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Development of immune complex trapping: experimental study of lymphoid follicles and germinal centers newly induced by exogenous stimulants in mouse popliteal lymph nodes

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Summary. The development of immune complex trapping in newly-induced lymphoid follicles of draining popliteal lymph nodes was investigated in young adult mice, which had been given bilateral injection of hemocyanin (KLH) or phytohemagglutinin (PHA), each absorbed onto alumina. HRP-anti-HRP immune complex was injected into the footpad 1 day before sacrifice. Using three series of semi-serial cryostat sections prepared from each popliteal node, the number of lymphoid follicles in each node was counted, and follicular localization of the *in vivo* injected and *in vitro* applied immune complexes in each follicle was determined. By day 5, a large germinal center had developed within each preexisting follicle. A large number of 'new' secondary follicles, each containing a small PNA-positive germinal center, appeared outside pre-existing follicles, from day 5 through day 11 in KLH-treated nodes, and from day 7 through day 14 in PHA-treated nodes. Shortly after their appearance, new secondary follicles showed no *in vitro* or *in vivo* trapping, but subsequently, many of the new follicles began to display *in vitro* trapping, at first weakly but later intensely. Occurrence of *in vivo* trapping in new follicles took some time and was first recognized when new follicles showed intense *in vitro* trapping. At day 21 or 25, many of the new follicles showed both *in vitro* and *in vivo* trapping. It was concluded that in lymph nodes treated with a stimulant, secondary follicles containing germinal centers can be formed de novo in the extrafollicular zone where the follicular trapping microenvironment is absent, but subsequently the microenvironment capable of trapping immune complexes develops at the site of formation of new follicles.

Key words: Immune complex trapping, Lymphoid follicle, Germinal center, Lymph node, Mouse

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Introduction

It is well known that if an appropriate stimulant is administered subcutaneously, the draining lymph nodes produce new lymphoid follicles with or without germinal centers outside pre-existing, normally developed lymphoid follicles (Sjövall and Sjövall, 1930; Conway, 1937; Hoshi et al., 1984, 1986). The mechanism underlying the formation of lymphoid follicles is still unclear, but it has been shown that the phenomenon is not antigen-dependent, although antigenic substances liable to be phagocytized are efficient inducers of follicle formation (Hoshi et al., 1986; Horie and Hoshi, 1989; Kaneko et al., 1994). We have therefore postulated that macrophages play a key role in triggering the phenomenon.

Lymphoid follicles comprise special stromal cells, called follicular dendritic cells (FDCs), which have the capacity to trap immune complexes (Nossal et al., 1968; Nossal and Ada, 1971), and which are thought to originate from reticulum cells (Humphrey et al., 1984). Studies on the ontogeny of the spleen and lymph node in rats and mice have shown that the early development of FDCs, of follicular antigen trapping, or both, concurs with the first appearance of primary follicles (Dijkstra et al., 1982, 1984; Imai et al., 1986; Kroese et al., 1987). On the basis of this and other observations, it has been postulated by some authors (Kroese et al., 1987; Nieuwenhuis et al., 1992) that the development of FDCs is independent of the presence of antigen and of follicular B cells, and that, when precursor FDCs develop into mature FDCs, they create a microenvironment at certain locations (termed the 'centrons' by Menzies, 1965) that permits primary follicle formation and formation of germinal centers within these structures.

In the present experiments, we studied the development of the antigen trapping capacity in newly-formed lymphoid follicles in draining popliteal lymph nodes of mice after injection of antigenic material into

the footpad. The follicular antigen trapping was assessed by two methods: 1) 24 h follicular localization of subcutaneously-injected HRP-anti-HRP immune complexes (*in vivo* trapping) (Chen et al., 1978; Dijkstra et al., 1982); and 2) incubation of cryostat sections with HRP-anti-HRP immune complexes (*in vitro* trapping) (van Rooijen, 1978; Dijkstra et al., 1983). Two substances, hemocyanin (KLH) and phytohemagglutinin (PHA), each absorbed onto gelatinous alumina, were chosen as stimulants for two reasons: first, they are much more effective in inducing follicle formation as precipitants than they are as simple solutions; and second, both are excellent inducers of germinal center formation (Hoshi et al., 1984, 1986). This second point is of particular interest, because it may give information as to whether newly-induced lymphoid follicles appear first as primary follicles without germinal centers, or as secondary follicles with germinal centers.

Materials and methods

Animals and injection of stimulants

Female C57Bl/6 mice aged 8 weeks were purchased from Charles River Japan Co. and kept under routine laboratory conditions.

Phytohemagglutinin-p (PHA, Sigma, St. Louis, MO, USA) and keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA, USA) were each absorbed onto gelatinous alumina prepared from 1% potassium alum (Williams and Chase, 1967). Each mouse was injected into both rear footpads with 40 μl of the alum-precipitated stimulant containing 40 μg dry weight of alumina and either 50 μg of PHA or 30 μg of KLH.

The animals were sacrificed at varying intervals from 3 to 49 days after the injection.

Follicular antigen trapping assays and immunohistochemistry

One day before sacrifice, animals treated with the stimulants were injected with 20-30 μl of HRP-anti-HRP immune complex solution (rabbit peroxidase-anti-peroxidase soluble complex, Dakopatts a/s, Glostrup, Denmark) into both rear footpads. The popliteal nodes of both sides obtained from each animal were frozen, using dry ice-acetone mixture and serially cut into 6 μm-thick sections. Three series of semi-serial cryostat sections were prepared, air-dried for 30 min, fixed in pure acetone for 10 min and air-dried again for at least 30 min. Popliteal nodes were also taken from control mice which had been given HRP-anti-HRP immune complex solution 1 day before sacrifice.

24 h localization of *in vivo*-injected HRP-anti-HRP immune complexes (*in vivo* trapping assay) (Chen et al., 1978; Dijkstra et al., 1982): one series of semi-serial cryostat sections prepared as above was stained for peroxidase activity for 10 min with 0.02% 3,3'-diaminobenzidine-tetrahydrochloride (DAB) in phosphate-buffered saline (PBS) containing 0.005% H_2O_2. The sections were then counterstained with methyl green.

