some of

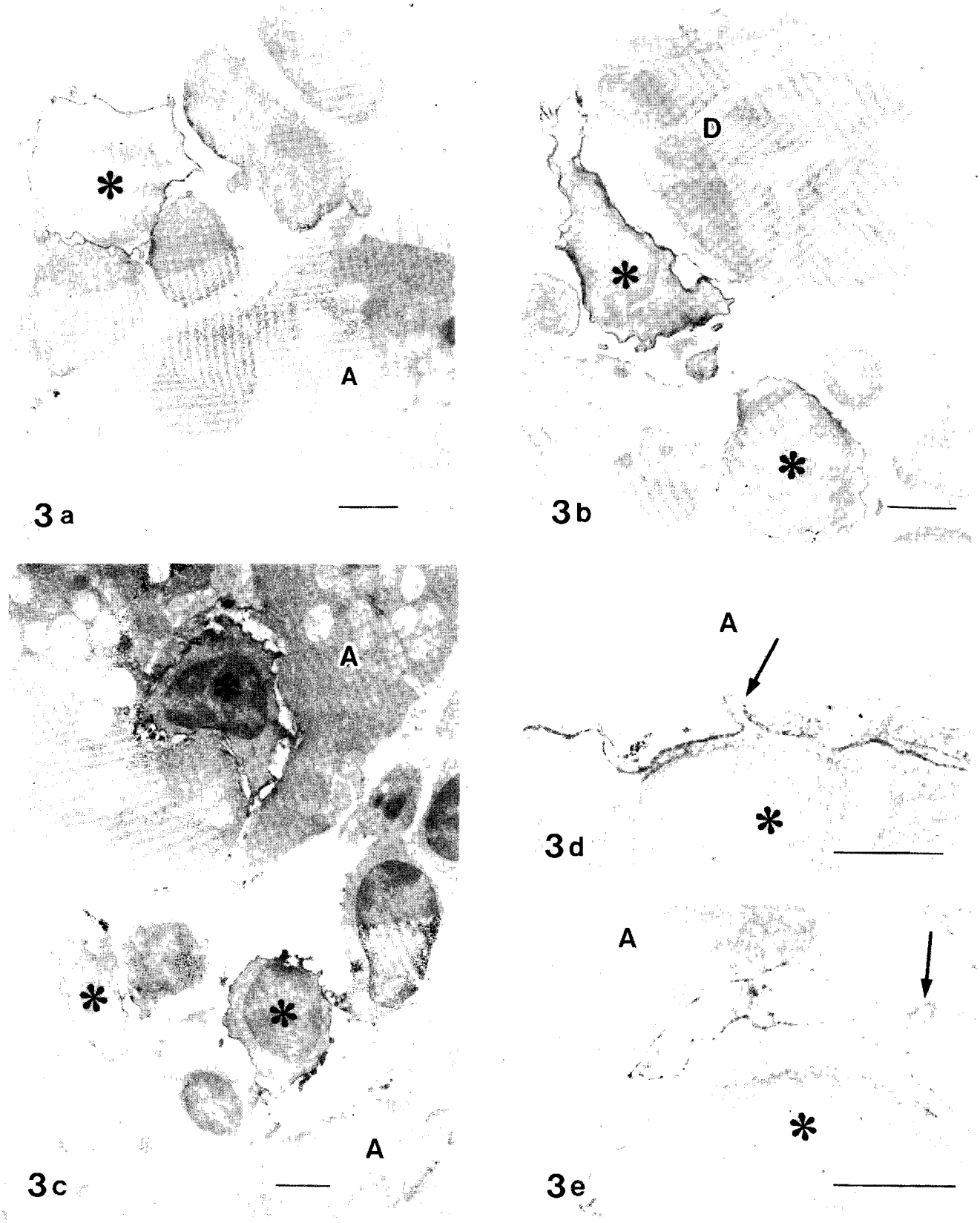

Fig. 3. Immunoelectron micrograph with Thy1.2 monoclonal antibody. **a.** A Thy1.2$^-$ cell (*) is seen around the acinus (A). × 5,300, Bar = 2 μm. **b.** Thy1.2$^+$ cells(*) are seen around the duct (D). × 6,000, Bar = 2 μm. **c.** Thy1.2$^+$ cells (*) are seen around and in the acinus (A). × 5,000, Bar = 2μm. **d, e.** A Thy1.2$^+$ cell (*) sends spike-like projection (arrow) into acinar cell (A). **d;** × 20,000, Bar = 1 μm. **e;** × 22,000, Bar = 1 μm.

