Formation of Lymph Follicles and Germinal Centers in the Somatic and Mesenteric Lymph Nodes of Growing Mice during Ontogenesis

By

Masato KUNIEDA, Masaki HIRAMOTO, Kaeko HORIE, Hidetsugu NAGATA,
Shin AIZAWA and Hajime HOSHI

Department of Anatomy, Nihon University School of Medicine, Tokyo 173-8610, Japan

– Received for Publication, June 4, 2002 –

Key Words: Lymph follicle, Germinal center, Immunohistochemistry, Lymph node, Ontogenesis

Summary: We investigated the age-dependent changes that occur in the numbers of lymph follicles and germinal centers in various lymph nodes in BALB/C and ICR mice aged between four days and 16 to 18 weeks. Young adult BALB/C mice have a relatively small body size, compared to ICR mice at the same stage, where there is a relatively large body size. In BALB/C mice somatic (popliteal, brachial, axillary, inguinal, submandibular and deep cervical) and mesenteric lymph nodes were examined. In ICR mice only the somatic (popliteal, brachial and axillary) lymph nodes were examined. In both BALB/C and ICR mice, the primary follicles were apparent in most somatic nodes by the 6th postnatal day. Up to 28 days of age, the number of follicles per node increased, reaching different levels in nodes from different locations. Thereafter, in most of the somatic nodes in BALB/C mice the number of follicles increased only slightly, although there was a substantial increase in ICR mice, reaching a peak or a plateau at 8 or 12 weeks of age. In the mesenteric (ileocecal) nodes in BALB/C mice, the primary follicles first appeared at 10 to 12 days, then there was a linear increase until a plateau level was reached at 8 weeks of age. Germinal centers appeared in the mesenteric nodes at 28 days and increased rapidly in number thereafter. In most somatic nodes germinal centers were scarcely observable until 8 weeks of age. Based on our observations we have three suggestions. Firstly, in BALB/C mice there were two different patterns of age-dependent changes in the numbers of lymph follicles in the somatic and the mesenteric nodes during ontogenesis. These different patterns are probably due to variations in the magnitude of the exogenous antigen stimulatory effect. Secondly, it seems likely that the variations in the numbers of lymph follicles that are produced in somatic nodes at different locations during the first 28 days after birth relate to the dimensions of the body regions that are drained by that particular somatic node at that stage of development. Thirdly, in the relatively small BALB/C mice, the ontogenetic production of lymph follicles in a somatic node is mostly completed during the first four weeks of life, whereas in the relatively larger ICR mice, this process may continue until the young adult stage of 8 weeks.

Lymph nodes contain a number of lymph follicles within the outer cortex. 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). FDCs are specialized non-lymphoid cells with long dendritic processes that form a web-like membranous network. These cells are intimately associated with B-lymphocytes, which reside temporarily in the interstices of the network. One feature of FDCs is that immune complexes become trapped on the surface of both the cell body and the dendritic processes, via Fc and complement receptors. These immune complexes are then retained for long periods of time (Nossal *et al.*, 1968; Mandel *et al.*, 1980;

Gerdes and Stein, 1982; Reynes *et al.*, 1985; van den Berg *et al.*, 1995). (Primary) lymph follicles develop 'spontaneously' within the peripheral lymphoid organs during ontogenesis. This event is thought to be antigen-independent, whereas the development of germinal centers within the lymph follicles is an antigen-dependent phenomenon. When adequately stimulated by exogenous antigens, lymph nodes produce new lymph follicles (with or without germinal centres) outside the existing ones. As a result of this, there is an increase in the number of follicles per node (Sjövall and Sjövall, 1930; Conway, 1937; Hoshi *et al.*, 1984, 1986, 1989; Horie *et al.*, 1999). Recent studies have suggested that the major function of the germinal centers is the generation of memory B-lymphocytes (Klaus *et al.*, 1980; Kroese

et al., 1987b; Gray, 1988; Gray *et al.*, 1991; Liu *et al.*, 1991, 1997; Nieuwenhuis *et al.*, 1992; MacLennan, 1994). However, the precise functional significance of the primary follicles remains ill-defined. In addition, the mechanism that underlies the formation of lymph follicles remains unclear.

Many investigators have studied the ontogenetic development of the secondary lymphoid organs in rats and mice. These authors have described the age at which primary follicles and germinal centers first appear not only in the mesenteric and popliteal lymph nodes but also in other lymphoid organs (Eikelenboom *et al.*, 1979; Groscurth, 1980; Dijkstra *et al.*, 1982, 1983; Sminia *et al.*, 1983; van Rees *et al.*, 1985, 1986; Kroese *et al.*, 1987a; Murakami *et al.*, 1989; Chen *et al.*, 1995). In previous studies in young adult C57B1/6 mice, we have shown that the popliteal lymph node, a very small node, contains 11–13 lymph follicles with only occasional germinal centers, whereas the brachial node, a relatively large node, contains approximately 40 follicles with several germinal centers (Hoshi *et al.*, 1986; Nagata *et al.*, 1996; Horie *et al.*, 1999). These findings indicate that the number of follicles per lymph node, at a given stage of development, varies widely between different locations. It is conceivable that the number of follicles present within a lymph node may reflect the lymph node activity at that time and might be associated with the size of the draining area of the node. This gives rise to several questions about the process whereby the number of follicles within a particular lymph node changes during ontogenesis, and to what extent the 'spontaneous' invasion of exogenous antigens in growing animals affects this process. In addition, it raises questions about the factors that govern the number of lymph follicles that develop within a node.

