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A change in the localization of the region trapping immune complexes in rat popliteal lymph nodes during development of germinal centers, with regard to the distribution of follicular dendritic cells

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Summary. A study was conducted to clarify changes in the relationship between the region of immune complex (IC) trapping by follicular dendritic cells (FDCs) and the distribution of FDC during reaction of germinal centers (GCs), and to examine the relationship between the tridimensional shape of the IC-trapping regions and their two-dimensional shape. Five-week-old rats were given footpad injections of sheep red blood cells, and then their popliteal lymph nodes were excised between days 0 and 42, 24 h after injection of peroxidase-antiperoxidase complex (PAP) as an IC. The specimens were immunostained for PAP trapping on serial paraffin sections, and for S-100 protein as a marker of FDCs. It was found that during the GC reaction, PAP trapping became weak and then disappeared on the basal side of developing GCs where S-100 protein-positive FDCs were still present. All of the 1933 lymph follicles examined were found to trap PAP. Whereas the tridimensional shapes of the trapping regions showed similar patterns according to the development of lymph follicles, their two-dimensional shapes varied. We suggest that FDCs in primary follicles may differentiate into FDCs in the light zone and FDCs in the dark zone in secondary follicles. To evaluate each of the compartments of a lymph follicle more accurately, investigators should pay attention to the tridimensional shape of the compartment.

Key words: Follicular dendritic cells, Immune complex trapping, S-100 protein, Rat lymph node

Introduction

Follicular dendritic cells (FDCs) are non-lymphoid cells present in lymph follicles, and play a role in immune reactions by trapping immune complexes (ICs) on their cytoplasmic membranes. Whereas some investigators use the term «FDCs» to delineate only cells with trapping ability and/or those showing typical morphological features in lymph follicles, particularly in the light zone (Heusermann et al., 1980; Imai et al., 1986), others use it to refer to all dendritic-shaped non-lymphoid cells in lymph follicles (Stein et al., 1982; Rademakers, 1992). Here we use it to indicate the latter.

It is widely accepted that the regions where FDCs trap ICs show a round shape in primary follicles and a crescent shape in secondary follicles, particularly in the light zone (Nossal et al., 1968; Imai et al., 1986). However, little is known about how the localization of the IC-trapping regions changes during reaction of germinal centers (GCs). Moreover, we have noticed that a few lymph follicles in some sections show no trapping. However, trapping in each lymph follicle cannot be evaluated without tridimensional observation. Thus, whether all lymph follicles show trapping remains unclear.

On the other hand, it has been proposed that FDCs show heterogeneity, i.e. there are morphological (Rademakers, 1992) and functional differences (Imai et al., 1993; Yoshida et al., 1993) between FDCs in the light zone and those in the dark zone. However, it is still unclear how these two FDC subpopulations are produced. In rats, FDCs express a strong immunoreaction for S-100 protein in their nuclei and cytoplasm within both GCs and the lymphocytic corona (Iwanaga et al., 1982; Cocchia et al., 1983). Recently we have studied changes in the distribution of rat FDCs during the GC reaction, using immunostaining for S-100 protein (Sato and Dobashi, 1995). However, little is known about how the relationship between FDC distribution and the IC-trapping regions changes during the GC reaction. Although IC trapping has been detected only on Vibratome (Kamperdijk et al., 1987) or cryostat (Dijkstra et al., 1983) sections, we have established a novel method for detecting ICs trapped by FDCs using

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conventional paraffin sections. This immunostaining for ICs and S-100 protein on paraffin sections enabled us to investigate the relationship between the trapping region and FDC distribution, thereby allowing the two FDC subpopulations to be surveyed.

In this study, we attempted to examine how the topological relationship between the region of IC-trapping by FDCs and the region of FDC distribution changes during the GC reaction, whether all lymph follicles trap ICs, and how the tridimensional shapes of the trapping regions relate to their two-dimensional shapes. For these purposes, we stimulated rats with sheep red blood cells (SRBCs) and then removed their popliteal lymph nodes at different intervals, 24 h after injection of peroxidase-antiperoxidase complex (PAP) as an IC. We then carried out immunostaining for trapped PAP on serial paraffin sections, and also for S-100 protein as a marker of FDCs. In addition, we examined whether or not the number of lymph follicles per whole popliteal lymph node increased after stimulation.

Materials and methods

1. Animals and tissue preparation

Fifty-three male Wistar rats (Charles River Japan Inc., Atsugi, Japan) aged five weeks were kept under specific pathogen-free conditions. Excluding 10 rats as negative controls, 27 of the remaining 43 rats received subcutaneous injection of 0.2 ml SRBCs at a concentration of $1x10^9$ cells/ml, in both hind footpads. For detection of the preadministered PAP, 0.2 ml of rabbit soluble PAP (DAKOPATTS, Glostrup, Denmark) diluted 1:5 with saline was administered into both hind footpads of these 43 rats 24 h before excision of the lymph nodes. The bilateral popliteal lymph nodes of the 27 stimulated rats were removed on days 1, 3, 5, 10, 21 and 42 after SRBC stimulation. Those of the remaining 16 rats without SRBC stimulation were also removed using the same procedures on days 0 (5-week-old rats), 3, 5, 10 and 21. The stimulated group on day 42, each of the unstimulated groups on days 3, 5 and 10, and each of the other groups contained two, two, and five rats, respectively. The excised lymph nodes were fixed in Zamboni's solution (Zamboni and De Martino, 1967) at 4 °C overnight, and then processed routinely for embedding in paraffin. We prepared at least 50 sections (contained step, serial, and mirror sections) per lymph node from 63 lymph nodes, and also all serial sections from 23 whole lymph nodes. For the negative control, 10 rats (day 0 and day 10 after SRBC stimulation) that had not received rabbit PAP were examined.

