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Ultrastructural localization of S-100 protein in rat popliteal lymph nodes, and very slight proliferative activity of follicular dendritic cells

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Summary. The purposes of this study were to examine the tissue distribution of S-100 protein in rat lymph nodes at the ultrastructural level with respect to the relationship between follicular dendritic cells (FDCs) and antigen transporting cells (ATCs), and to determine whether FDCs increase after secondary stimulation with sheep red blood cells (SRBCs). We examined the ultrastructural localization of S-100 protein in rat popliteal lymph nodes, and the density of S-100 protein-positive FDCs in lymphoid follicles, after secondary stimulation with SRBCs, on paraffin wax sections. We found S-100 protein expression in FDCs in all regions of lymphoid follicles, although FDCs in the central portion of lymphoid follicles showed stronger reactions than FDCs in the periphery. S-100 protein recognized ATCs weakly. At the border between the subsinus layer and the lymphoid follicles, ATCs were very close to FDCs. There were only two mitotic S-100 protein-positive cells in the lymphoid follicles of all specimens. The density of S-100 protein-positive FDCs in the lymphoid follicles in secondary stimulated rats was significantly lower than in primary stimulated rats. We suggest that S-100 protein expression reflects FDC development and supports a close relationship between FDCs and ATCs. FDCs may have only very slight proliferative activity, though the FDC density in the lymphoid follicles decreased after secondary stimulation.

Key words: Follicular dendritic cells, Antigen transporting cells, S-100 protein, Proliferative activity

Introduction

Follicular dendritic cells (FDCs) are non-lymphoid cells found only within lymphoid follicles, and play a role in immune responses by trapping immune complexes (ICs) on their cytoplasmic membranes. Their typical morphological features include euchromatic nuclei and scanty cytoplasm with intricate dendritic processes, particularly in the light zone (Szakal et al., 1983; Imai et al., 1986; Rademakers, 1992).

S-100 protein is one of the calcium-binding proteins, and is widely distributed in various cells although it had initially been assumed to be specific for the nervous system (for review, see Kligman and Hilt, 1988). Whereas in humans S-100 protein recognizes interdigitating cells within the paracortex and FDCs within germinal centers (GCs) (Carbone et al., 1985), in rats it recognizes FDCs within both GCs and lymphocytic coronas (Iwanaga et al., 1982; Cocchia et al., 1983). Furthermore, in our previous study of rat lymph nodes (Sato and Dobashi, 1995). S-100 protein was found to recognize not only FDCs but also antigen transporting cells (ATCs; Szakal et al., 1983), which are distributed in the subsinus layer (Sainte-Marie and Peng, 1985) between the subcapsular sinus and the lymphoid follicles. However, S-100 protein expression in ATCs remains to be investigated at the ultrastructural level.

Moreover, our previous study showed that the density of FDCs in the lymphoid follicles decreases after stimulation with sheep red blood cells (SRBCs). Thus, we obtained no evidence that the number of FDCs increased. However, in another study it was concluded that the number of FDCs in GCs of murine lymph nodes increased after secondary stimulation with SRBCs (Heinen et al., 1985). Thus, there is the possibility that the numbers of FDCs may change differently after secondary stimulation compared with after primary stimulation.

The purposes of the present study were to confirm and extend current knowledge on the tissue distribution of S-100 protein in rat lymph nodes at the ultrastructural level, with special reference to the relationship between FDCs and ATCs, and to determine whether the density of FDCs increases after secondary stimulation. First, we examined the ultrastructural localization of S-100 protein in rat popliteal lymph nodes. Secondly, we measured the density of S-100 protein-positive FDCs after secondary stimulation, using paraffin wax-

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embedded sections.

Materials and methods

1. Electron microscopical immunohistochemistry for S-100 protein

1) Animals and tissue preparation

Male and female Wistar rats (Charles River Japan Inc., Atsugi, Japan) aged six weeks to 28 months were maintained under specific pathogen-free conditions. Details of the animals used are presented (Table 1). Some rats received subcutaneous injections of 0.2 ml SRBCs (at a concentration of $1x10^9$ cells/ml) or lipopolysaccharide (LPS; at a dose of 100 µg; *E. coli* lipopolysaccharide B, lot 0111, B4; List Biological Lab, Inc., Campbell, CA, USA), diluted with saline, into the hind footpads. The stimulated and unstimulated rats were killed and their popliteal lymph nodes were harvested at the times shown in Table 1. The lymph node specimens were fixed in periodate-lysine-paraformaldehyde (PLP) containing 4% paraformaldehyde at 4 ºC for 4 h or in B5 at room temperature for 3 h, rinsed, and then sliced into 40 µm sections with a Vibratome (DTK-1000; Dosaka EM Co., Ltd., Kyoto, Japan).

