

Ontogeny of the antigen-reactive lymph follicle-forming capacity of the popliteal lymph node in neonatal mice

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Summary. The ontogenetic development of the reactive lymph follicle-forming capacity of the popliteal lymph node was investigated immunohistochemically in young mice which had received a single injection of hemocyanin (KLH) in a rear footpad at a predetermined age (between 1 and 21 days). The mice were sacrificed at various intervals after injection. In non-stimulated young mice, primary lymph follicles first appeared in the popliteal node at 11 days of age. When KLH was given to 7-day-old or older mice, each draining popliteal node showed a marked increase in B lymphocytes in the extrafollicular zone 3 days after injection and produced a number of 'new' lymph follicles outside the pre-existing follicles over the next few days. In mice injected at 2-4 days of age, these nodes showed an increase in B lymphocytes in the outer cortex and had produced several lymph follicles by 8 days of age. The number of lymph follicles produced by each node tended to increase in line with age at injection. These results indicate that neonatal popliteal nodes become able to produce lymph follicles in response to exogenous antigens some time before ontogenetically developing follicles appear. The formation of new lymph follicles observed in draining popliteal nodes after KLH injection at an early postnatal age is discussed in relation to the ontogenetic development of stromal cells (precursors of follicular dendritic cells) that are capable of interacting with B lymphocytes and the extent of B lymphocyte influx into the node induced by KLH stimulation.

Key words: Lymph follicles, Germinal centers, B lymphocytes, Follicular dendritic cells

Introduction

Lymph nodes contain a number of lymph follicles in their outer cortices. These nodular accumulations consist mainly of B lymphocytes but also contain some T lymphocytes, follicular dendritic cells (FDCs) and

macrophages (Nossal et al., 1968; Gutman and Weissman, 1972; Nieuwenhuis and Keuning, 1974; Imai et al., 1998; Fu and Chaplin, 1999). Primary lymph follicles develop in the peripheral lymphoid organs during ontogenesis, and this event is thought to be antigen-independent, whereas the development of germinal centers within lymph follicles is an antigen-dependent process. Recent studies showed that development of primary follicles and generation of mature FDCs in the lymph nodes and spleen require tumor necrosis factor (TNF) and lymphotoxin (LT) that are expressed by lymphocytes or other cell types (Pasparakis et al., 1997; Endres et al., 1999; Fu and Chaplin, 1999; Ngo et al., 1999; Tumanov et al., 2002) and CXCL13 (B-lymphocyte chemoattractant, BLC) produced by stromal cells that express receptors for TNF and LT (Ansel et al., 2000).

On the other hand, it is well known that if an appropriate stimulant is administered subcutaneously, draining lymph nodes reactively produce 'new' lymph follicles with or without germinal centers outside the pre-existing follicles, resulting in a significant increase in the number of follicles per node (Sjövall and Sjövall, 1930; Conway, 1937; Hoshi et al., 1984, 1986; Horie et al., 1999). However, it has been reported that deliberate antigenic stimulation did not alter the timing of the first appearance of FDC-containing lymph follicles in the lymph nodes and spleens of neonatal rats (Veerman, 1975; van Rees et al., 1986; Kroese et al., 1987a), even though germinal centers were present within some of the lymph follicles that appeared in the spleen.

We have previously studied age-dependent changes in the numbers of lymph follicles in various somatic lymph nodes in growing mice (Hoshi et al., 2001). Our observations showed that primary follicles first appear in these nodes at 6-12 days of age, then increase with increasing age. The number of follicles present in each node at 21-28 days of age varied widely among the different types of somatic lymph nodes, and this variation was found to reflect differences in the sizes of the body areas drained by particular lymph nodes at this stage of development. This finding indicated that the formation of lymph follicles in a somatic node is regulated by the amount of lymph transported from the

drainage territory into that node via the afferent lymphatics. Based on these observations, we concluded that lymph follicles that develop ontogenetically within somatic lymph nodes are reactive in origin. If this hypothesis is correct, it is possible that these nodes may develop their reactive lymph follicle-forming mechanism some time before the first appearance of primary follicles; this mechanism would be capable of responding to deliberate antigen stimulation and, once triggered, would lead to the formation of lymph follicles. The main goal of the present study was to examine this possibility.

In these experiments, young C57Bl/6N mice received a single injection of antigenic material into one of the rear footpads at different postnatal ages. The timing of the first appearance of, and successive changes in the numbers of, lymph follicles and germinal centers in the draining popliteal lymph nodes were then examined and compared with corresponding data obtained from untreated normal popliteal nodes.

Materials and methods

Animals

Young mice derived from breeding pairs of C57Bl/6N strain (obtained from Charles River Japan Inc.) and maintained in our laboratory animal unit were used in this study. All animals were kept under pathogen-free conditions. Young mice were weaned at 21–23 days of age. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nihon University School of Medicine.