2. Detection of preadministered PAP

The preadministered PAP was detected on serial and step sections by the indirect immunoperoxidase method. Inhibition of endogenous peroxidase activity was omitted because the immunoreactions for trapping showed the same pattern in lymph follicles regardless of inhibition. For proteolytic digestion, the sections were incubated with 0.1% trypsin (DIFCO Lab., Detroit, MI, USA) in 0.01M phosphate-buffered saline (pH 7.4) containing 0.02% $CaCl_2$ at 37 °C for 10 min. The sections were then sequentially incubated with 5% swine serum for 15 min and peroxidase-conjugated swine immunoglobulins anti-rabbit immunoglobulins (1/100; DAKOPATTS) for 1 h at room temperature. The peroxidase activity was developed in 0.03% 3,3'-diaminobenzidine in 0.05M Tris-HCl (pH 7.6) containing 0.006% H_2O_2. The sections were counterstained with methyl green or hematoxylin. For specificity control, the specimens were immunostained without antibody or proteolytic digestion.

3. Immunohistochemistry for S-100 protein

Sections were immunostained by the streptavidin-biotin (SAB) method as follows. Endogenous peroxidase activity was blocked with 5mM periodic acid dihydrate for 15 min. The sections were then sequentially incubated with 5% swine serum for 15 min, rabbit anti-ox S-100 protein antibody (1/400; DAKOPATTS) for 1 h, biotinylated goat anti-rabbit IgG antibody (1/200; Vector Labs., Burlingame, CA, USA) for 20 min, and peroxidase-conjugated streptavidin (non-diluted; Nichirei, Tokyo, Japan) for 10 min at room temperature. The subsequent procedures from the development of labeled peroxidase were the same as described for the detection of preadministered PAP. In addition, some sections were immunostained for S-100 protein after proteolytic digestion with trypsin as described above; these sections were sequentially incubated with periodic acid dihydrate, swine serum, 0.1% trypsin, and anti-ox S-100 protein, subsequently followed by the same procedures. As a negative control, the primary antibody was omitted, or normal rabbit serum as used instead of the primary antibody.

4. Mirror-sectioning technique

The aim of the study was to estimate the localization of PAP trapping and S-100 protein-positive FDCs in the same region. Although a preliminary examination showed successful double immunostaining for both trapping and S-100 protein using this experimental system, it was difficult to distinguish the weak staining for one marker (particularly, trapping) from the strong staining for the other. To overcome this difficulty, we adopted the mirror sectioning technique. Each of a pair of mirror sections was stained for trapping or for S-100 protein using the above procedures.

5. Tridimensional analysis

First, to determine whether all lymph follicles trap PAP, we traced the contours of lymph nodes, lymph follicles and GCs on every ninth serial section immuno-

stained for trapping onto tracing paper using a profile projector. The numbers of lymph follicles examined in each group were 316 for day 0 (5-week-old rats), 41 for day 1, 78 for day 3, 74 for day 5, 88 for day 10, 737 for day 21, 126 for day 42 after stimulation, and 473 for day 21 without stimulation. The total number of lymph follicles was 1993. On both day 0 and day 21 with/without stimulation, six whole lymph nodes from five rats were examined. On each of the other days, one whole lymph node from one rat was examined. We examined whether each lymph follicle showed trapping by comparing the immunoreactions for trapping in each section and individual lymph follicles drawn on tracing paper.

Second, to examine tridimensional shapes of the trapping regions in relation to their two-dimensional shapes, we selected randomly at least four of the lymph follicles drawn on the tracing paper in each of the groups for day 0, and days 3, 5, 10, 21 and 42 after stimulation. All serial sections of these selected follicles were photographed at final magnifications of x100 to x400, and the shapes of the trapping regions were examined tridimensionally using the photographs.

Finally, to determine whether the number of lymph follicles per whole lymph node increased after stimulation, we counted the numbers of lymph follicles and GCs per whole lymph node on day 0 (5-week-old rats), day 21 after stimulation (8-week-old rats), and day 21 without stimulation, using the tracing paper method described above. Each of these three groups contained six whole lymph nodes from five rats. Statistical analysis of the differences in the mean values was performed by t test.

Results

1. Immunohistochemical staining for trapped PAP and S-100 protein in rat popliteal lymph nodes

The localization of the regions trapping PAP changed during the GC reaction (Fig. 1). PAP trapping became weak and then disappeared on the basal side of developing GCs where S-100 protein-positive FDCs were still present. As a result, the regions where FDCs were distributed came to occupy larger areas than the trapping regions, particularly in well-developed secondary follicles. The expression of S-100 protein by FDCs showed the same pattern as that described previously (Sato and Dobashi, 1995).

1) Localization of the two markers in each group

On day 0 (5-week-old rats) and day 1 after SRBC stimulation, almost all follicles were primary follicles and showed immunoreactions for trapped PAP as an extensive reticular network pattern (Fig. 2). This staining pattern in primary follicles was also observed on all other days. Many histiocytes in the medulla phagocytosed PAP, thereby showing a positive reaction in their cytoplasm. A weak reaction for endogenous peroxidase was observed on these first two and all other days. On the other hand, immunoreactions for S-100 protein were recognized on FDCs in primary follicles, mainly in their nuclei, and partly in their cytoplasm. In the subsinus