Localization of HRP-anti-HRP immune complexes in cryostat sections (*in vitro* trapping assay) (Dijkstra et al., 1983): a second series of semi-serial cryostat sections was incubated for 3 h in a medium containing the HRP-anti-HRP complex (mouse PAP, 1:20 diluted in PBS; Dako Corp., Carpinteria, CA, USA) supplemented with 20% fresh mouse serum as a source of complement. After washing in PBS, peroxidase activity was visualized by incubating in a solution of DAB and H_2O_2 as described above.

Demonstration of B220-positive lymphocytes and peanut agglutinin (PNA)-positive germinal center cells (Rose et al., 1980): a third series of semi-serial cryostat sections was incubated for 1 h with peroxidase-conjugated peanut agglutinin (PNA) (1:150 diluted in PBS; EY Lab., San Mateo, CA, USA). The sections were stained for peroxidase activity with a solution of DAB and H_2O_2 for 10 min. After washing in PBS, sections were incubated for 1 h with the rat monoclonal antibody, anti-B220 (WRY/6,1; Coffman and Weissman, 1981), and washed and incubated for 30 min with alkaline phosphatase-conjugated goat anti-rat Ig (1:50 diluted in PBS; Organon Teknika N.V.-Cappel Products, West Chester, PA, USA). Alkaline phosphatase reaction was developed in a mixture of naphthol AS-MX phosphate and color reagent of fast blue BB salt (Burstone, 1958).

Demonstration of complement receptors: with regard to the popliteal nodes from some mice which were sacrificed from day 3 to 21 after injection of alum-precipitated KLH, four series of semi-serial sections were prepared. Two series were stained for the *in vitro* trapping of immune complexes and for B lymphocytes and germinal center cells as described above. The remaining two series were stained for complement receptors, using 8C12, a rat antibody against mouse C3b receptor (CR1) and 7G6, a rat antibody against both mouse C3b and C3d receptor (CR1/2) (Kinoshita et al., 1988, 1990).

Three-dimensional analysis of stained sections

Each semi-serial node section double-stained for B220-positive lymphocytes and PNA-positive germinal center cells was examined under light microscopy to verify the presence of lymphoid follicles and germinal centers. Each follicle with or without a germinal center was identified with a reference number and was traced three-dimensionally in subsequent sections. This reconstruction technique permitted determination of the total number of lymphoid follicles and germinal centers in each node.

Next, using the semi-serial sections stained for *in vivo* and *in vitro* trapped HRP-anti-HRP immune complexes and for complement receptors, immune complexes and/or complement receptors were identified

in each lymphoid follicle. At each time point, at least three animals were used for examination of the *in vivo* and *in vitro* trapping by follicles, and two mice treated with alum-precipitated KLH, for the *in vitro* trapping and complement receptors.

Histochemical electron microscopy

Mice treated with alum-precipitated PHA on day 0 were given bilateral footpad injections of 20-30 μl of HRP-anti-HRP immune complex solution on day 13. On day 14, after chloral hydrate anesthesia, the animals were perfused with physiological saline followed by perfusion fixation, using a mixture of 0.25% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid, buffered by 0.2M phosphate buffer (pH 7.4). The popliteal nodes were removed and post-fixed by immersion for 2 h at 4 °C in a solution of 4% paraformaldehyde and 0.2% picric acid in 0.2M phosphate buffer, pH 7.4, and then cut on a Vibratom into 40 μm-thick sections, which were incubated with a solution containing DAB and H_2O_2. Sections were washed, immersed in a 2% glutaraldehyde solution and after washing, postfixed in 1% OsO_4 in 0.2M phosphate buffer for 1 h at 4 °C. The postfixed sections were dehydrated with ethanol and embedded in an Araldite-Epon mixture. Ultrathin sections either stained with uranyl acetate or left unstained, were examined with a JEOL 100CX electron microscope.

Results

Normal popliteal nodes

Unstimulated normal animals were injected with rabbit HRP-anti-HRP immune complex into the rear footpad on the left side 1 day before sacrifice.

The normal popliteal node usually contained approximately 13 lymphoid follicles consisting largely of accumulations of B220-positive lymphocytes. Germinal centers consisting of PNA-positive cells were occasionally found in larger follicles. Most lymphoid follicles were located in the portion of peripheral cortex overlying the deep cortex, and only one or two smaller