Injection of test substances

Test substances employed as the stimulants were: 1) hemocyanin (KLH, Calbiochem. Co., 2 mg/ml of saline/kg body weight), 2) lipopolysaccharide (LPS, Westphal type, phenol extraction, Sigma, 2 mg/ml of saline/kg), 3) horseradish peroxidase (HRP, Sigma, 2 mg/ml of saline/kg) and 4) physiological saline. When administered subcutaneously to young adult animals, KLH efficiently induces 'new' lymph follicles and germinal centers in the draining lymph node, whereas soluble substances, such as LPS and HRP, do not induce the formation of new lymph follicles, even though they stimulate the germinal center development (Hoshi et al., 1984, 1986; Horie and Hoshi, 1989). Physiological saline was used as a non-specific stimulant.

Each mouse received a single injection of KLH into the rear footpad at 1, 2, 3, 4, 7, 9, 11, 12, 14 or 21 days of age. Other young mice aged 4, 7, 12 and 21 days received a single injection of LPS or HRP. The treated young mice were chloroformed generally 5 and 7 days after injection, and at 14, 21, 28 and/or 35 and 42 days of age. Some treated mice were also sacrificed 2, 3 and 4 days after the injection of the test solutions. Non-

stimulated normal mice aged from 1 to 42 days served as the normal control.

Antibodies and lectin

Rat anti-mouse CD45R/B220 antibody (RA3-6B2, Pharmingen, San Diego, CA), anti-mouse CD90 (Thy-1.2) (53-2.1, Pharmingen), HRP-conjugated peanut agglutinin (PNA) (EY Lab. San Maetro, CA) and anti-mouse CD35 and CD21/35 (CR1 and CR1/2 complement receptors) (8C12 and 7G6, Pharmingen) were used for detecting B cell follicles, T lymphocytes, germinal center cells and FDCs, respectively, in cryostat sections of lymph nodes. Rat monoclonal antibodies (mAbs) against mouse macrophages (Moma-1 and Moma-2, Serotec LTD, Oxford, U.K.), interdigitating cells (NLDC-145, Serotec LTD) and endothelial cells of the high-endothelial venules (HEVs) (Meca-79: Pharmingen) were used to identify the corresponding cells in the cryostat sections.

HRP-conjugated rabbit anti-rat IgG (Dako, Denmark) and alkaline phosphatase-conjugated goat anti-rat IgG (Cappel Product) antibodies were used as secondary antibodies.

Immunohistochemistry

At necropsy, the popliteal lymph nodes of both sides were excised from each animal, frozen using a dry ice-acetone mixture and cut into 6- μ m thick serial sections. The lymph nodes on the non-injected side served as additional controls. Three or 4 sets of (semi-) serial cryostat sections were prepared from each specimen, with each set containing one in every 3 or 4 consecutive sections, air dried, fixed in pure acetone for 10 min and air dried again.

One set of serial cryostat sections was immunostained for either PNA-positive germinal centers or Thy-1.2-positive T lymphocytes and then for B220-positive B lymphocytes. The second set was used to detect FDC-associated CR1/CR2 complement receptors. The third and fourth sets were stained for CR1 complement receptors of FDCs, macrophages, interdigitating cells or endothelial cells of HEVs. The specimens used for the above immunostaining were those obtained from at least three mice at each time point.

For single immunoenzymatic staining, the sections were incubated for 1 h with optimal concentrations of mAbs in phosphate-buffered saline (PBS) containing 1 % mouse serum. After washing with PBS, the sections were incubated with HRP-conjugated rabbit anti-rat IgG (1:100 dilution in PBS) for 30 min at room temperature, washed with PBS and then stained for 10 min with a solution consisting of 0.02% 3, 3'-diaminobenzidine-tetra HCl (DAB; Dojindo Co.) and 0.005% v/v hydrogen peroxide in PBS to determine peroxidase activity. After washing with PBS, the sections were counterstained with methyl green. For double immunoenzymatic staining,

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the sections were incubated for 1 h with either an anti-mouse mAb, followed by HRP-conjugated secondary antibody, or with HRP-conjugated PNA (1:150 diluted in PBS). After washing with PBS, the peroxidase activity was visualized as described above. These sections were then rewashed with PBS and incubated for 1 h with another type of mAb, before being washed and incubated for 30 min with alkaline phosphatase-conjugated goat anti-rat IgG (1:100 diluted in PBS; Organon Teknika N.V.-Cappel Products, West Chester, PA). The alkaline phosphatase reaction was developed in a mixture of naphthol AS-MX with fast blue BB salt as the color reagent (Burstone, 1958).

Three-dimensional analysis of immunostained sections

Each serial node section that was double-stained for either PNA-positive germinal center cells or Thy-1.2-positive lymphocytes plus B220-positive B lymphocytes was examined by light microscopy to verify the presence of lymph follicles and germinal centers. Each follicle (with or without a germinal center) was given a reference number and was traced three-dimensionally throughout the subsequent sections. This reconstruction technique ensured accurate determination of the total number of lymph follicles and germinal centers in each node.

Next, using the serial sections stained for complement receptors, presence of FDC-associated

complement receptor networks within each lymph follicle was identified. Macrophages, interdigitating cells and HEVs were detected in some lymph nodes, using one or two sets of serial sections stained with rat anti-mouse mAbs against the respective types of cells.