The present study was undertaken in an attempt to clarify these issues. We have previously conducted an immunohistochemical study on the postnatal development of various lymph nodes in growing C57B1/6 mice (Hoshi *et al.*, 2001). Although this study gave information that increased our understanding of the ontogenetic development of lymph follicles in lymph nodes, some uncertainty has remained as to whether the findings obtained in C57B1/6 mice are generally applicable to other murine strains. In order to gain more convincing information on this matter, we re-examined the postnatal development of several somatic and mesenteric (ileocecal) lymph nodes in growing mice. Two strains were chosen: BALB/C and ICR mice, BALB/C mice are similar to C57B1/6 mice, with a relatively small body size, whereas ICR mice are relatively large. Particular attention was given to the time of first appearance of the lymph follicles

and to the successive changes in the number of lymph follicles and germinal centers in particular lymph nodes during ontogenesis.

Materials and Methods

Animals

The mice used in this study were young mice that had been derived from breeding pairs of each of the BALB/C and ICR strains that were maintained in our laboratory animal unit (The breeding pairs of both strains were obtained from Charles River Japan Inc.). All the animals were kept under pathogen-free conditions. The young mice were weaned 21 to 23 days after birth.

Antibodies and lectin

We used rat anti-mouse CD45R/B220 antibody (RA3-6B2, Pharmingen, San Diego, CA), anti-mouse CD90 (Thy-1.2) (53-2.1, Pharmingen), peroxidase (HRP)-conjugated peanut agglutinin (PNA) (EY Lab. San Maetro, CA) and anti-mouse CD21/35 and CD35 (CR1/2 and CR1 complement receptors) (7G6 and 8C12, Pharmingen) to identify the B-cell follicles, the T-lymphocytes, the germinal center cells and the FDCs, respectively, in cryostat sections of lymph nodes. HRP-conjugated rat anti-mouse IgM (Cappel Product, Organon Teknika Corp., Westchester, PA) was used to detect IgM-synthesizing cells and sIgM-bearing B-lymphocytes.

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

Necropsy and tissue preparation

The animals that were selected for investigation in both strains were 4, 6, 10, 12, 14 and 21 days old. In addition BALB/C animals aged 4, 8, 12 and 16 weeks and ICR animals aged 4, 8, 13 and 18 weeks were selected. At least three test animals of each age were killed using chloroform. In all the BALB/C mice, selected somatic and mesenteric lymph nodes were excised (the popliteal, brachial, axillary, inguinal, submandibular, deep cervical and mesenteric nodes). In the ICR mice, the popliteal, brachial and axillary nodes were selected. In all cases, except for the mesenteric lymph nodes, the nodes were obtained from only one side (right or left) of each mouse. The mesenteric lymph nodes consisted of a chain of jejuno-ileal nodes and an ileocecal node, although the latter usually lay some distance from the jejuno-ileal nodes, as is generally the case in C57B1/6 mice. In this study, the ileocecal lymph node together with the distal portion of the jejuno-

ileal node chain was obtained from each mouse.

Immunohistochemistry

The specimens obtained at necropsy were frozen using a dry ice-acetone mixture and cut into 6- μ m serial sections. Four sets of (semi-) serial cryostat sections were prepared from each specimen, with each set containing every fourth consecutive section. These specimens were air dried, fixed in pure acetone for 10 min and then air dried again.

One set of serial cryostat sections was immunostained for either PNA-positive germinal centres or for Thy-1.2-positive T-lymphocytes and then subsequently stained for B220-positive lymphocytes. A second set was used to identify CR1/2 complement receptors expressed on the cell surfaces of FDCs. The third and fourth sets were stained for the CR1 complement receptors of FDCs and to show IgM-synthesizing cells, using the corresponding mAbs.