2) Immunostaining for S-100 protein

The specimens were immunostained by the peroxidase-anti peroxidase (PAP) method described by Cocchia et al. (1983). The endogenous peroxidase activity was inhibited by incubation of the sliced sections with 0.1% phenylhydrazine hydrochloride in 0.1M phosphate buffer (pH 7.4) at 37 ºC for 1 h. After incubation in 5% swine serum for 15 min at room temperature, the sections were sequentially incubated overnight at 4 ºC with rabbit anti-ox S-100 protein antibody (1/1000; prepared according to Zuckerman et al., 1970), swine immunoglobulins anti-rabbit immunoglobulins (1/100; Dakopatts, Glostrup, Denmark), and rabbit PAP reagent (1/100; Dakopatts). After incubation in 0.03% 3,3'-diaminobenzidine (DAB) in 0.05M Tris-HCl (pH 7.6) for 30 min, the labeled peroxidase was visualized with DAB in Tris-HCl containing 0.006% H_2O_2. Following postfixation with 0.5% glutaraldehyde for 15 min and 1% OsO_4 for 1 h at 4 ºC, the sections were dehydrated, and then embedded in Epon columns. For light microscopy, 2 µm-thick sections were stained with toluidine blue. Ultrathin sections were examined with an electron microscope (HS-9; Hitachi, Tokyo, Japan). For a negative control, the primary antibody was omitted.

2. Measurement of FDC density in the lymphoid follicles after stimulation with SRBCs

This experiment was designed with a similar age of animals and duration of SRBC stimulation in the previous study on mice (Heine et al., 1985). Twelve female Wistar rats (Charles River Japan Inc.) aged 10 weeks, under specific pathogen-free conditions, received bilateral hind footpad injections of 0.2 ml SRBCs at a concentration of $1x10^9$ cells/ml. Since in our preliminary study we had confirmed that young adult rats had many secondary follicles 3 to 4 weeks after SRBC stimulation, unstimulated rats were not used in this experiment. Thus, these 12 rats were divided into primary and secondary stimulated groups. The popliteal lymph nodes of six of the rats (primary stimulated group) were harvested at day 25 after stimulation by SRBCs. On the same day, the remaining six animals (secondary stimulated group) received a second bilateral hind footpad injection of the same amount of SRBCs, and their popliteal lymph nodes were removed at day 29 (4 days after the secondary stimulation). The removed nodes were fixed in B5 for 3 h at room temperature, and then embedded in paraffin wax. At least 60 sections per group were prepared.

The paraffin sections were immunostained by the avidin-biotin complex (ABC) method as follows. The sections were sequentially incubated with 5mM periodic acid for 15 min, 5% swine serum for 15 min, rabbit anti-ox S-100 protein antibody (1/5000) for 1 h, biotinylated goat anti-rabbit IgG antibody (1/200; Vector Lab., Burlingame, CA, USA) for 30 min, and ABC Elite reagents, which contain avidin DH and biotinylated horseradish peroxidase H reagents (Vector Lab.), for 30 min at room temperature. The labeled peroxidase was visualized with DAB in Tris-HCl containing H_2O_2. The sections were counterstained with methyl green.

The FDC density was determined as described previously (Sato and Dobashi, 1995). Briefly, about 100 lymphoid follicles in immunostained sections of each group were photographed randomly at a final

Table 1. The numbers of rats used in the ultrastructural study.