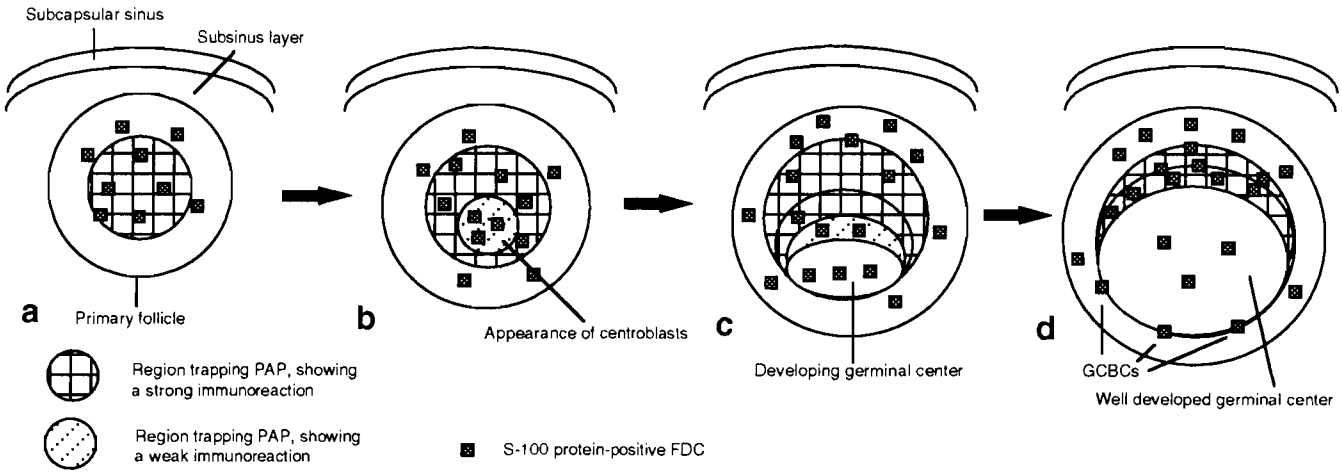

Fig. 1. Schematic diagrams showing the change in the relationship between the regions trapping PAP and the distribution of S-100 protein-positive follicular dendritic cells (FDCs) in rat popliteal lymph nodes during development of germinal centers (GCs). **a.** On day 0 (5-week-old rats), the trapping region occupies a large area in primary follicles, where FDCs are distributed (see Fig. 2). In the subsinus layer, antigen transporting cells (ATCs) show a weak reaction for S-100 protein. **b.** On day 3 after stimulation with SRBCs, the trapping region shows a strong reaction in the upper region of immature secondary follicles but a weak reaction on the basal side of immature GCs (see Figs. 3, 4a). FDCs are distributed in all regions of the lymph follicles (see Fig. 4b). **c.** On day 5 after stimulation with SRBCs, the trapping region shows a strong reaction in the upper region of developing secondary follicles but a weak reaction in the middle region of GCs (see Figs. 5, 6a). FDCs are distributed in all regions of the lymph follicles (see Fig. 6b). **d.** On day 10 after stimulation with SRBCs, the trapping region is localized in the light zone and adjacent corona of well developed secondary follicles, appearing as a crescent (see Fig. 7a). FDCs are distributed in all regions of the lymph follicles. At the border between the dark zone and adjacent corona, germinal center bordering cells (GCBCs) express a reaction for S-100 protein (see Fig. 7b).

layer (Sainte-Marie and Peng, 1985) between the subcapsular sinus and lymph follicles, antigen transporting cells (ATCs; Szakal et al., 1983) showed a weak reaction for S-100 protein on the first two and all other days, as described previously (Sato and Dobashi, 1995).

On day 3 after SRBC stimulation, foci of centro-

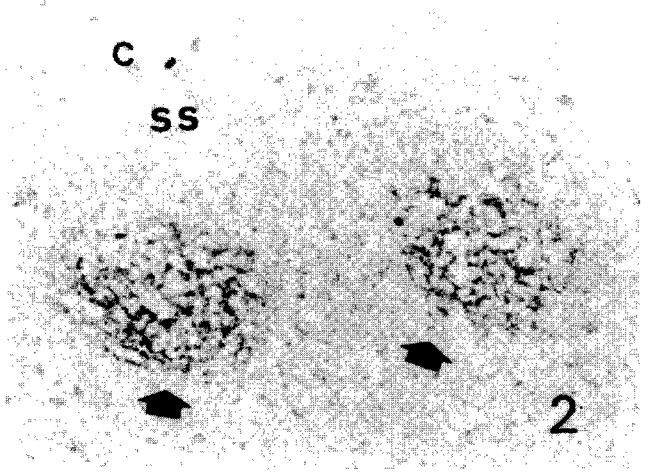

Fig. 2. Immunostaining of PAP trapping in a popliteal lymph node in a 5-week-old rat. Immunoreactions are distributed in two primary follicles (arrows). C: capsule; SS: subcapsular sinus. Counterstained with methyl green; indirect peroxidase method. x 190

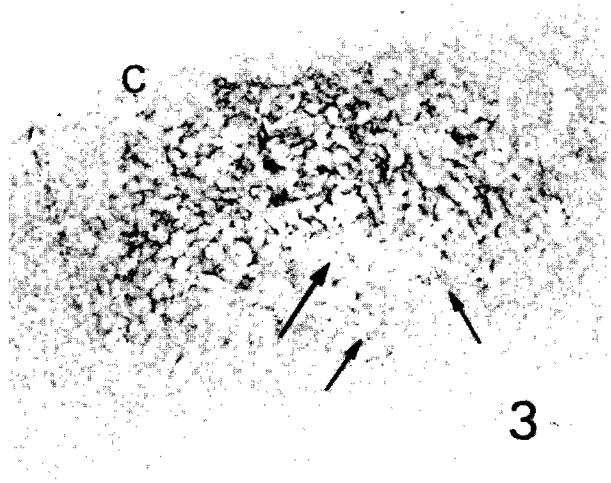

Fig. 3. Immunostaining of PAP trapping in a rat popliteal lymph node at day 3 after stimulation with SRBCs. Note strong reactions in the upper region of an immature secondary follicle, and weak reactions (arrows) in the lower region including the corona, particularly the basal side of a germinal center. C: capsule. Counterstained with methyl green; indirect peroxidase method. x 180