Results

Normal development of lymph follicles in neonatal popliteal lymph nodes (Table 1)

At 1 and 2 days of age, the popliteal nodes were extremely small, and very few lymphoid or other free cells were observed in the nodal parenchyma. By 4 days of age, the nodes had grown slightly, and very small numbers of T- and B-lymphocytes, together with accessory cells such as Moma-2-positive macrophages and NLDC-145-positive interdigitating cells (IDCs), were scattered throughout the nodal parenchyma (data not shown). Over the next few days, the number of T- and B-lymphocytes and accessory cells gradually increased, and these cells began to show preferential localization within the node: the B lymphocytes gathered in the outer cortex, while the T cells and IDCs congregated in the paracortex and the Moma-2-macrophages became diffusely distributed throughout the paracortex and medulla (Figs. 1, 2). HEVs with Meca-79 mAb-positive endothelium were seen in the medullary cords and deep cortex from 4 days onwards (data not

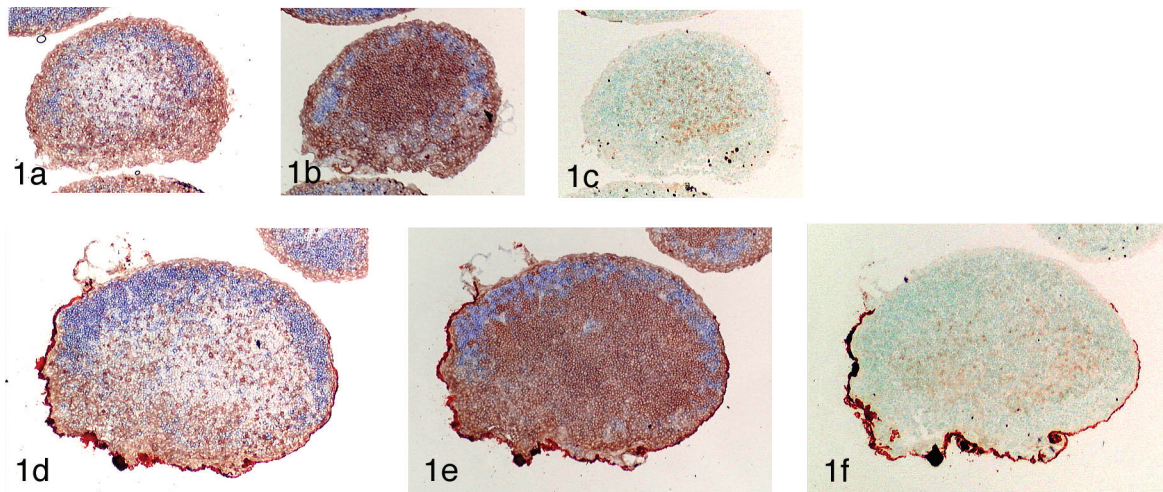


Fig. 1. Popliteal lymph nodes on both sides from a mouse injected with KLH in rear footpad unilaterally at 4 days of age and sacrificed at 7 days of age. **a.** Popliteal node on the contralateral (non-injected) side. Cryostat section double-stained for B220-positive B lymphocytes (blue) and Moma-2-positive macrophages (brown). A small number of B lymphocytes are scattered in the outer cortex, while macrophages are dispersed throughout the nodal parenchyma and accumulated in the medullary region. **b.** Section adjacent to section 1a, double-stained for B220-positive B lymphocytes (blue) and Thy-1.2-positive T lymphocytes (brown). The paracortex of this node is small and populated with T lymphocytes, while B lymphocytes are scattered in the outer cortex. **c.** Section adjacent to section 1b, showing NLDC-145-positive interdigitating cells scattered in the deep cortex. **d.** Popliteal node on the ipsilateral side. Section double-stained for B220-positive B lymphocytes and Moma-2-positive macrophages. **e.** Section adjacent to section 1d, double-stained for B220-positive B lymphocytes and Thy-1.2-positive T lymphocytes. **f.** Section adjacent to section 1e, stained for NLDC-145-positive interdigitating cells. Note that this popliteal node is enlarged and populated with more frequent numbers of B lymphocytes, T lymphocytes, macrophages and interdigitating cells than that on the contralateral side shown in Figures 1a-c. x 60

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shown). By 10 days of age, the B-lymphocytes had become diffusely distributed throughout the outer cortex, and tend to form nodular accumulations; however, no networks of FDC-associated CR1/CR2 complement receptors were detectable within any of the nodular accumulations of B lymphocytes or in any other areas of the nodal parenchyma (data not shown). At 11 days of age, 1-3 small primary follicles became visible in the outer cortex, each displaying a fine, fibrillar network of CR1/CR2 complement receptors (Table 1). Thereafter, the number of primary follicles with CR1/CR2 complement receptor networks increased slowly, reaching a plateau of approximately 11 at 21-28 days of age. No germinal centers were observed in any of the non-stimulated popliteal nodes during the observation period.

Effect of injection of KLH into the footpad (Table 1)

In this series of experiments, young mice were each given a single injection of KLH into one of the footpads at a predetermined postnatal age (between 1 and 21 days). Changes in the numbers of lymph follicles and germinal centers in the draining popliteal lymph nodes were then monitored, generally from 5 days after the injection until 21-42 days of age. The major findings are summarized in Table 1. In addition, earlier morphological and cellular alterations that occurred in the draining popliteal nodes after KLH injection were studied in some detail in representative mice that received the injection at 4 or 11 days of age.