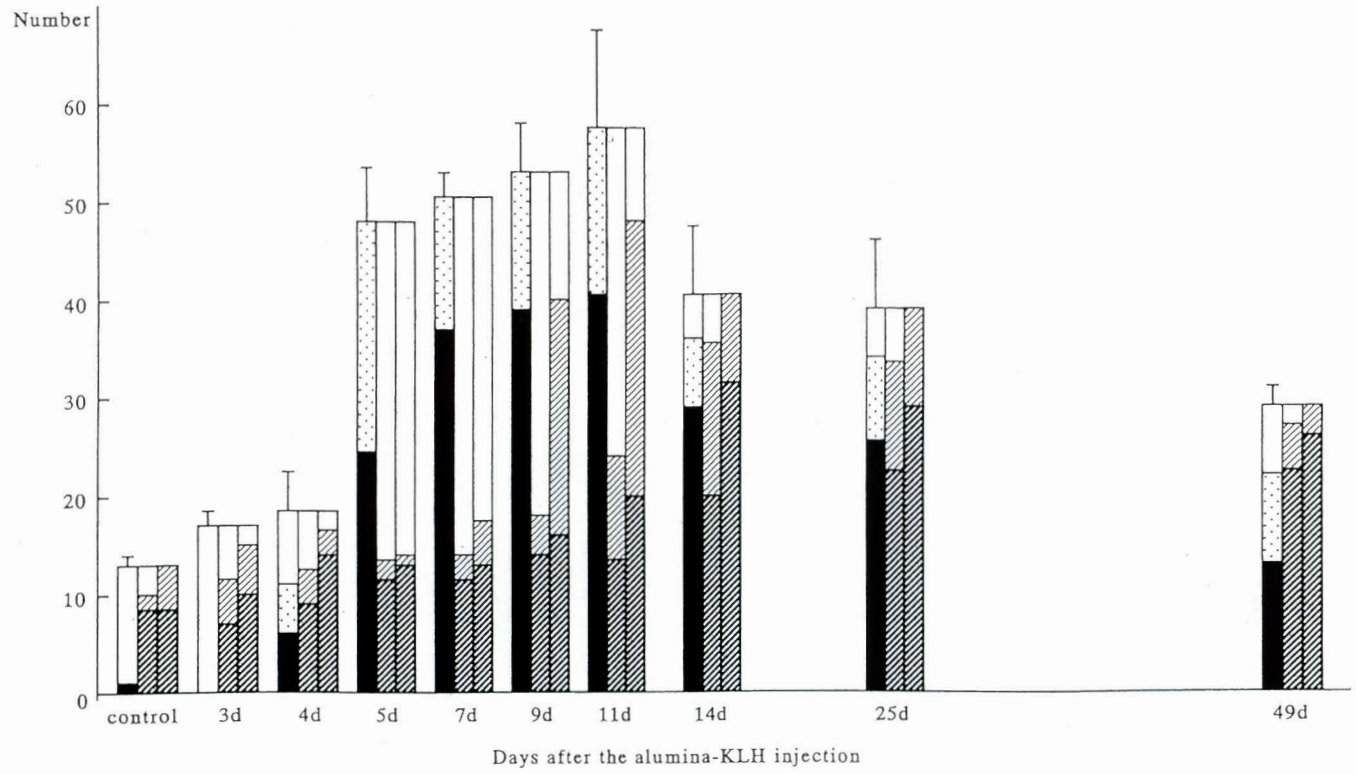


Fig. 1. Changes in the number of lymphoid follicles and germinal centers in each whole popliteal node and in the number of follicles showing localization of *in vivo*- and *in vitro*-applied HRP-anti-HRP immune complexes at various intervals after the alumina-KLH injection. The three columns at each time point represent the number of follicles with or without germinal centers per node (left column), the number of follicles showing localization of the *in vivo*-applied immune complex (middle column) and localization of the *in vitro*-applied immune complex (right column). At each time point, 3-5 lymph nodes were examined. The height of the left column indicates the mean number of follicles with or without a germinal center in each node, and the vertical bar, SD: the open part of the column indicates the number of primary follicles; the closed part, the number of secondary follicles containing germinal centers larger than 0.1 mm; the dotted part, the number of secondary follicles containing germinal centers smaller than 0.1 mm. In the middle and right columns, the hatched bars indicate the number of follicles showing intense localization of immune complexes; the shadowed bars, the number of follicles showing weak localization of the complexes, and the open bars, the number of follicles showing no localization.

follicles were present in the portion of the peripheral cortex extending beyond the deep cortex towards the hilus. Indeed, the latter portion of peripheral cortex together with the underlying medullary cords was underdeveloped with sparse lymphoid elements.

In the *in vivo* trapping assay, most follicles displayed a moderate degree of localization of the subcutaneously injected HRP-anti-HRP immune complexes. The complexes were seen in a dendritic pattern in the central portion of the primary follicle or with a beaded appearance with a crescentic pattern of distribution around the light region of the germinal center in the secondary follicle. More intense localization of HRP-anti-HRP immune complexes in the lymphoid follicles was observed when cryostat sections of the popliteal node on the right side were incubated with isologous HRP-anti-HRP immune complexes supplemented with fresh mouse serum (*in vitro* assay).

The *in vitro*-applied immune complexes were scanty in any lymphoid follicles when cryostat sections were incubated with isologous HRP-anti-HRP immune complexes without adding fresh mouse serum.

Popliteal lymph nodes after administration of alum-precipitated KLH

Mice injected into the footpad with alum-precipitated KLH on day 0 were sacrificed at intervals after the injection. The HRP-anti-HRP immune complex was injected into the footpad 1 day before sacrifice. Changes in the number of lymphoid follicles and germinal centers per node and of follicles displaying localization of the *in vivo*- or *in vitro*-applied HRP-anti-HRP immune complexes are summarized in Figure 1.

After KLH , the popliteal nodes hypertrophied, their weight and size reaching a peak at day 11. On day 3 after the injection, large numbers of B220-positive lymphocytes were seen around the high-endothelial venules in the deep cortex and medullary cords and in the extrafollicular zone, particularly in the portion of the peripheral cortex extending beyond the deep cortex. Lymphoid follicles were expanded with B220-positive lymphocytes. On day 4, B220-positive lymphocytes had been further accumulated in the extrafollicular zone and in places, formed nodular aggregations representing 'new' lymphoid follicles. At day 3, no germinal centers were visible in any follicles (Fig. 2), but at day 4, a PNA-positive germinal center had developed within expanded follicles, while new follicles displayed no germinal center development. The latter follicles were recongnizable as such by their smaller size and their failure to localize the *in vivo*- and *in vitro*-applied immune complexes.

On day 5, each expanded follicle then contained a prominent germinal center. In addition, a large number of small PNA-positive germinal centers appeared in the extrafollicular zone where B220-positive lymphocytes were distributed to form a layer of cells of variable thickness (Fig. 3). Newly-induced germinal centers had irregular outlines and were mostly less than 0.1 mm in any dimension, but by day 7, many newly-induced germinal centers had become rounded and were more than 0.1 mm in size. At days 5 and 7, these smaller germinal centers were some distance apart, and located either very close to, or deep to, the subcapsular sinus and surrounded by a mantle of B220-positive lymphocytes. Similar smaller germinal centers were sometimes seen in the periphery of the cap region of the expanded follicles.