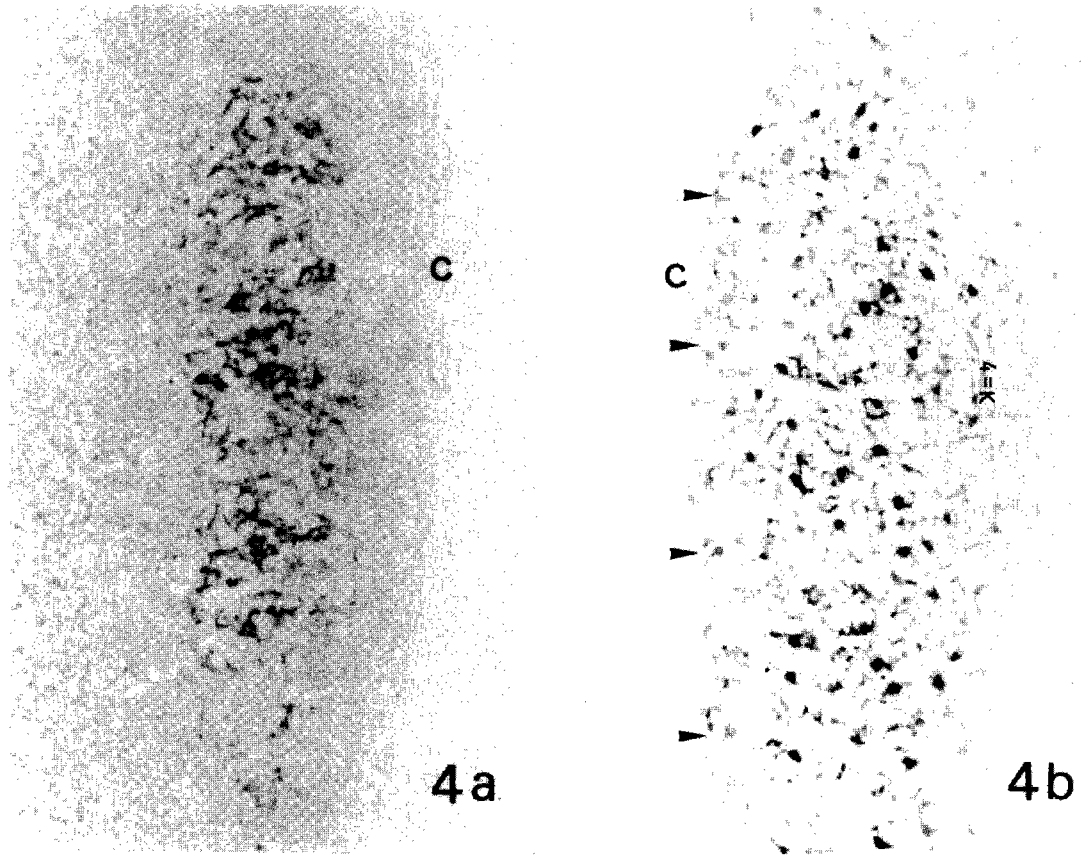

Fig. 4. Two photomicrographs showing mirror sections of an immature secondary follicle at day 3 after stimulation with SRBCs. 4a is stained for PAP trapping, and 4b for S-100 protein. a shows strong reactions, and weak reactions where centroblasts are distributed. b shows that S-100 protein-positive FDCs are distributed in all regions of the follicle. Weak reactions are also distributed in the subsinus layer (arrowheads). C: capsule. Counterstained with methyl green. a; indirect immunoperoxidase method. b; SAB method. x 240

blasts appeared in many follicles. Immature secondary follicles were recognized in one third of all lymph follicles. In these secondary follicles, there was strong immunostaining for trapping in the corona and upper portion of the follicles, but weak immunostaining where centroblasts had just appeared (Fig. 3, 4a). On the medullary side of the corona, no or only a weak positive reaction was observed. On the other hand, S-100 protein-positive FDCs were scattered in all regions of the lymph follicles (Fig. 4b). There was no decrease in the intensity of immunostaining for S-100 protein, even in regions of weak trapping.

On day 5 after SRBC stimulation, developing GCs were recognized in two thirds of all follicles. In secondary follicles, the immunostaining for trapping was strong on the apical side of GCs and the adjacent corona, but weak in the middle portion of GCs (Figs. 5, 6a). On the basal side of GCs, no or only a slight reaction was observed. However, S-100 protein-positive FDCs were distributed in all regions of the lymph follicles (Fig. 6b).

On days 10, 21 and 42 after SRBC stimulation, well-developed secondary follicles with light and dark zones were observed. The GCs on days 10 and 21 showed the strongest development, whereas the GCs on day 42 showed some decline. The immunostaining for trapping was strong in the light zone and adjacent corona, and its distribution was crescent-shaped (Fig. 7a). S-100 protein-positive FDCs were distributed in all regions of lymph follicles (Fig. 7b). The positive reactions for S-100 protein were found not only in the corona, light and dark zones, but also on the border between the dark zone and adjacent corona. At this border, slender spindle cells, which have been referred to as germinal center bordering cells (GCBCs) (Fossum, 1980), showed a positive reaction.

In unstimulated groups on days 3, 5, 10 and 21,

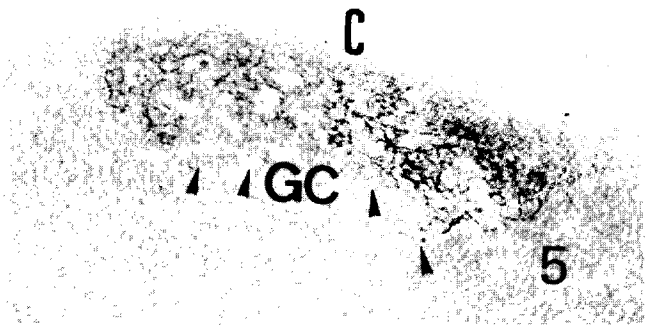

Fig. 5. Immunostaining of PAP trapping in a developing secondary follicle at day 5 after stimulation with SRBCs. Note strong reactions in the subcapsular side of a germinal center (GC) and adjacent corona, and weak reactions (arrowheads) in the middle region of the GC. C: capsule. Counterstained with methyl green; indirect peroxidase method. x 180