Injection of KLH into 4-day-old mice resulted in enlargement of the draining popliteal node and

Table 1. Number of lymph follicles and germinal centers in the draining popliteal lymph nodes at various ages after a KLH-injection to the rear footpad at different postnatal age in young C57Bl/6 mice

AGE AT INJECTION	AGE													
	6 d	7 d	8 d	9 d	10 d	11 d	12 d	14 d	16 d	18 d	21 d	28 d	35 d	42 d
<i>Untreated control</i>	0	0	0	0	0	1.8±1.5* 0**	3.2±0.5 0	5.4±2.4	/	7.3±1.1 0	8.2±2.8 0	10.5±1.6 0	10.5±1.6 0	11.1±2.4
<i>Saline-injection</i>														
4 d	/	0 0	0.5±0.6 0	1.2±1.5 0	2.7±1.0 0	3.3±1.2	/	5.0±0.6	/	/	11.0±1.5 0	12.3±1.1 0	11.7±2.1	/
12 d	/	/	/	/	/	/	/	/	/	/	/	11.0±1.0 0	11.5±2.5	/
<i>KLH-injection</i>														
1 d	/	0	/	/	/	/	/	5.0±1.0 0	/	/	9.3±1.5 0	/	/	/
2 d	0	1.2±0.8¶ 0	1.3±0.9 0	3.5±0.6¶	/	/	/	/	/	/	11.5±1.3 0	/	/	/
3 d	0	1.0±1.0¶ 0	4.0±1.9¶ 0	/	4.2±2.0	/	4.3±1.5 0.3±0.6	6.5±1.0	/	/	11.2±1.9 0	11.3±2.3	/	/
4 d	/	0 0	4.5±1.3¶ 1.2±1.5	5.8±1.8¶ 1.6±1.5	8.8±2.2¶ 1.5±1.7	8.3±2.1¶	/	7.8±0.7 3.3±1.0	/	/	12.0±1.2 2.2±1.6	13.0±1.2	/	/
5 d	/	/	0 0	3.6±0.6 0.8±1.3	8.3±2.8¶ 0.5±1.0	8.0±1.4¶	/	9.7±1.8¶ 4.0±3.0	/	12.2±2.3¶ 4.8±1.1	13.0±1.8¶ 4.7±1.2	15.7±4.0	/	/
7 d	/	/	/	/	/	/	10.3±1.5¶ 2.1±1.2	18.0±3.8¶ 7.2±1.9	/	/	15.7±2.6¶ 7.7±3.4	19.4±4.2¶	/	/
9 d	/	/	/	/	/	/	/	12.5±2.4¶ 7.5±1.9	18.1±2.9¶ 8.2±1.9	/	20.2±3.7¶ 13.5±2.6	23.7±2.7¶	/	/
11 d	/	/	/	/	/	/	/	/	14.7±2.7¶ 6.0±1.4	27.2±3.5¶ 16.2±3.9	27.6±2.7¶ 17.0±2.7	27.2±2.6¶	/	/
14 d	/	/	/	/	/	/	/	/	/	17.3±2.1¶ 2.0±2.0	26.7±2.9¶ 17.5±4.1	28.0±2.8¶ 20.7±4.1	26.3±2.8¶	/
21 d	/	/	/	/	/	/	/	/	/	/	/	18.7±3.0¶ 15.7±3.0	20.3±7.1¶ 17.7±6.1	19.0±4.0¶ 13.7±2.5

* Number of follicles : mean±S.D. ** Number of germinal centers: mean±S.D. /: not examined. ¶: the number of follicles at the age is significantly larger (p<0.05; t test) from that of untreated control and/or saline-treated nodes at the corresponding age. At each time point, lymph nodes from 3-6 mice were examined.