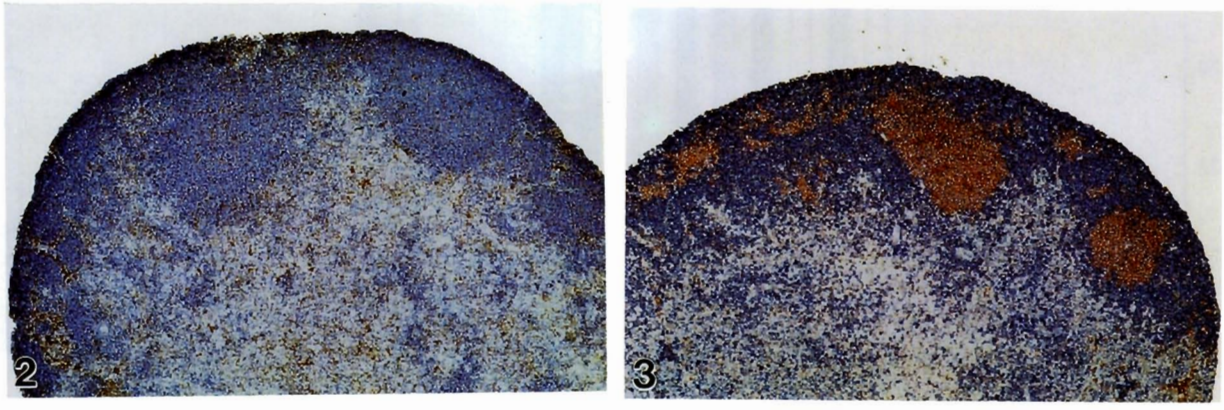


Fig. 2. Double immunostaining for B220-positive lymphocytes (blue) and PNA-positive germinal cells (brown) in a popliteal node at 3 days after alumina-KLH injection. Pre-existing follicles are expanded as a result of increased accumulation of B220-positive cells. The extrafollicular zone of the peripheral cortex is populated with numerous B220-positive lymphocytes. At this stage, no germinal centers consisting of PNA-positive cells are visible within the expanded follicles or in the extrafollicular zone. x 45

Fig. 3. Double immunostaining section of a popliteal node 5 days after injection of alumina-KLH, showing a large germinal center consisting of PNA-positive cells within the expanded follicles. Note that in the peripheral cortex outside the expanded follicles, newly developing secondary follicles can be seen, each containing a small PNA-positive germinal center surrounded by an ill-defined mantle of B220-positive lymphocytes. Examination of neighboring cryostat sections revealed that at this stage, the expanded follicles display definite localization of the *in vivo*- and *in vitro*-applied immune complexes, while the newly developing follicles show no localization of immune complexes. x 45

These small germinal centers together with surrounding B220-positive lymphocytes represented 'new' secondary follicles at an early stage of their development. New secondary follicles were at first in many cases ill-outlined, the interfollicular zone being populated with B220-positive cells, but with time, a truly follicular appearance of new secondary follicles developed. These could be recognized by three features: 1) the germinal center in many follicles increased in size and moved from the center towards the medullary pole of the follicles; 2) the high-endothelial venule appeared in the interfollicular zone between two apposing new follicles, and 3) B220-positive cells gradually disappeared from the interfollicular zone (Fig. 4). From day 7 to day 11, new secondary follicles continued to appear. The total number of follicles per node reached a peak on day 11; mean number of follicles per node was 58, 40 of which contained germinal centers bigger than 0.1 mm. Between days 5 and 7, the number of new secondary follicles containing germinal centers larger than 0.1 mm increased, but the number of these 'larger' germinal centers did not change significantly from day 7 through 11. This observation indicates that germinal centers which were small at day 7 had ceased growth by that stage. From day 11 to 14, some of the new secondary follicles had disappeared, so that the total number of follicles per node decreased to about 40, but this number was maintained up to day 25 (Fig. 6).

From day 3 to day 5, the follicles that showed *in vivo* and *in vitro* trapping were limited to those expanded follicles present in the node at the time of the KLH-injection. Indeed, at days 5 and 7, the *in vitro*- and *in vivo*-applied immune complexes were only sparsely localized in newly-induced secondary follicles. By days 9-11, many of the new secondary follicles started showing weak to intense localization of the *in vitro*-applied immune complexes (Fig. 5). On days 14 and 25, all follicles examined showed weak to intense *in vitro* localization (Figs. 7, 8). When weakly localized in the secondary follicles, the *in vitro*-applied immune complexes were seen in a dendritic pattern predominantly in the light region of the germinal center, but if intensely localized, the complexes were distributed throughout the germinal center and part of the cap region in the secondary follicle, with a higher concentration in the light region of the germinal center.

At any stage examined, those newly-formed follicles, as well as preexisting expanded follicles which displayed the *in vitro* localization, were found to display at the same time complement receptors, as was revealed by 8C12 (CR1) and 7G6 (CR1/2), with an intensity and distribution pattern quite similar to the localized immune complexes (not shown).

In comparison with *in vitro* trapping, development of the capacity to trap the *in vivo*-injected immune complexes in new secondary follicles proceded at a slower pace. *In vivo* localization was first recognized on day 9 in some of the new secondary follicles. On day 11, the number of new secondary follicles showing *in vivo* trapping increased only slightly, but on days 14 and 21 the majority of existing follicles showed *in vivo*-applied immune complexes. The density of localized immune complexes varied according to the follicles; in general, follicles containing larger germinal centers showed definite localization of the complexes, while follicles with smaller germinal centers showed weak localization (Fig. 9).

From day 25 onwards, germinal center activity declined, and the number of lymphoid follicles per node decreased. On day 49, the numbers of follicles and large germinal centers per node had fallen to 29 and 13, respectively. The persisting follicles displayed weak to

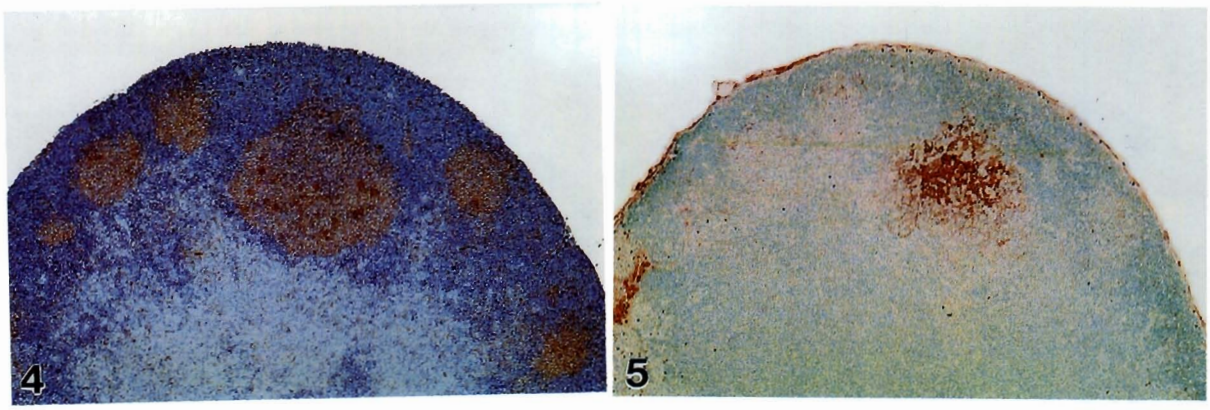


Fig. 4. Double immunostaining section of a popliteal node 9 days after injection of alumina-KLH, showing one large secondary follicle representing a pre-existing follicle, and five 'newly' formed follicles, each containing a relatively small germinal center. x 45