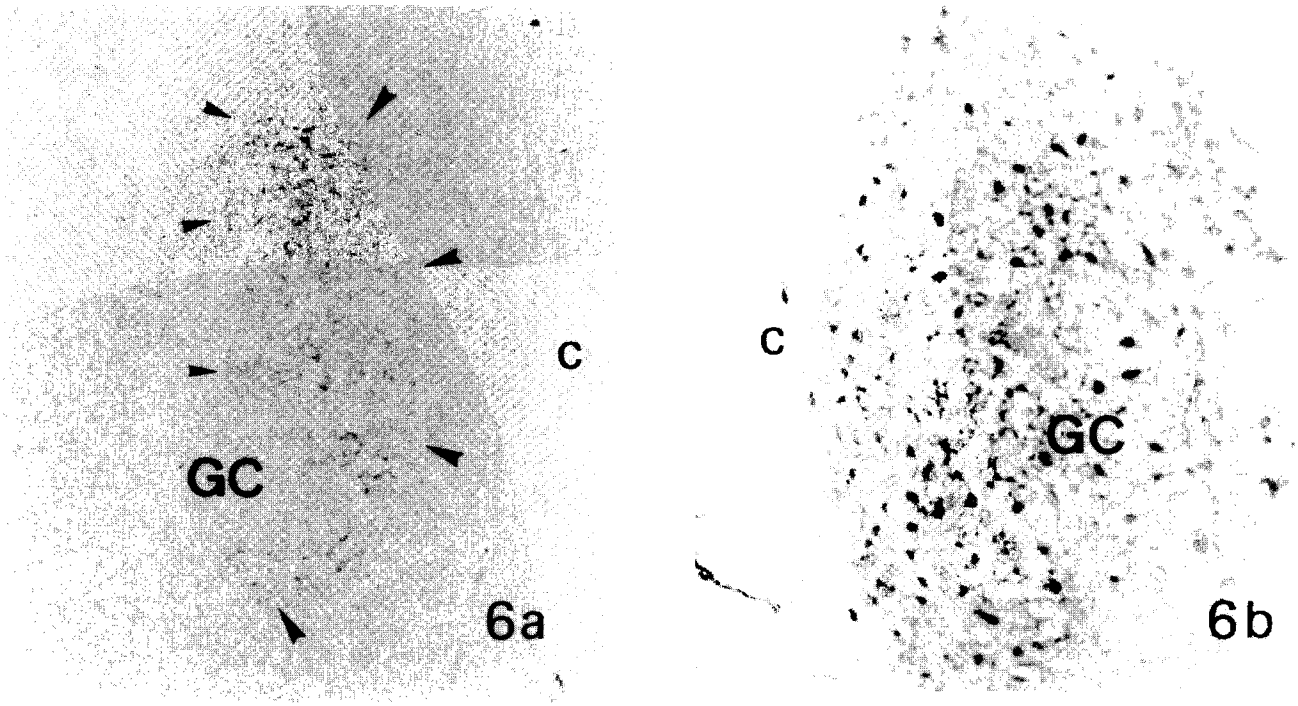

Fig. 6. Two photomicrographs showing mirror sections of a developing secondary follicle at day 5 after stimulation with SRBCs. a is stained for PAP trapping, and b for S-100 protein. a shows immunoreactions (arrowheads) in the upper region of the lymph follicle. b shows that S-100 protein=positive FDCs are distributed in all regions of the follicle. Weak reactions are seen in the subsinus layer. C: capsule; GC: germinal center. Counterstained with methyl green. a; indirect peroxidase method. b; SAB method. x 240

some small secondary follicles and primary follicles were observed. Few GCs were observed on days 3 and 5, but were appreciably evident on other days. The two markers showed similar patterns to those on day 0 and in the stimulated groups, according to the development of lymph follicles.

In the negative control group (not given PAP), the immunostaining for trapping was only faint due to endogenous peroxidase activity. Similarly, in the groups given PAP, omission of peroxidase-conjugated anti-rabbit immunoglobulins had the same effect. Moreover, staining for trapping without trypsin digestion showed little or no reaction. On the other hand, the negative control for S-100 protein also showed little or no reaction: the slight reaction that was evident was due to faint recognition of rabbit PAP by biotinylated anti-rabbit antibody. In contrast, the immunostaining for S-100 protein after proteolytic digestion was slight or absent, but a strong reaction for trapping of rabbit PAP was detected by biotinylated anti-rabbit antibody.

2) Relationship between trapping regions and the distribution of FDCs

In mirror sections, S-100 protein-positive FDCs at all stages were distributed not only in the regions trapping PAP but also in those not trapping PAP in lymph follicles (Figs. 4, 6, 7).

2. Tridimensional analysis of PAP-trapping regions

1) Number of lymph follicles with trapping ability

All of the 1933 lymph follicles which we were able to examine tridimensionally showed positive reactions for trapping

2) Tridimensional and two-dimensional shapes of the trapping regions

The trapping regions showed essentially similar tridimensional shapes in each group. On days 0 and 3 after SRBC stimulation, the trapping regions showed an almost spherical shape, occupying a large space in the lymph follicles. In contrast, on days 10, 21 and 42 after stimulation, they appeared cap-like, possessing a central hollow on the apical side of GCs, and occupying a small space in the follicles. Similarly, on day 5, they showed an incomplete cap-like shape with a small central hollow.