For single immunoenzymatic staining, the sections were incubated for 1 hour 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 for 30 minutes at room temperature, washed with PBS and then stained for 10 minutes with a solution of 0.02% 3,3'-diaminobenzidine-tetra HCl (DAB; Dojindo Co.) with 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, the sections were incubated for 1 hour with either anti-mouse mAb, followed by a peroxidase-conjugated secondary antibody, or with peroxidase-conjugated PNA (1:150 dilution in PBS). After washing with PBS, peroxidase activity was identified as described above. These sections were then rewashed with PBS and incubated for 1 hour with another type of mAb, before being washed and incubated for 30 minutes with alkaline phosphatase-conjugated goat anti-rat IgG (1:100 dilution in PBS). 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 had been double-stained for either PNA-positive germinal center cells or Thy-1.2-positive lymphocytes plus B220-positive B-lymphocytes was examined under light microscopy to verify the presence of lymph follicles and germinal centers. Each follicle (either

with or without a germinal center) was given a reference number and was traced three-dimensionally throughout the subsequent sections. This reconstruction technique enabled the total number of lymph follicles and germinal centers in each node to be determined.

Using the serial sections stained for complement receptors, FDC-associated networks of complement receptors were identified in each lymph follicle.

In each lymph node the IgM-synthesizing cells were identified in one set of the serial cryostat sections that had been stained with the anti-mouse IgM mAb. These stained sections, except for several sections that had been cut from the ends of each specimen, were all examined using light microscopy. The IgM-synthesizing cells in each section were counted, and the mean number of these cells per section was determined.

Results

In this study, young BALB/C and ICR mice of both sexes were used. Over the age of 21 days, only male mice were used with 3–4 mice being sacrificed at each time point. The body weight and the weights of the lymph nodes from different locations at several time points are shown in Table 1. The major findings are summarized in Tables 2–5.

The first appearance of primary follicles

BALB/C mice: By four days after birth, T-lymphocytes were present in the paracortex of the submandibular, deep cervical, brachial, axillary and inguinal lymph nodes, whilst B-lymphocytes were distributed diffusely throughout the outer cortex (not shown). By 6 days of age, the B-lymphocytes had become organized into primary follicles in some places within the outer cortex (Fig. 1a). In sections that had been stained with the anti-CD21/CD35 mAb, many of the primary follicles showed fibrillar FDC-associated networks of CR1/2 complement receptors (Fig. 1b). In this study, primary follicles that had no detectable complement receptor networks were not included in the total follicle count. Over the next few days, the primary follicles in the various lymph nodes were found to have increased in both number and size, and had denser networks of the complement receptors (Fig. 2a, b).

In the popliteal nodes, primary follicles with CR1/2 complement receptor networks first appeared in the outer cortex by 10 days after birth (Table 2).

In the mesenteric (ileocecal) nodes, primary follicles with CR1/2 receptor networks appeared spo-

Table 1. Age-dependent changes in body weight and weights of the lymph nodes from different locations in BALB/C and ICR mice*

BALB/C mice Age (days and weeks)	14 d	4 w	8 w	12 w	16 w
Body weight (g)	7.1 ± 0.5	16.2 ± 1.1	23.7 ± 0.9	27.0 ± 1.0	29.2 ± 1.5
Lymph node weight (mg)					
Popliteal	0.3	1.0 ± 0.3	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.3
Brachial	0.8	2.6 ± 0.4	2.6 ± 0.2	2.5 ± 0.2	2.6 ± 0.2
Axillary	0.5	1.7 ± 0.3	1.8 ± 0.4	1.8 ± 0.4	1.7 ± 0.3
Inguinal	0.5	1.7 ± 0.6	2.2 ± 0.5	2.4 ± 1.0	2.4 ± 0.5
Submandibular	0.5	1.5 ± 0.2	2.6 ± 1.1	2.4 ± 0.7	2.6 ± 0.6
Deep cervical	0.6	2.5 ± 0.3	2.3 ± 0.2	1.7 ± 0.1	1.9 ± 0.2
Ileocecal	0.7	2.9 ± 0.4	5.3 ± 1.1	5.1 ± 0.5	6.8 ± 0.4
ICR mice Age (days and weeks)	14 d	4 w	8 w	13 w	18 w
Body weight (g)	10.7 ± 0.6	24.5 ± 1.8	37.0 ± 3.5	39.7 ± 0.6	40.0 ± 3.5
Lymph node weight (mg)					
Popliteal	n.e.	1.1 ± 0.3	1.8 ± 0.4	1.3 ± 0.3	1.1 ± 0.2
Brachial	n.e.	3.1 ± 0.6	3.7 ± 0.4	3.8 ± 0.5	3.8 ± 0.3
Axillary	n.e.	2.6 ± 0.6	2.4 ± 0.3	2.6 ± 0.2	3.0 ± 0.4

At each time point, 3–6 animals were examined. * Mean ± S.D.