Fig. 5. The section adjacent to the one shown in Fig. 4, showing follicular localization of the *in vitro*-applied HRP-anti-HRP immune complex. The large follicle displays intense localization, while other 'newly' formed follicles show very weak localization. Examination of neighboring sections indicates that the large follicle also displays definite localization of the *in vivo*-applied immune complex, while the other follicles containing smaller germinal centers show no *in vivo* localization. x 45

intense localization of both the *in vitro*- and *in vivo*-applied immune complexes.

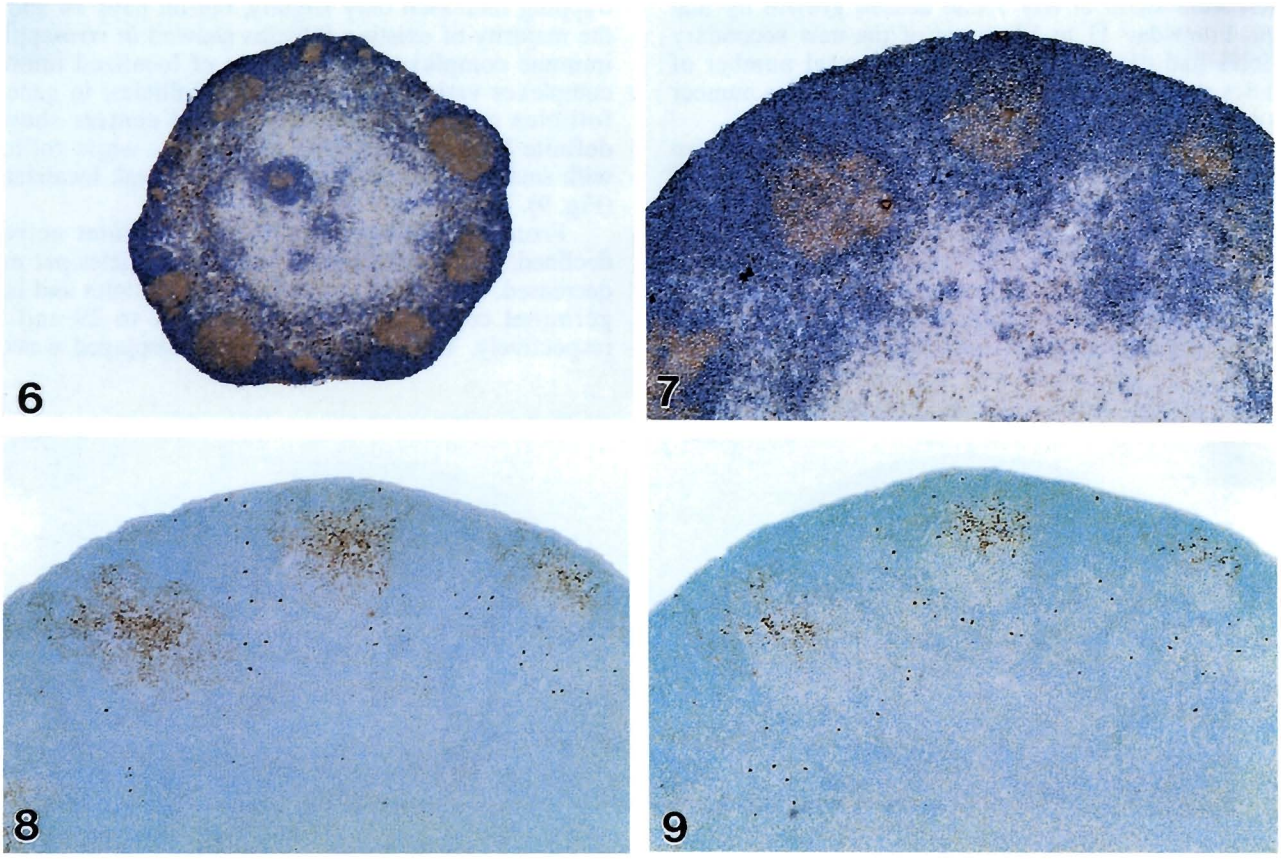
Popliteal lymph nodes after administration of alum-precipitated PHA

Mice were injected with alum-precipitated PHA into the rear footpad on both sides and sacrificed at intervals after the injection. At 1 day prior to sacrifice, animals were given rabbit HRP-anti-HRP immune complex into the rear footpad on both sides. Major findings of this series of experiments are summarized in Figure 10.

After footpad injection of alum-precipitated PHA, the draining popliteal nodes increased in size and weight with a peak weight at 14 days. On days 3 and 4 after injection, the treated popliteal nodes showed little change in morphology, and no germinal centers were recognized in any lymphoid follicles. At day 5, B220-positive lymphocytes were frequently found around the high-endothelial venules and tended to accumulate in the extrafollicular zone of the peripheral cortex. The lymphoid follicles had expanded, and a large PNA-positive germinal center had developed in the central to basal part of each expanded follicle. These expanded follicles showed localization of both the *in vivo*- and *in vitro*-applied immune complexes, and were regarded as the pre-existing follicles.

At day 7, a number of new lymphoid follicles, each containing a small PNA-positive germinal center, appeared in the peripheral cortex outside the expanded follicles which now contained a prominent germinal center. In the following days, new secondary follicles containing small germinal centers continued to be formed while the germinal centers in some new follicles developed so that secondary follicles containing larger germinal centers increased in number. At day14, the total number of follicles per node reached a peak, and this number was maintained at day 21.