	NUMBER OF RATS			
AGE/SEX	UNSTIMULATED	SRBCs (x1)	SRBCs (x2)	LPS
6 weeks				
male	1			
female	2			
10 weeks				
male	5			11
female	10			10
11 weeks				
female	5	5		
13 weeks				
female		6		
14 weeks				
female	6		11	
24 to 28 months				
male				2
female	1			3

SRBCs (x1): rats stimulated with SRBCs were sacrificed between days 10 and 25; SRBCs (x2): rats stimulated with SRBCs were stimulated with SRBCs again on day 25, and then sacrificed on day 29; LPS: rats stimulated with LPS were sacrificed on day 10.

magnification of x360. To measure the areas of the lymphoid follicles and GCs on the photographs, we traced their contours on to the digitizer of a computerized analysis system (DIANA98-III; Meiwa Shoji Co., Osaka, Japan). The area of each lymphocytic corona was obtained by substracting the area of the GC from the area of the lymphoid follicle. The number of S-100 protein-positive FDCs was recorded in each region. Finally, we calculated the density of FDCs (per $10^4\mu m^2$) in each region of the lymphoid follicle by dividing the total number of S-100 protein-positive cells in each region by its area. Statistical analysis of the differences in the mean values was performed using t-tests.

Results

1. Ultrastructural localization of S-100 protein in rat popliteal lymph nodes

Other than in FDCs, there were no immunoreactions within lymphoid follicles. S-100 protein-positive cells in tissues fixed with PLP for electron microscopy showed weaker reactions, particularly in their nuclei, than those in paraffin sections examined by light microscopy. Moreover, ultrastructurally these cells showed stronger reactions in B5-fixed tissues than those in PLP-fixed tissues, though the former tissues were damaged by B5. A possible explanation of these findings is that it may have been difficult for the antibodies used in this immunostaining to penetrate into the membranes, particularly the nuclear membranes, of these cells in 40-µm-sections. Thus, because of the damage, it may have been easier for tissues fixed in B5 to have been penetrated by the antibodies than those fixed in PLP.

FDCs expressed S-100 protein strongly in their cytoplasm, except for the endoplasmic reticulum and mitochondrial matrix, but faintly in their nuclei. Whereas young rats had well developed secondary follicles after stimulation with SRBCs or LPS, unstimulated young rats had less well developed secondary follicles. In aged rats, the lymphoid follicles were atrophic (detailed findings will be described in another paper).

In the light zone and adjacent corona, FDCs with dispersed chromatin showed strong positive reactions, including one in their intricate cytoplasmic dendritic processes. A few binuclear FDCs were observed (Fig. 1). Some FDCs, like pericytes, were observed around blood capillaries in lymphoid follicles. In the dark zone, several FDCs with elliptical nuclei and scant cytoplasmic processes showed positive reactions (Fig. 2). Whereas FDCs in primary follicles showed only moderate reactions for S-100 protein, FDCs in secondary follicles showed strong reactions, particularly in the light zone, as described previously (Sato and Dobashi, 1995). FDCs in the central portion of lymphoid follicles tended to show stronger reactions than those in the periphery.

S-100 protein also recognized fat cells and some fibroblasts in the capsules of lymph nodes (not shown). Furthermore, S-100 protein recognized ATCs in the subsinus layer, though the reactions in ATCs were weaker than those in FDCs (Figs. 3, 4). In the region near the subcapsular sinus, ATCs had round, lobular euchromatic nuclei with some chromatin at the margins. In the deeper regions, ATCs had quite well developed cytoplasmic processes. At the border between the subsinus layer and the lymphoid follicle, ATCs were very close to FDCs in the shallowest regions (Fig. 4). Negative control staining for S-100 protein showed no positive reaction.

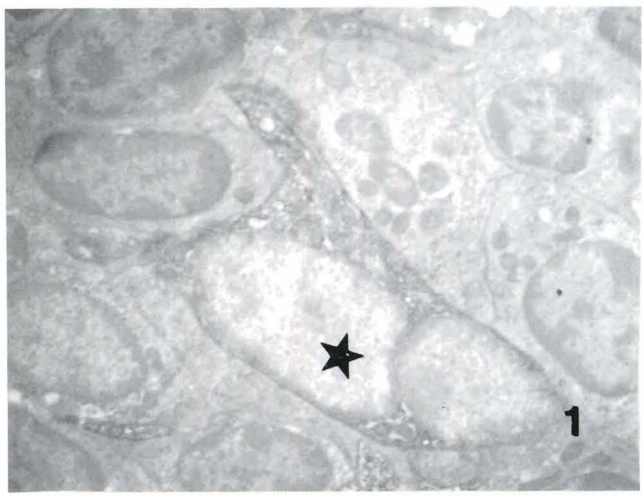


Fig. 1. Electron micrograph showing anti-S-100 protein labeling in the corona of a secondary follicle of a popliteal lymph node from a 14-week-old rat without stimulation. A binuclear FDC with euchromatic nuclei and dendritic processes shows a positive reaction in the cytoplasm (asterisk). Fixed in PLP, PAP method. x 4,000