Table 2. Number of lymph follicles and germinal centers in various lymph nodes of BALB/C and ICR mice at various postnatal ages

BALB/C mice		Age in days and weeks						
	6 d	10 d	14 d	3 w	4 w	8 w	12 w	16 w
Popliteal	0	1.7 ± 0.6*	5.0 ± 1.0	14.7 ± 3.0	14.3 ± 3.2	14.7 ± 3.3	15.3 ± 2.1	15.0 ± 4.0
	0**	0	0	0	0	0.5 ± 0.6	4.7 ± 7.2	2.0 ± 2.0
Brachial	5.3 ± 1.1	11.3 ± 4.5	18.0 ± 1.0	40.0 ± 4.0	37.0 ± 3.8	40.0 ± 2.7	43.0 ± 4.6	36.7 ± 2.1
	0	0	0	0	1.5 ± 3.0	1.5 ± 1.0	4.7 ± 3.5	9.0 ± 4.1
Axillary	4.3 ± 2.1	6.7 ± 2.1	13.3 ± 5.4	23.6 ± 1.5	23.5 ± 4.3	29.7 ± 3.2	23.0 ± 1.0	25.0 ± 6.9
	0	0	0	0	0	1.2 ± 1.9	0.8 ± 1.3	4.7 ± 2.1
Inguinal	4.0 ± 1.0	8.3 ± 0.6	12.0 ± 2.0	20.3 ± 5.6	21.3 ± 3.2	25.0 ± 4.8	25.3 ± 1.5	24.5 ± 5.1
	0	0	0	0	0	2.0 ± 1.1	6.0 ± 6.5	8.7 ± 4.8
Submandibular	4.0 ± 2.0	8.0 ± 2.6	10.7 ± 3.2	20.0 ± 2.0	22.0 ± 3.6	26.7 ± 4.5	32.0 ± 3.0	25.5 ± 4.8
	0	0	0	3.3 ± 3.3	5.7 ± 1.1	12.2 ± 3.9	19.7 ± 5.8	17.5 ± 1.7
Deep cervical	6.3 ± 2.3	11.7 ± 3.2	15.3 ± 2.1	31.9 ± 6.1	30.0 ± 5.3	35.5 ± 4.1	30.7 ± 1.1	29.7 ± 3.8
	0	0	0	1.0 ± 1.7	0	3.2 ± 2.6	2.7 ± 2.1	6.7 ± 4.7
Ileocecal	0	3.0 ± 4.3	14.0 ± 5.0	27.4 ± 9.1	33.5 ± 4.0	53.5 ± 1.7	53.7 ± 5.1	58.0 ± 7.3
	0	0	0	0.8 ± 1.5	26.0 ± 4.3	47.2 ± 4.3	51.7 ± 8.4	55.0 ± 12.1
ICR mice		Age in days and weeks						
	6 d	10 d	14 d	3 w	4 w	8 w	13 w	18 w
Popliteal	0	1.7 ± 1.1	7.7 ± 2.1	8.3 ± 0.6	12.3 ± 2.5	18.0 ± 3.0	17.7 ± 0.6	17.5 ± 5.1
	0	0	0	0	0	2.7 ± 3.8	1.3 ± 2.3	3.8 ± 3.3
Brachial	2.0 ± 2.0	10.0 ± 1.0	32.3 ± 4.9	28.3 ± 3.5	30.7 ± 3.0	45.3 ± 2.5	40.7 ± 5.7	41.5 ± 9.9
	0	0	0	0.7 ± 0.6	4.2 ± 2.0	1.3 ± 2.3	3.7 ± 4.0	18.2 ± 6.9
Axillary	2.0 ± 2.0	6.0 ± 3.0	20.0 ± 5.0	23.7 ± 2.1	19.0 ± 1.8	38.3 ± 7.1	34.3 ± 5.5	36.2 ± 6.9
	0	0	0	0	2.0 ± 3.3	3.4 ± 6.0	0.3 ± 0.6	14.8 ± 9.3

* Number of follicles: mean ± S.D. ** Number of germinal centers: mean ± S.D. At each stage, lymph nodes from 3–6 mice were examined.

Table 3. Statistic analysis of the age-dependent change in the number of lymph follicles in the respective lymph nodes

BALB/C mice	4 w against			8 w against	
	8 w	12 w	16 w	12 w	16 w
Popliteal	—	—	—	—	—
Brachial	—	—	—	—	—
Axillary	—	—	—	—	—
Inguinal	—	—	—	—	—
Submandibular	—	+	—	—	—
Deep cervical	—	—	—	—	—
Ileocecal	+	+	+	—	—

ICR mice	4 w against			8 w against	
	8 w	13 w	18 w	13 w	18 w
Popliteal	+	+	+	—	—
Brachial	+	—	—	—	—
Axillary	+	+	+	—	—

The number of follicles in a given lymph node at different ages shown in the Table was compared with that at 4 or 8 weeks of age. +, the number of follicles at the age is significantly different ($p < 0.05$; t test) from that at 4 or 8 weeks of age. —, no significant difference in the number of follicles between the 2 stages.

Table 4. Number of compartments in various lymph nodes in BALB/C and C57B1/6 mice

	Number of compartments	
	BALB/C mice	C57B1/6 mice
Popliteal	1	1
Brachial	3	3
Axillary	3	3
Inguinal	2	2
Submandibular	2	1 or 2
Deep cervical	2	1
Ileocecal	2	1

radically at 10 days and became three to seven in number by 12 days.