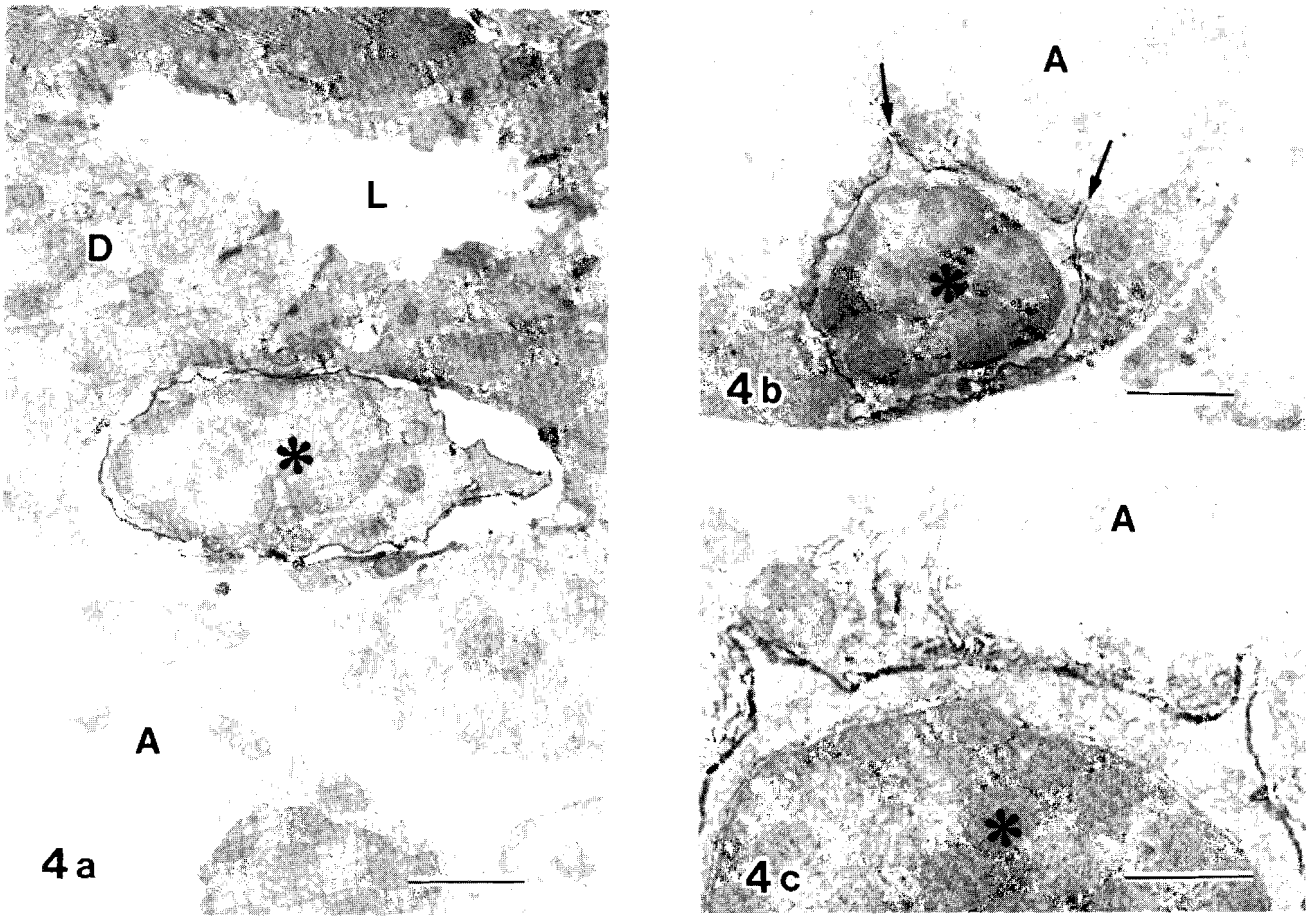

Fig. 4. Immunoelectron micrograph with Lyt1 monoclonal antibody. **a.** A Lyt1$^-$ cell (*) is seen in the duct (D). × 7,500, Bar = 2 μm. **b.** Lyt1$^+$ cell (*) is seen in the acinus (A). This Lyt1$^+$ cell sends spike-like projections (arrows) into acinar cell (A). × 7,000, Bar = 2 μm. **c.** Higher magnification of Fig. 4b. × 17,5000. Bar = 1 μm.

them sent projections into acinar and ductal cells. Furthermore we have observed a partial degeneration of acinar cells adjacent to the interepithelial lymphocytes. These results suggest that at least some of the interepielial lymphocytes present in the submandibular gland in NOD mice may participate in the destruction of acinar or ductal cells.