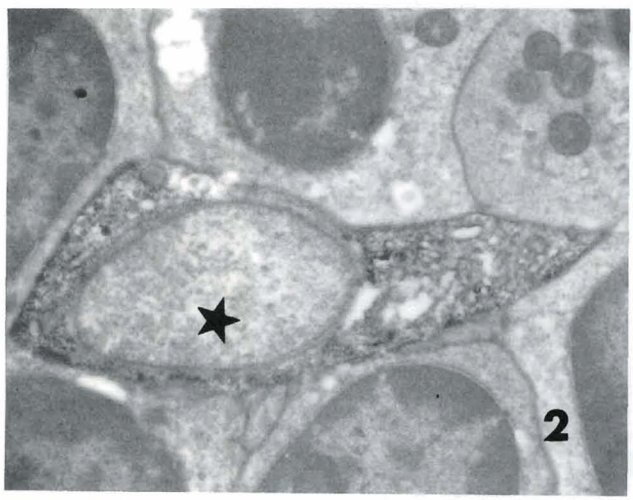


Fig. 2. Electron micrograph showing anti-S-100 protein labeling in the dark zone of a secondary follicle of a popliteal lymph node from a 14-week-old rat without stimulation. An FDC with few dendritic processes shows a positive reaction in the cytoplasm (asterisk). Fixed in PLP, PAP method. x 9,800

Contrary to our expectation that FDCs would show no mitosis, two instances were detected in S-100 protein-positive cells in lymphoid follicles out of all the specimens examined. These were in a GC of a 6-week-old rat without stimulation and a primary follicle of a 26-

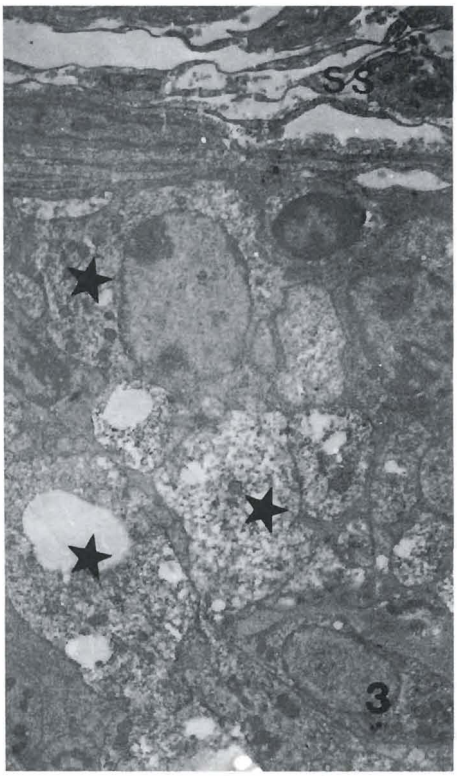


Fig. 3. Electron micrograph showing anti-S-100 protein labeling in the subsinus layer of a popliteal lymph node from a 14-week-old rat without stimulation. ATCs with lobed round euchromatic nuclei show weak positive reactions in the cytoplasm (asterisks). SS: subcapsular sinus. Fixed in PLP, PAP method. x 3,000