On day 7, newly-induced secondary follicles localized neither the *in vitro*-applied immune complexes nor the *in vivo*-applied ones, even though the expanded follicles containing a prominent germinal center showed both *in vitro* and *in vivo* trapping. On day 9, the *in vitro*-applied immune complexes were localized in many of



Figs. 6-9. Semi-serial sections of a popliteal node 14 days after injection of alumina-KLH. **Fig. 6.** Double staining for B220 and PNA, showing the presence of a number of secondary follicles with various sizes in the peripheral cortex at a low magnification. x 25. **Fig. 7.** Double staining for B220 and PNA. Three follicles with PNA-positive germinal centers can be seen in the peripheral cortex. x 55. Localization of HRP-anti-HRP complexes in these follicles is seen in **Fig. 8** (*in vitro* trapping assay) and in **Fig. 9** (*in vivo* trapping assay). Note that follicular localization of the *in vitro*-applied immune complex is more intense and extensive than that of the *in vivo*-applied complexes. x 55

the new secondary follicles, and the number of follicles showing *in vitro* localization increased in parallel with the increase in the total number of follicles. There was a tendency for secondary follicles containing germinal centers smaller than 0.1 mm to display weak *in vitro* lozalization, while follicles with larger germinal centers showed intense *in vitro* localization.

From day 3 to 9, localization of the *in vivo*-applied immune complexes was seen only in pre-existing expanded follicles. From day 11 on, follicles localizing the *in vivo*-applied immune complexes increased in number, but this increase took place at a slower rate. In general, *in vivo* localization occurred in those secondary follicles which contained larger germinal centers and which displayed intense *in vitro* localization. At day 21, the number of follicles showing *in vivo* trapping reached a peak (approximately 30), but *in vivo*-applied immune complexes were not seen in the remaining 12 follicles.

From day 21 on, the number of follicles per node decreased, and the germinal center activity declined. Between days 21 and 35, several follicles had disappeared and a number of germinal centers had disappeared from the persisting follicles. Since the number of follicles displaying *in vivo* trapping at day 35 was comparable to that at day 21, the follicles that had vanished during this period may be those which contained smaller germinal centers and which showed no trapping or only weak *in vitro* trapping at day 21.

Electron microscopy

Twenty-four hour localization of the *in vivo*-injected immune complexes in newly-induced lymphoid follicles was examined by electron microscopy in the popliteal nodes obtained from mice given alumina-PHA on day 0, followed by footpad injection of rabbit HRP-anti-HRP immune complex on day 13 and sacrificed on day 14.

HRP-anti-HRP complexes can be recognized at the ultrastructural level as extracellularly-located electron-dense material (Chen et al., 1978) in secondary follicles of various sizes in the treated node. Large follicles generally located in the peripheral cortex overlying the deep cortex are regarded as representing pre-existing follicles. Those smaller secondary follicles located in the portion of the peripheral cortex extending beyond the deep cortex are regarded as representing newly-induced follicles, since in untreated nodes, few lymphoid follicles are present in the corresponding portion of the peripheral cortex, and when new lymphoid follicles are induced in the popliteal node by exogenous stimuli, they

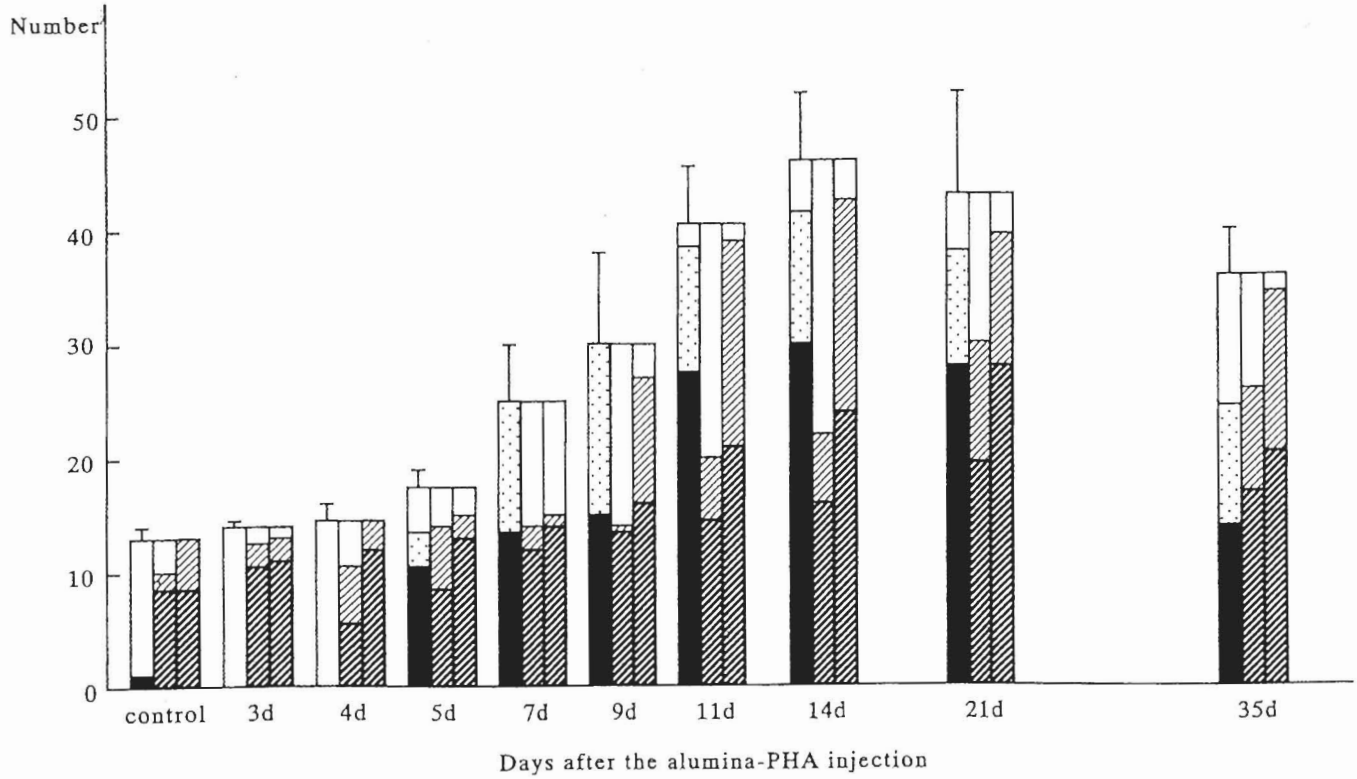


Fig. 10. Changes in the number of lymphoid follicles and germinal centers in each whole popliteal lymph node and in the number of follicles showing localization of in vivo- and *in vitro*-applied HRP-anti-HRP complex at vaious intervals after injection of alumina-PHA. The three columns at each point represent the number of follicles with or without germinal centers in each node (left column), the number of follicles showing localization of *in vivo*-applied immune complexes (middle column) and localization of *in vitro*-applied immune complexes (right column). At each time point, 3-5 lymph nodes were examined. For explanation of the columns, see the legend to Fig.1.

occur preferentially in that portion of the peripheral cortex (Ahn et al., 1990).