It has been suggested that T-cells with different functions have distinct surface markers of the Ly series (Shiku et al., 1976). The surface phenotype of the T-cell subset that destroys the allogeneic target cell is Lyt1$^-$, 2$^+$ (carrying only Lyt2 antigen) (Cantor and Boyse, 1975). Shimizu et al. (1987) indicated by an immunoelectron microscopic method that cells expressing Lyt2 marker alone are involved in the ß-cell destruction in the pancreatic islet in NOD mice. However, we have observed that not only cells expressing Lyt2 marker but also cells expressing Lyt1 marker invaded the acini and ducts, came into close contact with acinar and ductal cells and that some of them sent spike-like projections into them. It has been suggested that the phenotype of T-cells capable of destroying hapten- or virus-modified syngeneic cells or tumor cells are Lyt1$^+$,2$^+$ (they carry both Lyt1 and Lyt2 antigens) (Shiku et al., 1976; Stutman et al., 1977). It is therefore probable that some of the cells expressing the Lyt1 marker (Lyt1$^+$, 2$^+$) are involved in the destruction of the submandibular gland parenchyma. It is not possible to identify by an ordinary immunoelectron microscopic method whether the cells expressing Lyt1 marker are Lyt1$^+$, 2$^-$ or Lyt1$^+$, 2$^+$. However, the cells expressing the Lyt1 marker and also invading the acini and ducts and sending spike-like projections into acinar and ductal cells might be Lyt1$^+$, 2$^+$ cells, although the question remains whether or not the spike-like projections are at all related to cytotoxic effector cells.

The interactions between lymphocytes and acinar cells observed in the present study were similar to those reported previously between lymphocytes and pancreatic ß-cells of NOD mice *in vivo* (Shimizu et al., 1987; Maruyama et al., 1988) and *in vitro* (Maruyama et al., 1988). A similar interaction have also been observed between effector lymphocytes and target cells *in vitro* (Able et al., 1970; Leopardi et al., 1984). The ultrastructural features of cell-mediated cytotoxicity (CMC) have been investigated in several model systems in which effector T cells, killer (K) cells, and natural killer (NK) cells interact with suitable target cells that