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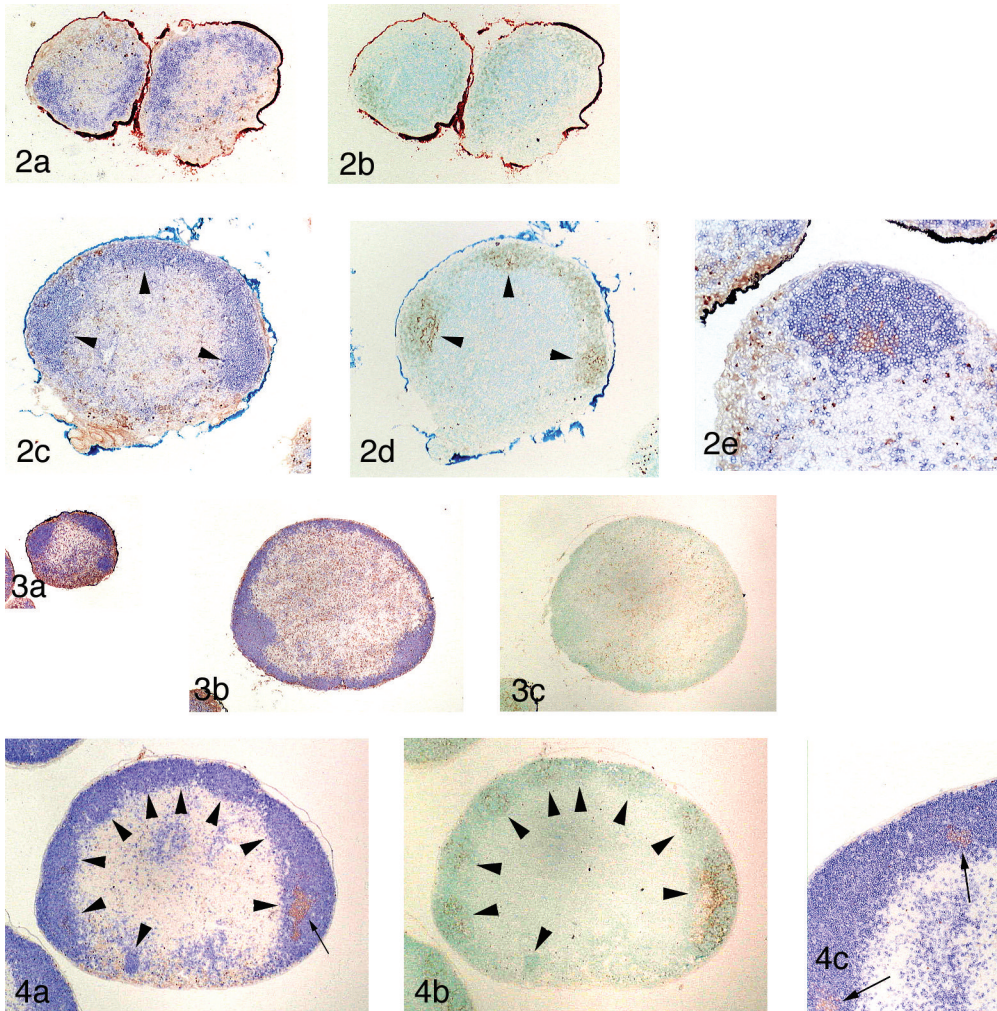


Fig. 2. Popliteal lymph nodes on both sides from mice injected with KLH into the rear footpad unilaterally at 4 days of age and sacrificed at 8 days of age. **a.** Two popliteal nodes on the contralateral (non-injected) side. Cryostat section double-stained for B220-positive lymphocytes (blue) and PNA-positive germinal center cells (brown), showing B lymphocytes diffusely distributed in the outer cortex of these two nodes. $\times 40$. **b.** Section adjacent to section 2a, stained for FDC-associated CR1/CR2 complement receptors. No networks of CR1/CR2 complement receptors can be seen anywhere in the nodal parenchyma of these nodes. $\times 40$. **c.** Popliteal node on the ipsilateral side. Section double-stained for B220-positive B lymphocytes and PNA-positive germinal center cells. Three lymph follicles (indicated by arrowheads) can be seen in the outer cortex. No germinal centers are visible within these follicles. $\times 40$. **d.** Section adjacent to section 2c, showing CR1/CR2 complement receptor networks (arrowheads) developing within each of the follicles shown in 2c. $\times 40$. **e.** Part of a popliteal lymph node on the ipsilateral (injected) side from a mouse injected with KLH at 4 days of age and sacrificed at 10 days of age. Cryostat section double-stained for B220-positive B lymphocytes (blue) and PNA-positive germinal center cells (brown). A small germinal center can be seen within a lymph follicle. $\times 90$

Fig. 3. Popliteal lymph nodes on both sides from a mouse injected with KLH into the rear footpad unilaterally at 11 days of age and sacrificed at 14 days of age. **a.** Popliteal node on the contralateral (non-injected) side at the same magnification as the popliteal node shown in Figures 3b and 3c. Cryostat section double-stained for B220-positive B lymphocytes and PNA-positive germinal center cells. Three lymph follicles (two being relatively large, while the other, very small) can be seen in the outer cortex of this node. $\times 20$. **b.** Popliteal node on the ipsilateral side. Section double-stained for B220-positive B lymphocytes and PNA-positive germinal center cells. Note that this popliteal node is much enlarged in comparison with the one on the contralateral side shown in Figure 3a. Two lymph follicles without germinal centers can be seen in the periphery of the node. Also note the widening of the extrafollicular zone of the outer cortex, and the diffuse distribution of B lymphocytes in a thin layer in the periphery of the extrafollicular zone. $\times 20$. **c.** Section adjacent to section 3b, stained for NLDC-145-positive interdigitating cells. This type of cell can be seen scattered throughout the expanded deep cortex. $\times 20$

Fig. 4. Popliteal lymph nodes on the ipsilateral (injected) side from mice injected with KLH at 11 days of age and sacrificed at 18 days of age. **a.** Cryostat section double-stained for B220-positive B lymphocytes and PNA-positive germinal center cells. Nine lymph follicles (indicated by arrowheads) can be seen in the periphery of the node. Some of the follicles contain small germinal centers (indicated by arrows). $\times 20$. **b.** Section adjacent to section 4a, stained for CR1/CR2 complement receptors. Note that each lymph follicle displays the CR1/CR2 complement receptor network. $\times 20$. **c.** Another section stained in a similar manner as section 4a, showing lymph follicles at higher magnification. Small germinal centers (indicated by arrows) can be seen within lymph follicles. $\times 40$