Whereas the tridimensional shapes of the trapping regions showed similar patterns according to the development of each lymph follicle, their two-dimensional shapes and sizes varied. This was

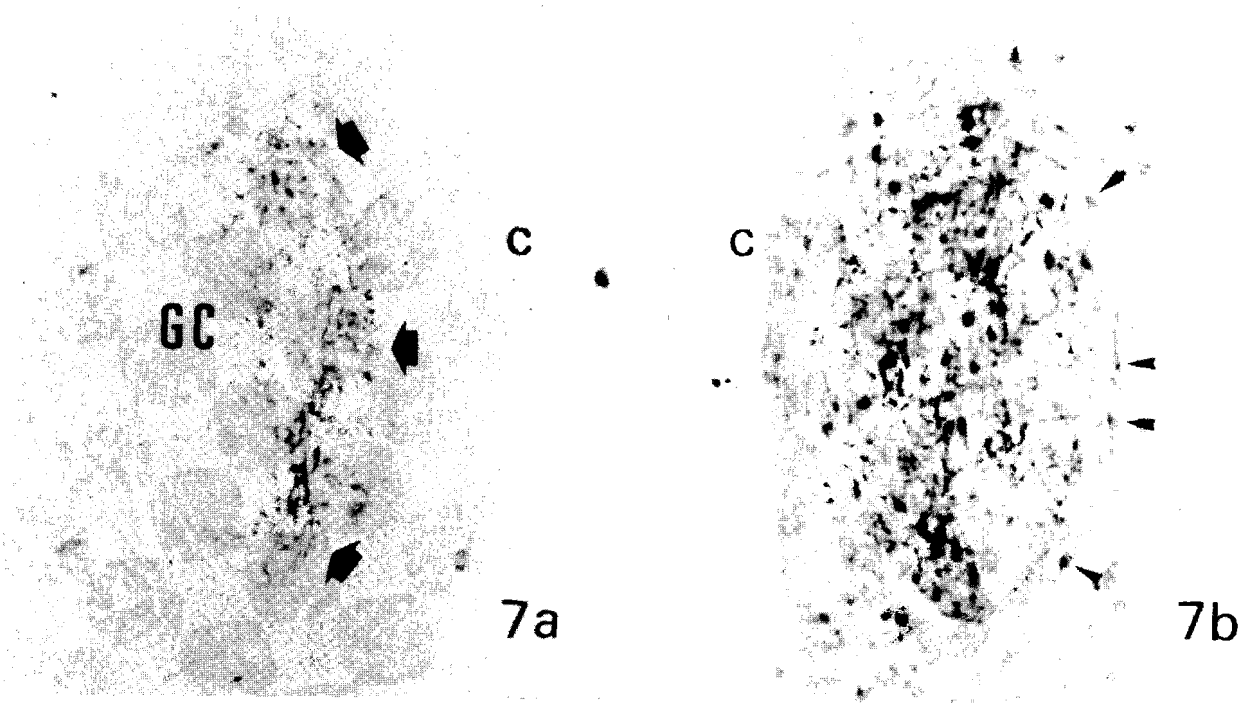

Fig. 7. Two photomicrographs showing mirror sections of a well developed secondary follicle at day 10 after stimulation with SRBCs. 7a is stained for PAP trapping, and 7b for S-100 protein. a shows immunoreactions in the light zone and adjacent corona, in the shape of a crescent (arrows). b shows that S-100 protein-positive FDCs are distributed in all regions of the follicle. Positive reactions are also seen at the border between the dark zone and adjacent corona (arrowheads). Weak reactions are observed in the subsinus layer. C: capsule; GC: germinal center. Counterstained with methyl green. a; indirect peroxidase method. b; SAB method. x 290