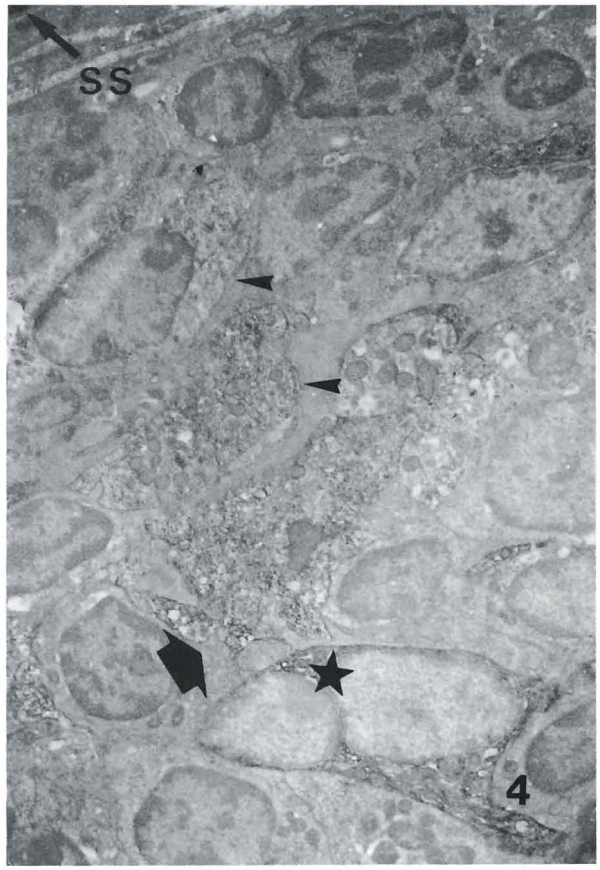
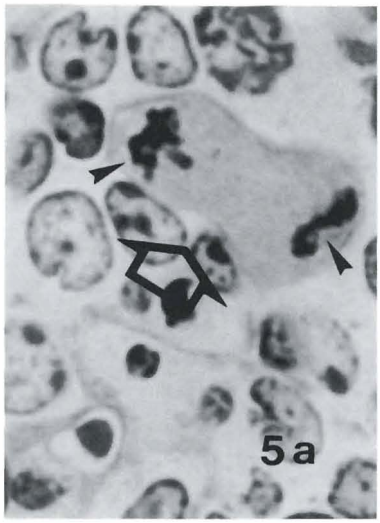


Fig. 4. Electron micrograph showing anti-S-100 protein labeling in the subsinus layer of a popliteal lymph node from a 14-week-old rat without stimulation. ATCs (arrowheads) show weak positive reactions in their cytoplasm. At the border between the subsinus layer and a secondary lymphoid follicle, the cytoplasm of an ATC (arrow) is located close to an FDC (asterisk) in the corona. SS: subcapsular sinus. Fixed in PLP, PAP method. x 3,300



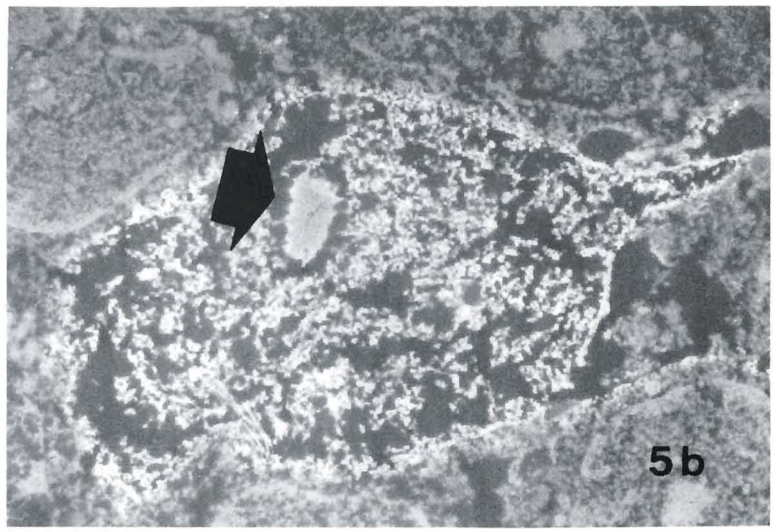


Fig. 5. Micrographs showing anti-S-100 protein labeling in the germinal center of a popliteal lymph node from a 6-week-old rat without stimulation. **a.** Light micrograph showing an S-100 protein-positive cell (arrow) with mitosis (arrowheads). Although in this photograph the cell shows weak staining in the cytoplasm (arrow), in the original photograph, which is a color transparency, it shows strong staining (not shown). **b.** Electron micrograph of the same cell as that shown in 5a. The S-100 protein-positive cell shows mitosis (arrow), although this tissue is damaged by B5. Fixed in B5, PAP method. a: counterstained with toluidine blue. a, x 2,300; b, x 6,000

month-old rat stimulated with LPS (Figs. 5, 6).