Within large secondary follicles, well-developed follicular dendritic cells (FDCs) with slender cytoplasmic processes forming a web-like structure were seen in the light region of the germinal center and the inner part of the cap region. The surface of FDCs consists of an intricate system of plasma membrane invaginations embedded in the homogeneous, electron-dense HRP-anti-HRP complexes. In smaller secondary follicles, the injected immune complexes were seen extracellularly in the light region of the germinal center and the inner margin of the cap region, in association with typical FDCs and their slender cytoplasmic projections. The nuclei of these cells had an irregularly indented outline, a euchromatic structure with dispersed heterochromatin, and contained one or more prominent nucleoli. In places their slender cytoplasmic extensions formed a web-like structure, although this structure was less intricate than the corresponding FDCs seen in large secondary follicles (Fig. 11).

Discussion

The present report describes the development of the capacity to trap immune complexes in relation to the formation of lymphoid follicles newly induced in lymph nodes after subcutaneous injection of antigenic stimulants. Our observations showed that under the experimental conditions employed, 'new' lymphoid follicles first appeared mostly as secondary follicles containing a small PNA-positive germinal center in the extrafollicular zone of pre-existing follicles, and that the capacity to trap HRP-anti-HRP immune complexes began to develop a few days later.

After subcutaneous injection of the stimulants, the draining popliteal nodes produced a large number of new secondary follicles in the peripheral cortex outside pre-existing follicles. However, there was a difference in the manner of appearance of new follicles between nodes treated with alumina-KLH and those treated with alumina-PHA: in the former, new follicles, containing a germinal center, appeared in the peripheral cortex largely between days 5 and 9, whereas in the latter they

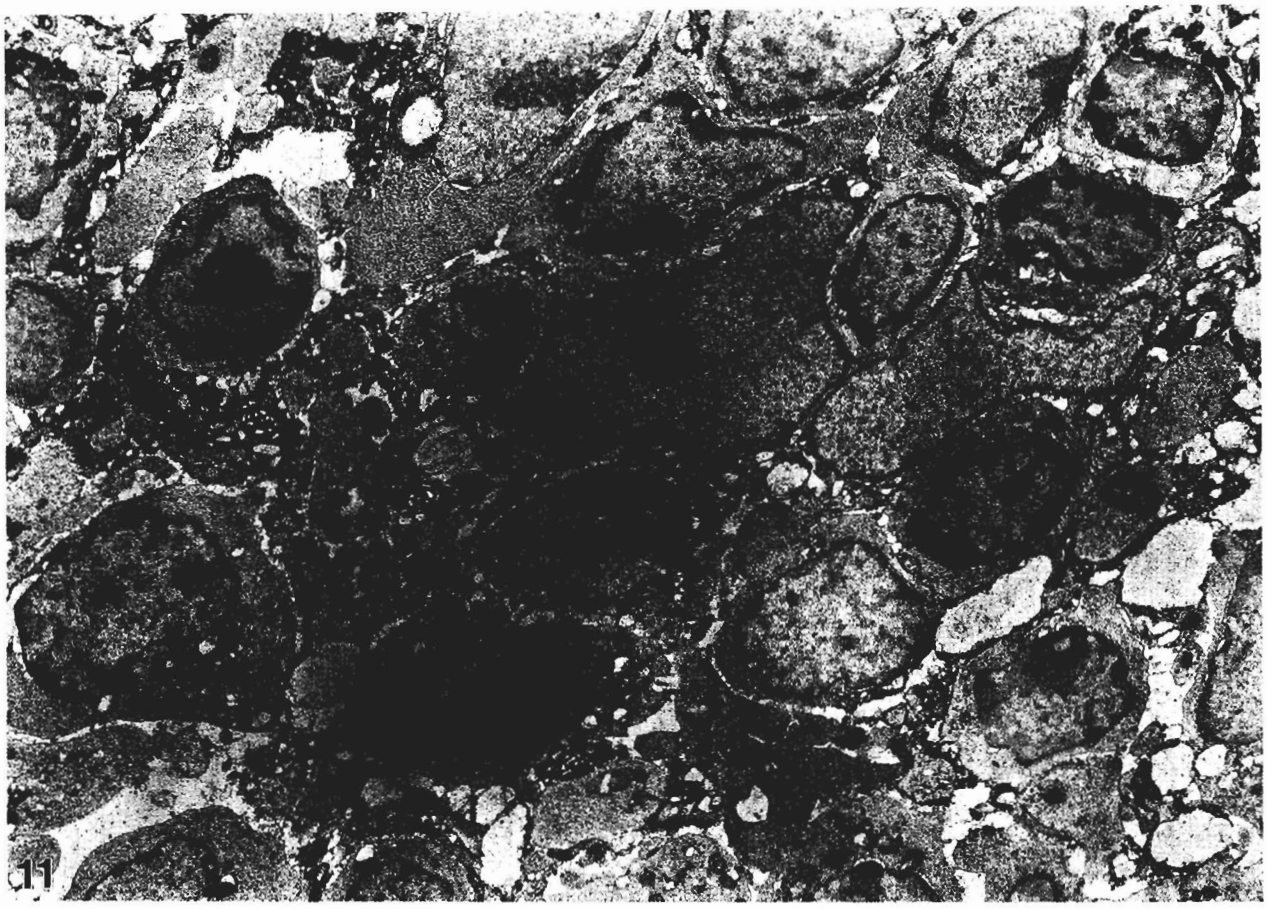


Fig. 11. Electron micrograph of FDC in a small 'newly' formed secondary follicle located in the portion of peripheral cortex extending beyond the deep cortex in a popliteal node obtained from a mouse which was given alum-PHA on day 0, injected with HRP-anti-HRP immune complex on day 13 and sacrificed on day 14. FDC is well differentiated, and the cell surface shows an intricate system of invaginations embedded in HRP-anti-HRP complexes. x 4,500

appeared gradually from day 7 through day 14. This indicates that the time schedule of reactive formation of new lymphoid follicles varies according to the nature of the stimulant concerned.