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expansion of the paracortex by 4 days after injection (8 days of age). During this period, the numbers of T and B lymphocytes, Moma-2-positive macrophages and NLDC-145-positive cells increased to a greater extent in the draining popliteal nodes on the treated side than in the contralateral nodes; the T lymphocytes and NLDC-145-positive cells were located in the expanded paracortex, the B lymphocytes in the outer cortex and the Moma-2-positive macrophages in the paracortex and medullary region (Figs. 1, 2). At 8 days of age, lymph follicles (typically 4-5 in number) displaying fine, fibrillar networks of CR1/CR2 receptors were first observed, in the outer cortex of each draining node (Fig. 2). The number of such follicles had increased to about 8 by 10 days of age (Table 1). At 21-28 days of age, the number of follicles per node was within the same range as in the controls. From 9 days of age onwards, a few small PNA-positive germinal centers were observed within the developing lymph follicles in the draining nodes (Fig. 2).

The morphological changes that occurred in the draining popliteal nodes after KLH injection at 2 or 3 days of age were somewhat similar to those seen when KLH was given at 4 days of age. B lymphocytes became more numerous than in the controls at 3 days after injection (5 or 6 days of age), and one or two lymph follicles with faintly stained networks of CR1/CR2

receptors appeared at 7 days of age. The number of follicles increased to some extent over the next few days (Table 1). No germinal centers were detected in the draining nodes at any stage. In mice that received KLH at 1 day of age, the draining popliteal nodes showed no increase in the B lymphocyte population or early appearance of lymph follicles at 7 days of age.

When mice were injected with KLH at any time between 7 and 21 days of age, the number of follicles per node increased significantly in the ipsilateral popliteal node compared with contralateral node by 5 days after injection and increased further to reach a temporal plateau by 7 days after injection, although the number of follicles present at this time varied depending on the age at which the KLH injection had been given (Table 1). Some PNA-positive germinal centers had developed within the lymph follicles by 5 days after injection and this number had increased further by 7 days after injection. In mice injected with KLH between 4 and 14 days of age, the numbers of lymph follicles and germinal centers produced in the draining nodes tended to be proportional to the age of the animal. However, in mice injected with KLH at 21 days of age, fewer new lymph follicles were produced in each draining node by 7 days after injection than when the injection was administered between 7 and 14 days of age.

Early cellular changes in the draining popliteal

Table 2. Number of lymph follicles and germinal centers in the draining popliteal lymph nodes at various ages after injection of physiological saline and soluble substances (LPS (w) and HRP) to the rear footpad at different postnatal age in young C57Bl/6 mice.

SUBSTANCES INJECTED AND AGE AT INJECTION	AGE				
	14 d	21 d	28 d	35 d	42 d
Untreated control	5.4±2.4* 0**	8.2±2.8 0	10.5±1.6 0	10.5±1.6 0	11.1±2.4 0
LPS(w)-injection					
4 d	5.0±1.7 0	11.0±2.8 0	10.3±2.1 0	/	/
12 d	/	13.4±2.3¶ 4.8±0.8	15.4±2.1¶ 6.0±1.7	16.7±1.1¶ 6.3±1.5	/
21 d	/	/	/	12.0±1.0 6.7±0.6	11.7±2.3 5.0±2.6
HRP-injection					
4 d	4.5±1.7 0	12.5±1.7 0	13.3±2.5 0	/	/
7 d	/	14.0±1.0¶ 0	16.7±2.3¶ 0.3±0.6	/	/
12 d	/	20.5±1.3¶ 4.6±3.9	19.5±2.3¶ 4.2±2.3	19.0±1.7¶ 2.7±3.0	/
21 d	/	/	/	13.0±2.0 2.2±1.6	12.8±2.6 1.0±0.9

* Number of follicles: mean±S.D. ** Number of germinal centers: mean±S.D. /: not examined. ¶: the number of follicles at the age is significantly different ($p < 0.05$; t test) from that of the control at the corresponding age. At each stage, lymph nodes from 3-6 mice were examined.

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nodes after KLH injection were also studied in some detail in mice that received the injection at 11 days of age. During the first 3 days after injection, enlargement of each node was noted, and this was associated with considerable increases in the populations of various cell types in the nodal parenchyma. In these mice, the T lymphocytes and NLDC-145-positive cells were located in the paracortex, whilst the Moma-2-positive macrophages in the paracortex and medulla, and the B-lymphocytes were distributed diffusely throughout the extrafollicular zones between the existing follicles (Fig. 3). From 4 through 7 days after injection, the B lymphocytes in the extrafollicular zones organized themselves into nodular accumulations to form new lymph follicles; consequently, the total number of follicles per node increased, reaching a plateau at 7 days after injection (Fig. 4, Table 1). At this stage, the newly developed follicles were distinguishable from the existing ones because the former contained weakly stained, fine, fibrillar networks of FDC-associated CR1/CR2 complement receptors, whilst the latter contained definitely stained, well developed networks of these receptors (Fig. 4). From 5 through 7 days after injection, PNA-positive germinal centers became evident and increased in number within the existing follicles, as well as in many of the newly formed follicles (Fig. 4, Table 1).