2. Quantitative analysis of the FDC density in the lymphoid follicles of rat popliteal lymph nodes

There was no evidence of any increase in FDC density in the lymphoid follicles after secondary stimulation by SRBCs (Table 2). The density of S-100 protein-positive cells in the lymphoid follicles of the secondary stimulated group was significantly lower than that of the primary stimulated one ($p<0.01$). On the other hand, almost all follicles in the primary (Fig. 7) and secondary stimulated (not shown) groups were secondary follicles (Table 2). In addition, no mitosis was found in S-100 protein-positive FDCs in any specimens in these two groups. However, as expected, the measured lymphoid follicles in the secondary stimulated group were significantly larger than those in the primary one ($p<0.01$): mean areas (±SD) of the measured lymphoid follicles in the secondary and primary stimulated groups were 58932±26794 and 46480±17555 μm^2 respectively.

Discussion

This study produced the following findings: 1) S-100 protein recognized not only FDCs but also ATCs; 2)

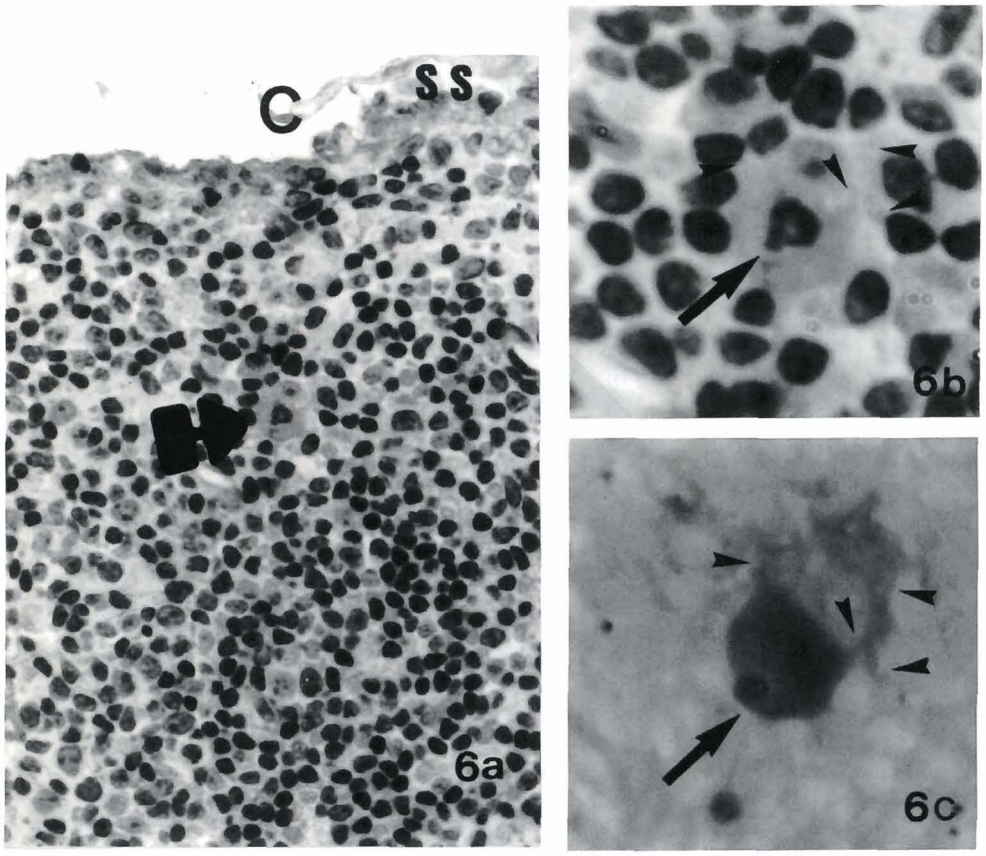


Fig. 6. Light micrographs showing anti-S-100 protein labeling in a primary follicle of a popliteal lymph node from a 26-month-old rat after stimulation with LPS. **b** and **c** are high-power views. **a** and **b** show mitosis (arrows) of a cell with a dendritic process-like morphology (arrowheads) in the primary follicle. Fig. 6c was obtained using an interference filter for immunohistochemistry (IF436; Olympus, Tokyo, Japan). Anti-S-100 protein recognizes the same cell (arrow) with dendritic process-like morphology (arrowheads) shown in 6b. Fixed in PLP, PAP method, counterstained with toluidine blue. a, x 600; b-c, x 1,500