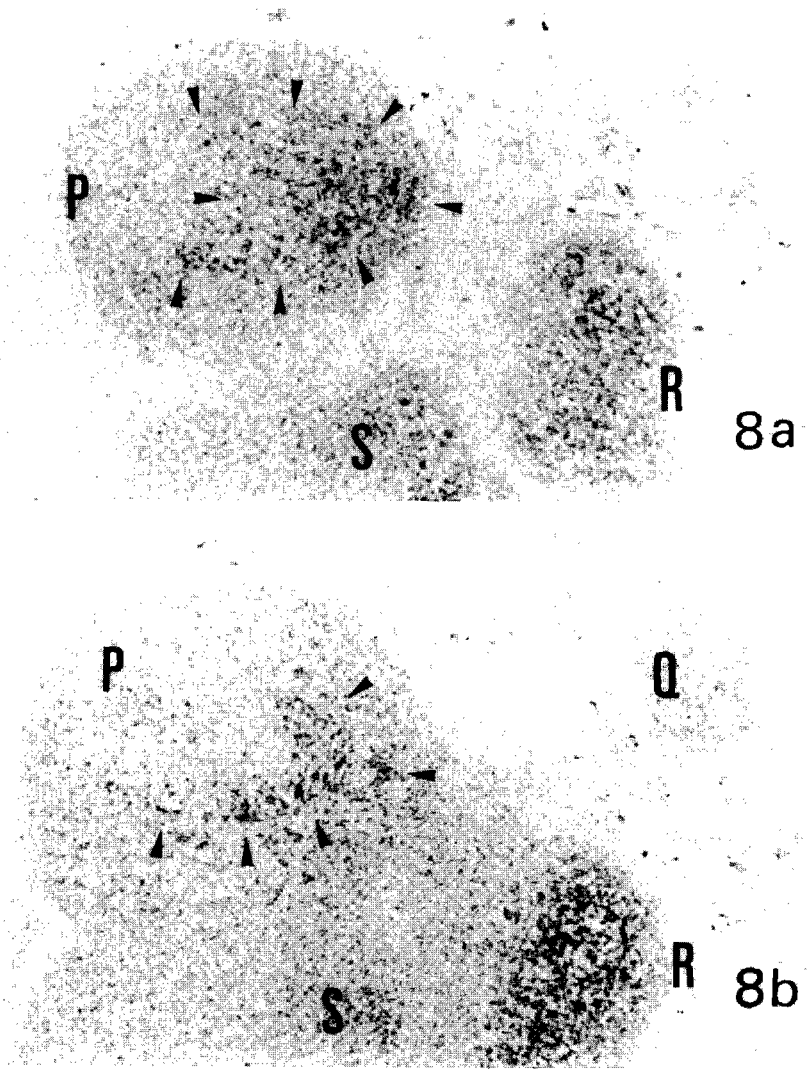

Fig. 8. Three photomicrographs showing immunostaining of PAP trapping in the same lymph node on day 21 after stimulation with SRBCs (Fig. b and c are the ninth and 18th sections from Fig. 8a, respectively). Each of the corresponding letters (P, Q, R and S) indicates the same lymph follicle. All of the regions trapping PAP in these lymph follicles have a crescent shape in secondary follicles on other sections. **a.** R and S appear to be two primary follicles, and their trapping regions show a round shape. P is a secondary follicle, and its trapping region is irregularly shaped (arrowheads). **b.** R is a secondary follicle, but its trapping region is round. The trapping region of P shows an irregular shape (arrowheads) in the secondary follicle, but the shape is different from that in Fig. 8a. Q appears to be a primary follicle without trapping. **c.** The trapping region of R shows a ring shape in the secondary follicle. P appears to be a secondary follicle showing no trapping. Q shows trapping. GC: germinal center. Counterstained with methyl green. Indirect peroxidase method. x 90

Table 1. Numbers of lymph follicles and germinal centers per whole popliteal lymph node of rats, in unstimulated 5-week-old, unstimulated 8-week-old, and stimulated 8-week-old groups.

	LYMPH FOLLICLES		GERMINAL CENTERS	
5-week-old	52.7±8.6	*	1.2±2.2	**
Unstimulated (8-week-old)	78.8±18.2	**	20.8±5.8	**
Stimulated (8-week-old)	122.8±33.5	*	89.5±23.2	**

Mean±SD. Three groups contain six lymph nodes each. *: $p<0.05$; **: $p<0.01$.

attributable to observation of only partial regions encountered incidentally in sections. In particular, well-developed secondary follicles showed diversity in their two-dimensional shapes as follows: 1) A crescent shape in secondary follicles when the light zone, dark zone and corona were encountered in a section (Fig. 7a). 2) A round shape in apparent primary follicles when only the corona with trapping was encountered (Fig. 8a). 3) A round shape in secondary follicles when the light zone and corona were encountered (Fig. 8b). 4) A ring shape at the periphery of GCs when the dark zone and basal side of the light zone were encountered (Fig. 8c). 5) An irregular shape, such as a moth-eaten profile, in secondary follicles when the periphery of the trapping region was encountered (Figs. 8a,b). 6) No trapping in apparent primary follicles when only the periphery of the corona was encountered (Fig. 8b). 7) No trapping in secondary follicles when the dark zone and corona were encountered (Fig. 8c).

3) Numbers of lymph follicles and germinal centers per whole popliteal lymph node

The numbers of lymph follicles and GCs per whole lymph node in the stimulated 8-week-old group (day 21 after stimulation) were significantly larger than those in the unstimulated 8-week-old and 5-week-old groups (Table 1). Moreover, the numbers of lymph follicles and GCs per whole lymph node in the unstimulated 8-week-old group were significantly larger than those in the 5-week-old group.

Discussion

This study produced the following findings: 1) The localization of the regions trapping PAP changed during the GC reaction, although S-100 protein-positive FDCs were distributed in all regions of lymph follicles. 2) All of the lymph follicles examined trapped PAP. 3) The tridimensional shapes of the trapping regions showed similar patterns according to the development of each lymph follicle. 4) The number of lymph follicles per whole lymph node increased after stimulation. We discuss these four findings and some points related to the immunohistochemistry used in this study.

We confirmed the previous finding (Nossal et al., 1968; Imai et al., 1986) that the regions trapping ICs show a round shape in primary follicles (Fig. 2) and a crescent shape in secondary follicles (Fig. 7a). Furthermore, we have extended the previous finding because we have found a change in the localization of the trapping regions during the GC reaction: 1) PAP trapping became weak and then disappeared on the basal side of immature GCs (Figs. 1-3, 4a, 5, 6a, 7a), and 2) S-100 protein-positive FDCs were distributed not only in the regions trapping PAP but also in those not trapping PAP (Figs. 1, 4b, 6b, 7b). These findings are useful when considering the differentiation of FDCs during the GC reaction.

Although FDCs are considered to be derived from mesenchymal cells such as fibroblastic reticulum cells or bone marrow cells (for review, see Heinen and Bosseloir, 1994), their origin is still unsettled. Moreover, it has been proposed that FDCs have two functional subpopulations in the light and the dark zones (Imai et al., 1993; Yoshida et al., 1993). Since their origin remains unclear, it is very difficult to discuss the differentiation of FDCs. However, from the viewpoint of the two FDC subpopulations, two possibilities can be considered: 1) All FDCs in primary follicles differentiate into FDCs in the trapping regions (the light zone and adjacent corona), and some special cells differentiate into FDCs in the dark zone. 2) Some FDCs in primary follicles differentiate into FDCs in the trapping region, whereas others differentiate into FDCs in the dark zone. The findings shown in Fig. 1 seem to support the latter possibility, for the following reasons.

First, in the case of the former possibility, if some cells flow into the dark zone and then become FDCs, it would be expected that 1) a distinct border would be evident between the trapping region and non-trapping region throughout the GC reaction, 2) few S-100 protein-positive FDCs would be present in the non-trapping region in the early phase of GC formation, and 3) an increase in the number of S-100 protein-positive FDCs would be evident in the non-trapping region during GC formation. However, none of these features were not recognized. On the other hand, the gradual decrease in the intensity of trapping on the basal side of GCs and the continuous presence of FDCs in this region (Fig. 1) support the latter possibility.

Second, under similar experimental conditions, we have recently observed that the density of FDCs in the lymph follicle decreases after stimulation, and suggested that FDCs have little or no proliferative activity (Sato and Dobashi, 1995). Thus, it seems unlikely that many precursors of FDCs flow into lymph follicles during the GC reaction, and therefore, we suggest that FDCs in primary follicles may mature and differentiate into FDCs in the light zone and into FDCs in the dark zone.