ICR mice: By 6 days of age, several primary follicles were seen in the outer cortex of the brachial and axillary nodes. Some of these follicles had fibrillar CR1/2 receptor networks, whilst in others this type of network was barely detectable (not shown). Over the next few days, primary follicles with CR1/2 receptor networks increased in number in these nodes.

In the popliteal nodes from 10-day old mice, one to three primary follicles were apparent (Table 2).

Age-dependent changes in lymph follicle numbers

Age-dependent changes in the number of lymph follicles within the various lymph nodes were monitored from the date of their first appearance

Table 5. Appearance of IgM-synthesizing cells per section in various lymph nodes of BALB/C mice at various postnatal ages

Ages	2 w	3 w	4 w	8 w
Lymph nodes				
Popliteal	—, —, —	±, ±, ±	±, ±, +	±, +
Brachial	±, ±, ±	+, +, +	+, +, +, +	+, +, +
Axillary	±, ±, ±	+, +, +, +	+, +, +, +, +	+, +, +, +
Inguinal	—, ±, ±	±, ±, ±	+, +, +, +	+, +, +, +, +
Submandibular	±, ±, ±	+, +, +, +	+, +, +, +, +	+, +, +, +, +
Deep cervical	±, ±, ±	+, +, +	+, +, +, +, +	+, +, +, +, +
Ileocecal	+, +, +, +	+, +, +, +, +	+, +, +, +, +, +	+, +, +, +, +, +

—, none; ±, 0–4; +, 4–15; ++, 15–30; +++, >30.

At each stage, lymph nodes from 2–3 animals were examined.

Fig. 1. Deep cervical lymph node from a BALB/C mouse at 6 days of age.

(a) Cryostat section double-stained for B220-positive B lymphocytes (blue) and PNA-positive germinal center cells (brown). Three primary follicles can be seen in the outer cortex. (b) Section adjacent to *a*, showing two of the three follicles shown in section *a* at higher magnification. Note that the fine-fibrillar networks of FDC-associated CR1/2 complement receptors are developing within these follicles.

Fig. 2. Deep cervical lymph node from a BALB/C mouse at 4 weeks of age.

(a) Cryostat section double-stained for B220-positive B lymphocytes (blue) and PNA-positive germinal center cells (brown). Several primary follicles without germinal centers can be seen in the periphery of the node. Note that this lymph node contains two compartment, each of which has one paracortex. (b) Section adjacent to *a*. Well developed networks of CR1 complement receptors can be seen within each of the follicles that are shown in section *a*.

up to 16 weeks of age. The major findings are summarized in Tables 2 and 3.

BALB/C mice: Primary follicles first appeared in the somatic nodes at 6 to 10 days of age. The number of lymph follicles per node then increased rapidly in the larger lymph nodes, whilst slowly in the smaller nodes. Therefore, during the first four weeks, the number of follicles varied according to the location of the lymph node (Table 2). Thereafter, in all of the somatic nodes that were examined, except for the submandibular node, the number of follicles per node showed only slight change until 16 weeks of age (Table 3).

The pattern of age-dependent changes in the

numbers of follicles that was seen in the ileocecal nodes differed from that seen in the somatic lymph nodes. In the ileocecal nodes, the lymph follicles appeared at about 10 days of age, and then the numbers increased linearly, reaching a plateau level at about 8 weeks of age (Tables 2 and 3).

ICR mice: The primary follicles appeared in the brachial, axillary and popliteal nodes around 6 to 10 days of age and then increased in number up to four weeks of age, with the number of follicles per node varying according to the location of the node. Over the next four weeks, the number of follicles per node further showed a substantial (statistically significant) increase, reaching a peak at 8 weeks of

Fig. 3. Brachial lymph node from a BALB/C mouse at 4 weeks of age, in a section double-stained for B220-positive B lymphocytes (blue) and PNA-positive germinal center cells (brown). Several primary follicles with no germinal centers can be seen in the periphery of the node. Note the architecture of this node, which is compartmentalized into 3 portions, each containing a single paracortex (indicated as P1, P2 and P3).

Fig. 4. Ileocecal lymph node from a BALB/C mouse at 4 weeks of age, in a section double-stained for B220-positive B lymphocytes (blue) and PNA-positive germinal center cells (brown). Germinal centers (indicated by arrowheads) can be seen within some of the lymph follicles in the periphery of the node. Also note that this lymph node contains two compartments, with one paracortex each (indicated as P1 and P2).

Fig. 5. Brachial lymph node from a BALB/C mouse at 4 weeks of age. Cryostat section stained with anti-IgM mAb. In this section, two of the three compartments of the node can be seen, each fused with the other at the medullary region. Note that IgM-synthesizing cells (intense brown, indicated by arrow), though not frequent in number, are scattered in the medullary region.