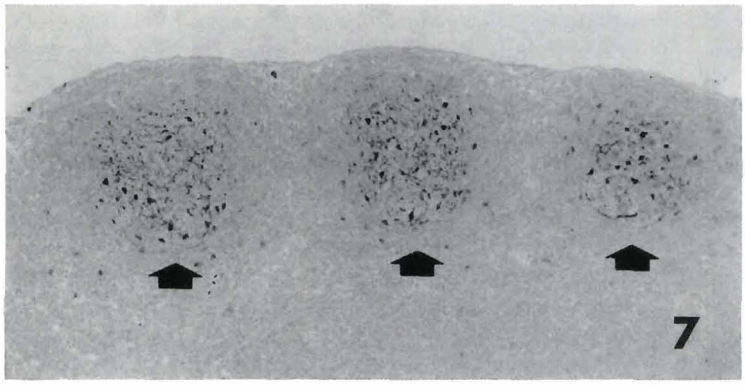


Fig. 7. Light micrograph showing immunoreactivity of anti-S-100 protein in a lymph node from a rat at day 25 after SRBC stimulation. Three secondary follicles show strong positive reactions (arrows). Fixed in B5, counterstained with methyl green, ABC method. x 100

Table 2. The mean densities (±SD) of S-100 protein-positive cells in the lymphocytic coronas, germinal centers, and total regions within lymphoid follicles of two stimulated groups (/10^4 μm^2)

	PRIMARY STIMULATED GROUP	SECONDARY STIMULATED GROUP
Coronas	4.4±2.9	3.9±2.5
Germinal centers	13.3±3.7	9.5±4.0*
Lymphoid follicles	8.3±2.5	6.3±2.5*

The primary stimulated group contains 121 lymphoid follicles including 111 secondary follicles. The secondary stimulated group contains 114 lymphoid follicles including 102 secondary follicles. «Coronas» include the regions of primary follicles and lymphocytic coronas in secondary follicles. *: the density of S-100 protein-positive cells in the lymph follicles or the germinal centers in the secondary stimulated group is significantly lower than that in the corresponding primary stimulated group ($p<0.01$).

only a few mitotic S-100 protein-positive cells were found in lymphoid follicles; 3) the density of FDCs in the lymphoid follicles decreased after secondary stimulation. On this basis, we discuss the significance of S-100 protein immunostaining in rat lymph nodes, and the likelihood of FDCs having very slight proliferative activity.

In the present study, S-100 protein recognized FDCs in the corona and the light and dark zones within lymphoid follicles at the ultrastructural level (Figs. 1, 2). Thus, S-100 protein seems to recognize all FDCs within lymphoid follicles. Moreover, FDCs showed stronger reactions for S-100 protein in secondary follicles than in primary follicles, and in the central portion of lymphoid follicles than in the periphery. These findings indicate that S-100 protein recognizes developed FDCs very strongly. Thus S-100 protein expression may reflect FDC development.

Recently, in mice, the immune-complex-coated body (iccosome) theory has been proposed: iccosomes are considered to be derived from FDC dendrites and to be endocytosed by GC cells, thereby processing antigen and presenting it to T cells (Szakal et al., 1988). However, iccosomes were not found in the present study. There have been only a few comments about iccosomes in rats (Terashima et al., 1991, 1992). Although FDC dendrites including labyrinth-like structures have a close relationship to iccosomes, they are poorly developed in rats (Terashima et al., 1991). Moreover, it has been reported that it is not easy to detect iccosomes in rats (Terashima et al., 1992). Thus, it is not surprising that we failed to find iccosomes in rat lymph nodes. However, the present study was not designed to focus on iccosomes, and therefore our finding awaits further confirmation through further investigation considering differences in experimental design, such as the species used and the method of stimulation employed.