On this basis we speculate that FDCs differentiate as follows. Some FDCs in primary follicles are compressed toward the apical side by enlargement of the lymph follicles, thereby differentiating into FDCs in the light

zone and adjacent corona; others in primary follicles remain on the basal side of the follicles, thereby differentiating into FDCs in the dark zone and GCBCs (Fossum, 1980) on the most basal side of GCs. However, whether or not FDCs in each region of lymph follicles remain in the same region during immune responses remains unknown. Moreover, although GCBCs express S-100 protein, it is still unclear whether FDCs include GCBCs, as discussed previously (Sato and Dobashi, 1995).

FDCs in the light zone have trapping ability, but those in the dark zone do not. It is known that immune complex trapping requires Fc receptors (Heinen et al., 1985) and/or complement receptors on FDCs (Stein et al., 1982; Imai et al., 1993), and complement components (Zwirner et al., 1989; Van den Berg et al., 1992). The two subpopulations of FDCs have distributions different from each other in terms of receptors, complement components, adhesion molecules, and FDC-specific surface antigens: FDCs in the light zone usually express more of these molecules than those in the dark zone (Stein et al., 1982; Maeda et al., 1992, 1995; Imai et al., 1993). Thus, the restricted localization of trapping in the light zone may reflect the differences in microenvironments between the light and dark zones. Considering the present observations (Fig. 1) and our suggestion above, it is likely that trapping ability is reduced when some FDCs in primary follicles differentiate into FDCs in the dark zone.

On the other hand, we cannot completely exclude the former possibility that all FDCs in primary follicles differentiate into FDCs in the trapping regions. In this case, our finding that PAP trapping became weak and then disappeared on the basal side of developing GCs (Fig. 1) would be explainable if newly accumulated special cells such as FDCs on the basal side showed weak trapping ability in the early phase of GC formation, and then lost their ability due to changes in the microenvironment of secondary follicles. However, our finding of S-100 protein-positive FDCs at least suggests that most newly accumulated FDCs flow into developing GCs at the time of GC formation. Further studies of this aspect are necessary, because almost nothing is known about the differentiation of FDCs.

In this study, all of the lymph follicles examined tridimensionally actually trapped PAP. At least under the present experimental conditions, this finding strongly suggests that all lymph follicles in lymph nodes show trapping.

In this study, the tridimensional shapes of the trapping regions showed similar patterns according to the development of each lymph follicle, whereas their two-dimensional shapes varied (Figs. 7a, 8). These findings indicate that when investigators observe each of the compartments of the lymph follicle in lymph nodes, they should pay attention to the tridimensional shape of the compartment. For example, when conducting an immunohistochemical study to estimate the distribution of a certain molecule in a compartment such as the light zone, interpretation of the data could be erroneous without an understanding of the tridimensional shape; in this respect, the ring- and irregular-shaped staining patterns revealed in this study (Fig. 8) might have been mistaken for unique findings.

Table 1 shows that the numbers of lymph follicles and GCs per whole lymph node increased after stimulation, in accord with previous data for popliteal lymph nodes of mice (Hoshi et al., 1986). These observations imply that stimulation of SRBCs induced some novel lymph follicles. Furthermore, all the lymph follicles examined showed trapping. Hence we conclude that the total number of FDCs showing trapping in whole lymph nodes increases after stimulation. However, this conclusion does not necessarily mean that the number of FDCs in each lymph follicle increases after stimulation, because there is no evidence that increase in the number of FDCs in each lymph follicle is related to the induction of lymph follicles. Moreover, our recent study found no evidence of an increase in the number of FDCs in lymph follicles (Sato and Dobashi, 1995). Thus, whether or not the number of FDCs in each lymph follicle increases remains unknown. On the other hand, the numbers of lymph follicles and GCs per whole lymph node in the unstimulated 8-week-old group were larger than those in the 5-week-old group (Table 1), suggesting that even rats not injected with SRBCs may receive some exogenous stimulation physiologically.

PAP has been widely used as an IC because it can be easily detected. Although many studies on PAP trapping in lymph follicles have been done, the injected PAP, to our knowledge, has never been detected on paraffin sections. In this study we were able to detect the trapping on paraffin sections by selecting an adequate fixation method and appropriate procedures for immunohistochemistry. Our method is more useful for observing a wide range of specimens, and for preparing specimens easily than the previous method using sections sliced with a Vibratome (Imai et al., 1986; Kamperdijk et al., 1987). Moreover, our method has the advantage of better morphological preservation, compared with the previous method using cryostat sections (Dijkstra et al., 1983).

In preliminary examinations, we were able to detect PAP trapping on Vibratome and frozen sections by the DAB/H_2O_2 method, whereas we were unable to do so using paraffin sections. Moreover, the staining pattern of the trapping was the same as that in the present study. Thus, the peroxidase activity of rabbit PAP seems to have been destroyed during the procedure of processing to paraffin sections, although it was detected due to the antigenicity of rabbit immunoglobulins constituting the rabbit PAP.

Whereas the staining for S-100 protein with proteolytic digestion showed a reduced reaction, the staining for trapping without digestion showed little or no reaction. These findings indicate that proteolytic digestion is required in order to stain trapping, but must be avoided in order to stain S-100 protein, at least under

the present experimental conditions. Hence, staining for S-100 protein in rats given PAP can be achieved with little interference. Although staining for S-100 protein without proteolytic digestion occasionally reveals faint staining for trapping of rabbit PAP detected by biotinylated anti-rabbit antibody, this faint staining does not disturb judgment of staining for S-100 protein, since the two markers are expressed at different sites in FDCs: S-100 protein is detected on both the nuclei and cytoplasm (Cocchia et al., 1983), whereas the trapping is detected only on the cytoplasmic membranes (Kamperdijk et al., 1987). Furthermore, the localization of FDCs can be estimated mainly by their nuclear staining.

In summary, this study has revealed the process of differentiation of FDCs during the GC reaction, and highlights the necessity of understanding the tridimensional shape of a compartment, particularly the light zone, in the lymph follicles of lymph nodes.

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