Fig. 6. Submandibular lymph node from a BALB/C mouse at 4 weeks of age. Cryostat section stained with anti-IgM mAb. A large number of IgM-synthesizing cells (intense brown) are present in the medullary region.

age (Tables 2 and 3).

Compartmentalization of the various lymph nodes

In BALB/C mice, all the somatic and ileocecal nodes, except for the popliteal nodes, were compartmentalized into two or three portions (Fig. 2–4,

Table 4). This was also found in the ICR mice. As has been reported previously (Nagata *et al.*, 1996; Hoshi *et al.*, 1997, 2001), the interior of the brachial node was divided into three portions (compartments), each of which contained a single paracortex or 'deep cortical unit' according to the terminology

of Bélisle and Saint-Marie (1981a, b). In general, the axillary node contained three paracortices, one of which was relatively large, while the other two were smaller. The inguinal, submandibular, deep cervical and ileocecal nodes consisted of two compartments with one paracortex each. The degree of lymph node compartmentalization that was noted in the BALB/C mice that were used in this study showed some variation from that previously found in C57B1/6 mice, and these differences are summarized in Table 4.

The larger, heavier lymph nodes, regardless of the time stage, tended to contain a larger number of lymph follicles than the smaller, lighter lymph nodes (see, Tables 1 and 2). In addition, in compartmentalized lymph nodes, the compartments with larger paracortices generally contained more follicles than those with smaller paracortices. For instance, at 8 weeks of age, the average number of follicles in the brachial node was 40, but within the three individual compartments the range was 10 to 18.

Germinal center development (Table 2)

BALB/C mice: In the first four weeks after birth, few, if any, germinal centers were seen in the somatic lymph nodes except the submandibular node. By 8 weeks of age, small numbers of germinal centers were visible in these nodes.

In the submandibular node, germinal centers were first observed at 21 days of age, although these were scarce. From this age onwards, germinal centers were always more prevalent in this node, compared to the other somatic nodes at all time points between four and 16 weeks of age.

In the ileocecal nodes and the distal portion of the jejunio-ileal node chain, germinal centers were seen sporadically at 21 days of age (not shown) but thereafter the numbers increased rapidly (Fig. 4, Table 2). From 8 weeks of age, almost every follicle contained a germinal center.

ICR mice: In three types of lymph nodes examined, germinal centers became apparent at four weeks of age. However, between four and 13 weeks of age, their appearance was unstable: small numbers of germinal centers were present within some nodes, while absent within others (Table 2).

Appearance of IgM-synthesizing cells in various lymph nodes of BALB/C mice

The results showing the frequency of occurrence of IgM-synthesizing cells per section at different postnatal ages are summarized in Table 5. IgM-synthesizing cells were readily distinguishable from

sIgM-bearing B-lymphocytes. The latter appeared as small round cells with expression of IgM on the surface and were either localized in the lymph follicles or were scattered around the cortico-medullary junction area. In contrast, the IgM-synthesizing cells were recognizable as round or spindle-shaped cells with a densely IgM-positive cytoplasm and were generally seen in the medullary region.

In all the nodes examined, except for the mesenteric lymph nodes, there were few IgM-synthesizing cells at 14 days. From 21 days onwards, the IgM-synthesizing cells increased slowly in number in the somatic lymph nodes. The highest concentration of this type of cell in the somatic nodes, at all the time stages, was in the submandibular nodes (Figs. 4, 5, Table 5).

In the mesenteric lymph nodes, IgM-synthesizing cells were rather frequent at 14 days, and increased rapidly in number thereafter (Table 5).

Discussion

In this study we have investigated the timing, and the order of appearance, of the primary follicles in various lymph nodes in young BALB/C mice and in three types of somatic lymph nodes in ICR mice. We have found that in most somatic nodes (i.e., brachial, axillary, inguinal, submandibular and deep cervical lymph nodes) the primary follicles expressing the FDC marker (networks of CR1/2 complement receptors) first become apparent at six days of age. However, in the mesenteric and popliteal lymph nodes the primary follicles displaying the FDC marker are first recognizable 10–12 days after birth. These findings are consistent with our previous findings in young C57B1/6 mice and may be generally applicable to other strains of mice.