After injection of the stimulants, new secondary follicles were formed in the extrafollicular zone of pre-existing follicles, where large numbers of B220-positive B lymphocytes were already present. Under certain experimental conditions, new lymphoid follicles can occur in the form of primary follicles without germinal centers (Hoshi et al., 1986; Horie and Hoshi, 1989), and in such cases, we have also observed an increase in the B lymphocyte population in the extrafollicular zone prior to the first appearance of new lymphoid follicles. Thus, an increased population of B lymphocytes in the extrafollicular zone, probably as a result of increased influx of circulating B lymphocytes via high-endothelial venules into the node, appears to be a prerequisite for the reactive formation of new follicles. In cases where new follicles occur in the form of secondary follicles, as in the present experiments, small newly-induced germinal centers appear at several sites in the extrafollicular zone among the large numbers of B lymphocytes either as soon as, or shortly before, these B lymphocytes accumulate in the new follicles. It is possible that small germinal centers occurring among B lymphocytes may act as formation centers for new lymphoid follicles; thus each germinal center may locally accumulate B lymphocytes.

Early new follicles, mostly containing a germinal center, occurred in close proximity to, or some distance away from pre-existing follicles. Irrespective of where they appeared, early new follicles showed neither *in vitro* nor *in vivo* trapping. This finding is of particular interest, because it indicates that new germinal centers and new follicles are formed in the peripheral cortex, unrelated to the pre-existing follicle stromata comprising the FDC. Subsequently, however, new follicles showed localization of *in vitro*-applied immune complexes, at first weakly but later intensely. *In vitro* trapping of immune complexes has been shown to be mediated by complement receptors expressed on the cell surface of FDCs (Yoshida et al., 1993; Imai et al., 1998). Development of *in vitro* trapping in new follicles, therefore, represents the development of functional FDCs expressing complement receptors. To support this, the present study showed that early new follicles in which the *in vitro*-applied immune complexes were localized also displayed complement receptors (CR1 and CR2) with a distribution pattern similar to that of the localized immune complexes. Several lines of evidence imply that FDCs originate from the stromal reticulum cells in the lymphoid organ (Humphrey et al., 1984; Gray et al., 1991; Imazeki et al., 1992). Recently, however, it has been suggested that they are derived from hematopoietic cells (Kapasi et al., 1994). Whatever the origin of FDCs, we believe that when new secondary follicles are reactively produced, the germinal centers developing in these follicles stimulate the development of FDCs at the site.

Development of *in vivo* trapping took some time, and it occurred in new follicles at about the time when they showed intense *in vitro* trapping. This observation indicates that FDCs developing in early new follicles are needed to achieve further differentiation before they have the capacity to trap *in vivo*-applied immune complexes.

De novo formation of germinal centers in existing primary and secondary follicles in association with immune complex trapping by FDCs in these follicles has been well documented (Nossal et al., 1964; van Rooijen, 1974; Klaus et al., 1980; Kosco et al., 1988; Szakal et al., 1990). In contrast, however, recent studies have shown that virgin B lymphocytes recruited to the lymphoid organ develop in the T cell region into B blasts, which then move to lymphoid follicles and differentiate into centroblasts forming the germinal center (Gray, 1988; MacLennan et al., 1990; Liu et al., 1991; Jacob and Kelsoe, 1992). Taking this recent interpretation into consideration, the present observation can be explained as follows: following subcutaneous injection of the effective stimulant, virgin B lymphocytes were mobilized into the nodes from the circulation and differentiate into B blasts in the extrafollicular zone; some B blasts migrated into pre-existing follicles to form their germinal centers, while those remaining in the extrafollicular zone developed into small germinal centers, and then into new follicles containing germinal centers, in this zone. Since the reactive formation of lymphoid follicles containing germinal centers in the lymph nodes has been observed in animals treated with antigenic and mitogenic substances liable to be phagocytosed, we assume that macrophages taking up the effective stimulant may play a key role in inducing the germinal center development in the extrafollicular zone of preexisting follicles.

In the course of ontogeny of the lymph nodes and spleen in rats and mice, FDCs, detectable by a mAb specific to FDC (van Rees et al., 1985) and/or by a functional marker of FDC (*in vivo* trapping of the immune complex, Dijkstra et al., 1982, 1984; Imai et al., 1986), are found at a time when primary follicles are also recognized. Furthermore, deliberate immunization does not lead to accelerated development of FDCs or lymphoid follicles, even though some early follicles appear in the form of secondary follicles containing a germinal center (Kroese et al., 1987). These observations have led some authors to suggest that the development of the follicular microenvironment is independent of the presence of antigen and of follicular B cells; when precursor FDCs develop into mature FDCs, they create at certain locations a microenvironment allowing primary follicle formation. In contrast to these ontogenic observations, the present study showed that when new lymphoid follicles are reactively induced by exogenous stimuli, the follicular microenvironments capable of trapping the immune complexes develop some time after the formation of new lymphoid follicles. Lymphoid

follicles may therefore be formed through two different mechanisms: lymphoid follicle formation preceded by or following the development of the follicular microenvironment. In this connection, observations on B-cell-depleted mice and SCID mice have shown that B cells in the former, and B and T cells in the latter, are necessary for the appearance of FDCs in follicles (Cerney et al., 1988; Kapasi et al., 1993). Furthermore, our recent observations on the ontogeny of Peyer's patches in the rat have suggested that the first occurrence of lymphoid follicles in the form of accumulations of sIgM-bearing B lymphocytes precedes by several days the first appearance of OX2-positive FDCs at the same sites (Chen et al., 1995). The relationship between lymphoid follicle formation and the development of the follicular microenvironment warrants further elucidation.

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