Effect of injection of other substances into the footpad (Tables 1 and 2)

The above observation showed that when KLH was given at any day between 7 and 14 days of age, the number of new follicles produced by each draining node was larger than the case when KLH was given at 21 days. This may be due to the fact that macrophages have heightened sensitivity to exogenous antigens during the second week of life. To examine this possibility, we employed soluble antigens, which are known to be ineffective in inducing the reactive formation of new lymph follicles in young adult animals. If the macrophages in the skin and lymph nodes have augmented sensitivity to exogenous antigens during the second week of life, these cells would respond to soluble antigens and initiate the formation of new lymph follicles in the draining lymph nodes.

Physiological saline

In 4-day-old mice injected with saline, a few primary follicles with fine, fibrillar networks of CR1/CR2 appeared in each ipsilateral popliteal node between 4 and 6 days after injection (Table 1). From 14 days of age onwards, the number of follicles in the node on the treated side remained at the control level. In mice injected with saline at 12 or 21 days of age, the number of follicles per node on the treated side was comparable with that of control nodes at all stages examined (Table 1).

LPS and HRP

LPS (Westphal type) and HRP are known to be inefficient inducers of the formation of new lymph follicles in the lymph nodes of young adult mice (Kaneko et al., 1994; Imaseki et al., 1997). In mice injected with HRP or LPS at 4 days of age, 1-3 primary follicles displaying fine, fibrillar networks of CR1/CR2 complement receptors were observed in each draining popliteal node at 4 days after injection (data not shown). The number of follicles per node remained unchanged for the next few days, then returned to a normal level at 21-28 days of age (Table 2). No germinal centers were observed in the draining nodes during the observation period.

When HRP or LPS was injected at 7 or 12 days of age, the number of follicles in each ipsilateral draining popliteal node was significantly higher than in the corresponding node on the contralateral (non-injected) side at 21, 28 and/or 35 days of age (Table 2). At these ages, some germinal centers were usually present in the draining nodes. In contrast, injection of the test substance at 21 days of age did not alter the number of lymph follicles in the draining node when examined at 35 and 42 days of age (Table 2), although some germinal centers were visible in each node.

Discussion

In this study, we demonstrated that subcutaneous injection of appropriate stimulants into young mice could not only advance the appearance of, but also enhance the formation of, lymph follicles in draining lymph nodes when compared with the corresponding lymph nodes on the non-stimulated side.

In newborn mice, the popliteal node begins to be populated with T and B lymphocytes from around 4 days of age. In mice that received KLH at 2 or 3 days of age, the B lymphocyte populations of the draining popliteal nodes showed a slight increase on days 5 and 6, resulting in the advanced appearance of lymph follicles (at 7 rather than 11 days of age). In contrast, mice that received KLH at 1 day of age showed neither an increase in the B lymphocyte population nor advanced lymph follicle formation. These observations suggest that, during the ontogeny of a lymph node, circulating and recirculating lymphocytes are unable to populate the node until its parenchyma has become able to support the lymphoid elements. An additional explanation could be that effective follicle formation only occurs after the HEV have switched from solely expressing mucosal addressin MAdCAM-1 to the expression of peripheral lymph node addressins PNA_d (Mebius et al., 1996). PNA_d expressed on HEVs can permit the entry of L-selectin expressing Lymphocytes. We described the appearance of PNA_d on HEV in the popliteal lymph node from 4 days of age onwards using anti-PNA_d antibody Meca-79. However, once a lymph node develops this capacity, it becomes able to accept not only

cells immigrating in the normal manner but also those recruited due to exogenous antigen stimulation, and therefore becomes able to increase the production of lymph follicles.

The numbers of newly produced lymph follicles (outside the pre-existing ones) in the draining popliteal nodes seen after KLH injection varied according to the timing of the injection. The number of new lymph follicles that will be induced after a single injection of KLH at a certain postnatal age were roughly determined using the following simple formula: (Number of newly produced follicles) = (Total number of lymph follicles in the draining node on the seventh day after injection) – (Number of lymph follicles in the non-stimulated control node at the corresponding age). In young mice that received a single injection of KLH at 3, 4, 7, 11, 14 or 21 days of age, the approximate numbers of lymph follicles that had been produced in the draining lymph nodes during the 7-day period after injection were 4, 8, 13, 20, 20 and 10, respectively. The number of new lymph follicles produced in a draining node may be largely determined by the number of B lymphocytes that are mobilized from the circulation into the node by KLH stimulation. Judging from previous observations on lymph nodes and other peripheral lymphoid organs, the circulating and recirculating B lymphocyte pool in newborn mice is very small, but undergoes rapid expansion during the suckling period (Hoshi et al., 2001; Kunieda et al., 2002). During this period, therefore, the size of the B lymphocyte pool at the time of KLH injection may be an important factor that affects the extent of B lymphocyte influx into, and successive formation of new lymph follicles within, a draining node. In mice receiving KLH between 3 and 14 days of age, the number of newly produced follicles in each draining node increased in line with age at the time of injection. This increase may be related to the age-dependent enlargement in the size of the B lymphocyte pool during this period.