We have confirmed our previous findings about ATCs in rat lymph nodes and extended them to the ultrastructural level (Figs. 3, 4). FDCs and ATCs have been described as having similar morphological features and a common function of IC transportation (Szakal et al., 1983). Moreover, ATCs have been suspected to be FDC precursors (Szakal et al., 1983; Maeda et al., 1995). In the present study, ATCs in the deepest region were very close to FDCs in the shallowest region (Fig. 4), consistent with the above hypothesis. Furthermore, ATCs were labeled weakly with anti-S-100 protein, whereas it labeled FDCs intensely, which could be explained by ATCs maturing into FDCs. However, the origin of FDCs is still unclear: they seem to be derived from mesenchymal or bone marrow cells, or ATCs (for review, see Heinen and Bosseloir, 1994). Thus, the hypothesis that they are derived from ATCs has not yet been proved. However, these findings at least indicate that S-100 protein expression may reflect a close relationship between FDCs and ATCs.

Contrary to expectation, two mitotic S-100 protein-positive cells were observed in lymphoid follicles (Figs. 5, 6). This implies that FDCs have mitotic activity, because no other cells in the lymphoid follicles expressed S-100 protein in the present study. Furthermore, the mitotic S-100 protein-positive cells had a dendritic process-like morphology (Fig. 6). Naturally, we cannot absolutely rule out the possibility that other cells in the lymphoid follicles acquire new positive reactions for S-100 protein during mitosis, though to our knowledge, there is no report of S-100 protein-positive mitotic cells in lymphoid follicles of rat lymphoid tissues. However, the following findings support the hypothesis that FDCs have mitotic activity. First, mitosis of FDCs, which have been described as fibroblastic reticulum cells, has been reported in the primary follicles of rat lymph nodes (Villena et al., 1983). Secondly, human FDCs in culture can proliferate in the presence of granulocyte-macrophage colony-stimulating factor (Clark et al., 1992). Naturally, FDC-like cell lines derived from human tonsils can proliferate (Kim et al., 1994; Lindhout et al., 1994). Finally, although tumors of FDCs are rare, several cases of mitotic FDC sarcomas have been reported (for review, see Chan et al., 1994).

However, the presence of mitosis does not mean that FDCs have any significant proliferative activity, for the following reasons. First, there have been few reports on mitosis in FDCs (Villena et al., 1983). Secondly, only two cases of mitosis in FDCs were found in the present study. Thirdly, it has been reported that human FDCs in long-term culture show no proliferation (Tsunoda et al., 1990). Finally, in our previous study, the FDC density in rat lymphoid follicles decreased after SRBC stimulation (Sato and Dobashi, 1995). These findings suggest that FDCs show no or little proliferation under normal conditions. From these findings and the presence of mitosis of S-100 protein-positive cells in lymphoid follicles (Figs. 5, 6), we suggest that FDCs have only very slight proliferative activity.

As shown in Table 2, the density of S-100 protein-positive cells, i.e. FDCs, in the lymphoid follicles in the secondary stimulated group was lower than in the primary one. Thus we conclude that the FDC density in

the lymphoid follicles decreased after secondary stimulation with SRBCs, with no evidence that the number of FDCs increased. However, in the previous study it was concluded that the number of FDCs in the GCs at various sites within the lymph nodes of young adult mice increased after secondary stimulation with SRBCs, injected intraperitoneally (Heinen et al., 1985), which differs from the findings of the present study.

This discrepancy can be explained by differences in the cells which were counted as FDCs, as discussed in our paper (Sato and Dobashi, 1995): it would have been difficult to identify FDCs in the previous study, because they are identified by their morphological features in semi-thin Epon sections (Heinen et al., 1985). However, we cannot absolutely exclude the possibility that the discrepancy may have been due to differences in experimental design such as the species used, the sites of the lymph nodes studied, and the method of SRBC injection. Moreover, in the present study the measured lymphoid follicles in the secondary stimulated group were larger than those in the primary group. Thus there remains another possibility, that enlargement of lymphoid follicles after secondary stimulation masked a slight increase in the number of FDCs. Rare occurrence of mitosis in S-100 protein-positive cells (Figs. 5, 6) may support this possibility. On the other hand, it has been reported that some murine FDCs degenerate at the secondary response, and are then phagocytozed by tingible body macrophages (Terashima et al., 1991). This report implies that numbers of FDCs might decrease, explaining the present result of a decreased density of FDCs. However, it is unknown whether new FDCs are produced in the response, so whether the total number increases or decreases remains to be investigated.

In conclusion, S-100 protein expression may support a close relationship between FDCs and ATCs. Moreover, FDCs may have only very slight proliferative activity.

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