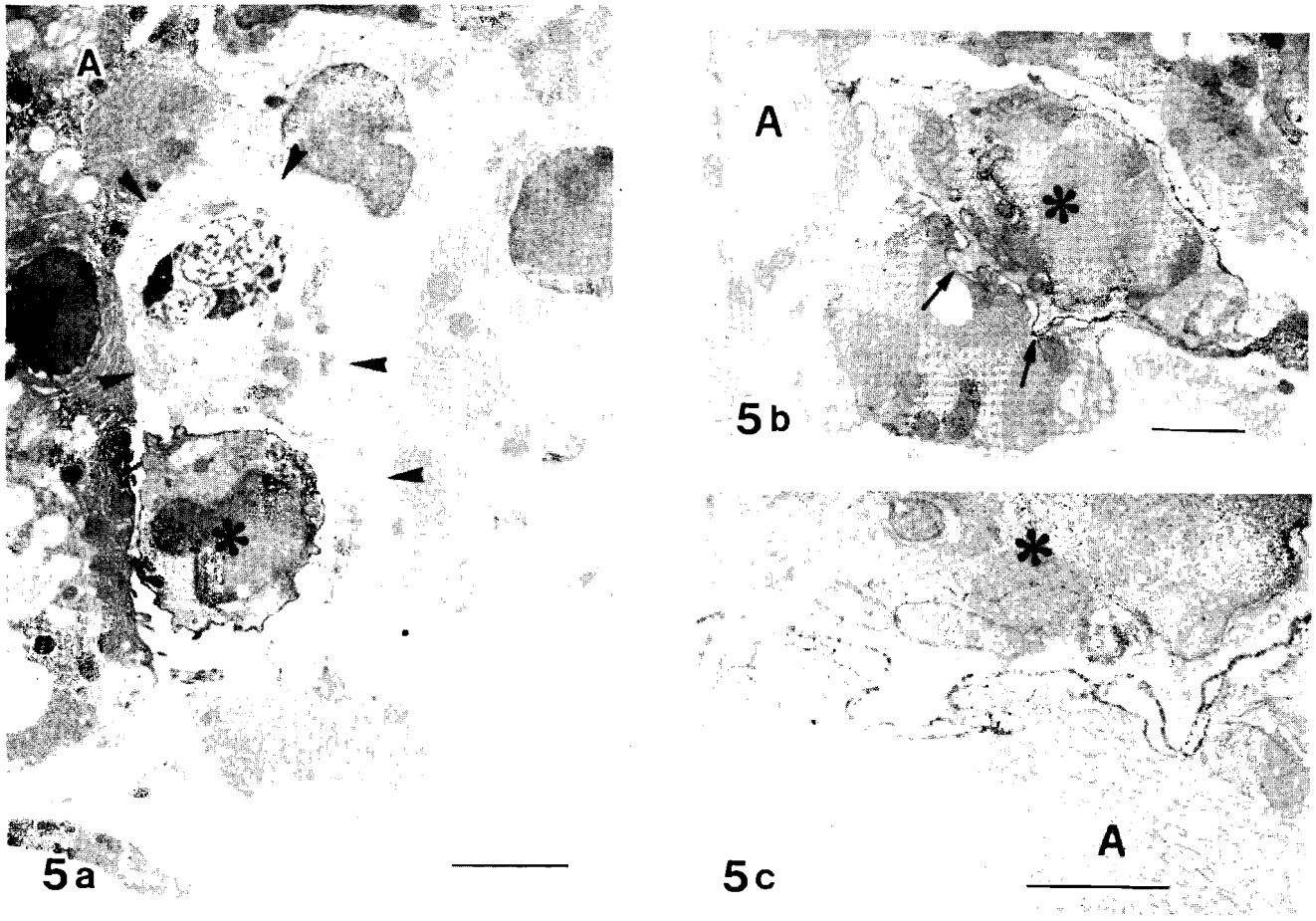

Fig. 5. Immunoelectron micrograph with Lyt2 monoclonal antibody. **a.** Lyt2$^-$ cell (*) is seen in the acinus (A). The partial degeneration of acinar cell (arrow heads) adjacent to the Lyt2$^+$ cell is seen. × 7,900, Bar = 2 μm. **b.** Lyt2$^+$ cell (*) is seen in the acinus (A). This Lyt2$^+$ cell sends spike-like projections (arrows) into acinar cell (A). × 6,700, Bar = 2 μm. **c.** Higher magnification of Fig. 5b. × 18,700, Bar = 1μm.

are usually of a tumor cell line (Able et al., 1970; Sanderson, 1982; Leopardi et al., 1984). In these model systems, effector cells adhere to the target cell and send spike-like projections into it and this is followed by a latent period after which the target cell undergoes «boiling» of its surface, cytoplasmic vacuolation, alterations in cytoplasmic organelles, and rapid lysis (Sanderson, 1982). The manner of contact between parenchymal cells and T-cells in the NOD mouse submandibular gland is similar to that between effector cells and target cells in the CMC model systems. This suggests that a lymphocytic cell-mediated cytotoxic mechanism may be involved in the phenomenon of the «submandibulitis» in NOD mice.

The molecular mechanisms of CMC are still largely unknown. It has been suggested that when cytotoxic cells recognize their target, they release a lymphotoxin and that this toxin destroys its target (Sanderson, 1982). However, it has not been established that lymphotoxin is the only, or the most important, mediator of cell-mediated acinar cell destruction. Recently, attention has been drawn to the significance of pore-forming protein (perforin) in target cell destruction (Podack and Dennert, 1983; Marx, 1986; Young et al., 1986). The cytotoxic T-cells and NK-cells are thought to have perforin in their cytoplasmic granule and this protein may play an active role in the cytolysis through pore forming on the target cells. In this study, we could not find any obvious structural changes in acinar or ductal cell plasma membrane adjacent to the invading lymphocytes. However, it is still possible that the cytotoxic cells introduce pores into the acinar and ductal cell membrane that cannot be detected by the ultrathin sections, as has been suggested by Abo et al. (1982).

It remains also to be clarified how T-cells recognize acinar or ductal cells as not-self. Fujita et al. (1984) have discovered type C retrovirus-like particles in pancreatic ß-cells in NOD mice. It is conceivable that viruses are involved in the development of autoimmunity in NOD mice. We therefore attempted to find virus-like particles, but without success.

In lymphoproliferative lesions in the submandibular glands of NOD mice, B-lymphocytes are also observed in the periphery of the infiltrate although the number is less

in comparison with T-lymphocytes (Miyagawa et al., 1986). At present we cannot find any record available in the literature concerning the function and/or significance of the infiltrating B-lymphocytes. It remains as a possibility that humoral immunity participates in the development of «submandibulitis» in NOD mice.

Nevertheless, it is suggested, this time by immunoelectron microscopy, that T-lymphocytes might play an important role in the direct acinar and ductal cell destruction in the submandibular glands of NOD mice.

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