Of interest is the finding that, when KLH was injected at 21 days of age, the number of new follicles produced by each draining node was somewhat diminished compared with the numbers produced when KLH was given at 7 or 14 days of life. This reduction can be explained by the macrophage response. In a previous study, we presented evidence for the view that, following activation by effective antigens, macrophages mediate the reactive formation of new lymph follicles in lymph nodes (Tanaka et al., 1998). Based on this, we postulate that in suckling mice, particularly during the second week of life, the macrophages in the skin and lymph nodes have heightened sensitivity to exogenous antigens; however, this sensitivity normalizes towards the end of the suckling period and after weaning. To confirm this assumption we employed soluble antigens, including LPS (Westphal type) and HRP, as stimulants. Soluble antigens are known to be ineffective in inducing the reactive formation of new lymph follicles in young adult animals: they are effective, however, if given in a

‘phagocytizable’ form, that is, adsorbed onto insoluble substances such as gelatinous alumina or sephadex (Hoshi et al., 1984, 1986; Horie and Hoshi, 1989). If the macrophages present in the lymph nodes and their draining territories do indeed have augmented sensitivity during the second week of life, these cells would be able to respond to soluble antigens administered during this period, become activated and initiate the formation of new lymph follicles in the draining nodes. As expected, when LPS or HRP was injected into 7- and/or 12-day-old mice, each draining popliteal node showed a significant increase in the number of follicles at 7-14 days after injection, whilst 21-day-old mice given such treatment failed to do so.

It is known that germinal centers are generated in the lymphoid organs as a result of interactions between virgin B cells, helper T cells and dendritic cells (interdigitating cells) after antigen stimulation, and that both virgin B and helper T cells are required to mount a specific response to a particular antigen (Gray et al., 1986; MacLennan and Gray, 1986; Kroese et al., 1987b; Gray, 1988; Liu et al., 1991; Liu and Arpin, 1997; Fu and Chaplin, 1999). In this study, when KLH was injected at 1-3 days of age, the draining popliteal nodes produced no germinal centers. The earliest appearance of small germinal centers, albeit only one or two, in these nodes was noted at 9 days of age in mice which received KLH at 4 days of age. Thereafter, the number of germinal centers induced by KLH increased in line with the age at injection. The failure of mice receiving KLH at 1-3 days of age to develop germinal centers in their draining lymph nodes can be explained by the paucity of virgin B and helper T cells available for recruitment into the nodes after the injection. Similarly, the injection-age-associated increase in the numbers of germinal centers observed in mice that received KLH at 4 days or later may be related to the age-associated increase in the numbers of virgin B and helper T cells mobilized in response to the injection. These observations strongly suggest that the neonatal popliteal node develops its reactive lymph follicle-forming capacity in tandem with its germinal center-forming capacity.

Recent studies revealed that TNF and LT derived from B cells and other cell types are required for compartmentalization of peripheral lymphoid organs into distinct B-cell and T-cell areas and development of B-cell follicles (Endres et al., 1999; Fu and Chaplin, 1999; Ngo et al., 1999; Tumanov et al., 2002), and that development of stromal cells expressing CXCL13 is essential for the formation of primary follicles and the generation of mature FDCs (Ansel et al., 2000). In neonatal murine lymph nodes, stromal cells in the outer cortex initiate to express a low level of CXCL13 in a B-cell-independent manner during the early postnatal ages (Cupedo et al., 2004). Subsequently, CXCL13 expressed on stromal cells induces immigrating B cells to express membrane LT, which in turn promotes production of CXCL13 by stromal cells, thus creating a positive feedback loop (Ansel et al., 2000) that leads to the

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formation of primary follicles containing mature FDCs (Cupedo et al., 2004). In connection with our observation, it is likely that in neonatal popliteal nodes, stromal cells expressing a low level of CXCL13 may develop in the outer cortex by around 4 days of age, indifferently of exogenous antigen stimulation, and thereby the node may become ready for initiating the lymph follicle formation. However, in order for a neonatal lymph node to do actually start the mechanism of lymph follicle formation by way of establishing a positive feedback loop described by Ansel et al. (2000), localization of a sufficiently large number of B lymphocytes in the outer cortex is necessary. This may explain the reason why an increased influx of B lymphocytes into the neonatal popliteal node after KLH injection at an early postnatal age resulted in the advanced appearance of primary follicles.

On the other hand, it is possible that stromal cells capable of differentiating into mature FDCs may be persistently present within the FDC-deficient extrafollicular areas since after appropriate antigen stimulation, 'new' lymph follicles containing FDC networks are produced in the FDC-deficient extrafollicular areas. Such stromal cells may lack the expression of CXCL13, or may express a low level of this chemokine (Ansel et al., 2000). Once the extrafollicular areas are populated with a sufficiently large number of B cells and other cells, as seen after KLH-injection in this study, the stromal cells in these areas may be induced, or promoted, to produce CXCL13, which in turn activates the expression of membrane LT on B cells, thus creating a positive-feedback loop that leads to formation of lymph follicles and generation of mature FDCs. This study does not exclude, however, the possibility that in the case extrafollicular areas are populated with a large number of B cells, CXCL13-positive, CR1-negative FDCs that are reportedly present in the peripheral part of each preexisting follicle (Ansel et al., 2000) may migrate to the extrafollicular areas and subsequently organize 'new' lymph follicles containing CR1-positive FDCs in these areas. Further analysis is required for understanding cellular interactions taken place during the reactive formation of lymph follicles in the lymph nodes after antigen stimulation.

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