FDCs constitute the major stromal element in the lymph follicles. They express Fc and complement receptors on their surfaces, and are therefore able to bind immune complexes (Gerdes *et al.*, 1982; Reynes *et al.*, 1985; Radoux *et al.*, 1985; Yoshida *et al.*, 1993; van den Berg *et al.*, 1995; Imai *et al.*, 1998). These receptors are thus useful immunocytochemical markers of FDCs. We have reported previously that when new follicles are induced in murine lymph nodes by deliberate antigen stimulation, the CR1 complement receptor networks of FDCs become apparent within the new follicles earlier than the networks of the Fc receptors (CD23 and CD16/32) appear (Horie *et al.*, 1997, 1999). In addition we have found that the CR1/2 receptor networks are the first to appear in

the developing primary follicles, whilst the CR1 receptor networks appear some time later (Hoshi *et al.*, 2001). In this current study, the CR1/2 receptor network has been identified as a FDC marker, enabling monitoring of the development of the FDC within the early primary follicles. Several authors have reported that the initial appearance of the primary follicles coincides with the development of FDCs (Dijkstra *et al.*, 1982; van Rees *et al.*, 1985; Imai *et al.*, 1986; Kroese *et al.*, 1987a). However, our findings suggest that FDC markers are present shortly after new follicles become recognizable, but do not occur in area without nodular accumulations of B-lymphocytes (Tanaka *et al.*, 1998; Horie *et al.*, 1999). It seems likely that during the early stages of differentiation from precursors, FDCs begin to express complement receptors only after interacting with the nodular accumulations of the B-lymphocytes that are present in the early primary follicles.

In this study, we estimated the possible impact of exogenous antigen stimulation on lymph nodes at different locations in BALB/C mice using two parameters: the germinal center development and appearance of IgM-synthesizing cells. In somatic lymph nodes, except for the submandibular lymph nodes, there were small numbers of IgM-synthesizing cells and few, if any, germinal centers during the first four weeks of life. This suggests that these lymph nodes do not receive effective exogenous antigen stimulation during this period. After the first month of life, there were only a limited number of germinal centers in most somatic lymph nodes at all the stage examined, suggesting that there is only a low level exogenous antigen stimulation up to 16 weeks of age. By contrast, in the mesenteric lymph nodes a certain number of IgM-synthesizing cells were present at 14 days of age and these numbers had increased significantly by 21 days. A considerable number of germinal centers had begun to appear by 28 days of age and increased thereafter. Maternal breast milk containing secretory IgA and other protective substances has been shown to protect newborn human infant from invading the exogenous antigens such as microbial flora and food proteins (Crabbé *et al.*, 1968, 1970; Cebra *et al.*, 1980; Hanson *et al.*, 1985; Parrott and MacDonald, 1990; Mackenzie, 1991; Kramer and Cebra, 1995). Our findings suggest that, despite the protective effects of maternal milk, some exogenous antigens, possibly derived from the intestinal lumen, reach the mesenteric lymph nodes during the suckling period, and their arrival at these nodes enhances after weaning.

The primary concern of this study was to understand the age-dependent changes in the numbers of

lymph follicles that occur in various lymph nodes during ontogenesis. In BALB/C mice, there were noticeable differences in the patterns of age dependent changes between the somatic and the mesenteric (ileocecal) lymph nodes. In general, in the somatic nodes primary follicles first appeared by six days of age, then increased in number rather sharply in larger nodes, while slowly in smaller ones, up to 28 days. The number of follicles per node at this stage therefore varied according to the location of the particular node. Apart from the submandibular nodes, it appears that none of the somatic lymph nodes received effective exogenous antigen stimulation during the first 28 days after birth. Our conclusion is that during this period the mechanism of development of the primary follicles in these lymph nodes is antigen-independent. Between 4 and 16 weeks of age, the number of lymph follicles in most of the somatic nodes showed no statistically significant change, although the mean number of follicles per node tended to exhibit a peak at 8 or 12 weeks of age. From these observations, it may well be said that in this murine strain, the ontogenetic formation of lymph follicles in the somatic nodes is largely completed within the first 4 weeks of life.

In the mesenteric (ileocecal) lymph nodes, the number of follicles increased during the suckling period and continuously after weaning to reach a plateau at 8 weeks of age. As mentioned above, after weaning the mesenteric nodes appear to receive effective exogenous antigens from the intestinal lumen. These antigens may stimulate germinal center development in the existing primary follicles, and also induce the formation of new follicles, resulting in a continuous increase in numbers that reaches a plateau at 8 weeks of age.

There was wide variation in the number of follicles that were present in the different somatic node types by 28 days of age. The number of follicles was roughly associated with the volume or weight of the lymph node, and this in turn appeared to be closely associated with the size of the area drained by that particular lymph node. Tilney (1971) has identified the body regions that are drained by individual somatic lymph nodes in rats. In addition, Sainte-Marie and colleagues (Bélisle and Sainte-Marie, 1981a, b; Sainte-Marie *et al.*, 1982) have documented that the size of the paracortex (termed the 'deep cortical unit' by these authors) is mainly regulated by the amount of lymph carried to the node via the afferent lymphatics. We have previously shown, in our studies in C57B1/6 mice, that the brachial and axillary nodes are generally compartmentalized into three portions, each of which contains one paracortex and drains a dif-

ferent body region (Fig. 1) (Nagata *et al.*, 1996; Hoshi *et al.*, 1997). There was some variability between the BALB/C and the C57B1/6 mice in the degree and pattern of compartmentalization in the different types of lymph nodes. In the C57B1/6 mice, the deep cervical and ileocecal nodes, and sometimes the submandibular node, consisted of a single compartment with a single paracortex, whereas in BALB/C mice, these lymph nodes, as well as the inguinal node, were composed of two compartments with one paracortex each. From the present and previous observations on BALB/C and C57B1/6 mice, it is possible to roughly predict the number of lymph follicles that will be produced in a somatic lymph node during the first 28 days of life. When a somatic node consists of a single compartment, the larger the size of the paracortex, the larger the number of lymph follicles produced. Similarly, when a somatic node contains two or three compartments, the larger the paracortex in a particular compartment, the larger the number of follicles that will be produced in that compartment. From these observations, it is evident that the number of lymph follicles that are produced in a particular somatic lymph node during the first 28 days of life is closely related to the size of the area drained by that lymph node at that stage of development. This suggests that the production of primary follicles during the ontogenesis of a somatic node is mainly regulated by the amount of afferent lymph transported from the draining area into that node, or by the quantity of certain cellular and humoral elements contained within that afferent lymph. On the basis of these findings, we conclude that the primary follicles that develop ontogenetically within the somatic lymph nodes during the first 28 days of life are antigen-independent but, nevertheless, 'reactive' in origin.

Three types of somatic nodes that were examined in ICR mice showed somewhat different patterns of age-dependent changes in the numbers of lymph follicles during ontogenesis, in comparison with the case of the somatic nodes in BALB/C mice. In ICR mice, a certain number of follicles were produced in each type of somatic nodes during the first 28 days of life. This was followed by a substantial production of follicles over the subsequent four weeks, with the number of follicles per node reaching a peak at 8 weeks of age. In the somatic lymph nodes in the ICR mice, as well as in most somatic nodes in the BALB/C mice, only a small numbers of germinal centers developed between four and 8 weeks of age. This is thought to indicate that these nodes received only a low level of exogenous antigen stimulation during this period. However, since the somatic nodes in the

BALB/C mice that received this level of exogenous antigen stimulation did not show a statistically significant increase in the number of follicles during this period, it is extremely unlikely that a similar low level of exogenous antigen stimulation would result in a significant increase in the number of follicles in the somatic nodes in ICR mice. We therefore assume that the ontogenetic production of lymph follicles in somatic nodes in ICR mice, probably mediated by certain cellular and/or humoral element(s) contained in the afferent lymph, may occur not only during the first four weeks of life, but also continue up to the young adult stage of 8 weeks of age. The reason why the ontogenic formation of lymph follicles in the somatic nodes in BALB/C mice is almost completed during the first four weeks of life, while this process continues up to 8 weeks of age in ICR mice, is currently unclear. It is possible that the number of lymph follicles that are produced in a particular somatic lymph node during the first 4 weeks of life may be closely related to the dimension of the body region being drained by that lymph node at that stage of development. Over the next 4 weeks, the size of the draining area of a lymph node may enlarge in parallel with overall body growth (as expressed by gain in body weight). Between 4 and 8 weeks of age, male BALB/C mice gained approximately 7.5 g in body weight, whilst male ICR mice gained 12.5 g (Table I). Since BALB/C mice have a relatively small body size, the increase in the dimension of the draining area of a somatic node during this period may not be insufficient to cause a substantial increase in the number of follicles per node, although this may be sufficient in ICR mice. We have previously reported that the primary follicles in the popliteal node of growing Wistar rats first appear at around 18 days of age, then increase in numbers, reaching approximately 40 follicles by four weeks of age (Murakami *et al.*, 1989; Suzuki *et al.*, 1996). The follicles continued to increase in number until a plateau (80–90) is reached at about 10–12 weeks of age. The body weight of male rats is about 50 g at four weeks and about 300 g at 10–12 weeks. In considering these observations, we postulate that in relatively small murine strains, such as BALB/C and C57B1/6, the ontogenetic formation of lymph follicles in a somatic node is largely achieved during the first 28 days of life. By contrast, in murine strains where there is a relatively large body size, such as ICR mice, this process occurs not only during this early period but also continues up to the young adult stage at 8 weeks of age.

References

- 1) Blisle C and Sainte-Marie G. Tridimensional study of the deep cortex of the rat lymph node. I: Topography of the deep cortex. *Anat Rec* 1981a; **199**:45–59.
- 2) Blisle C and Sainte-Marie G. Tridimensional study of the deep cortex of the rat lymph node. II: Relation of deep cortex units to afferent lymphatic vessels. *Anat Rec* 1981b; **199**:61–72.
- 3) Burstone MS. Histochemical comparison of naphthol AS-phosphates for the demonstration of phosphatase. *J Natl Cancer Inst* 1958; **20**:601–615.
- 4) Cebra JJ, Gearhart PJ, Halsey JF, Hurwitz JL and Shahin RD. Role of environmental antigens in the ontogeny of the secretory immune response. *J Retl Soc* 1980; **28** (suppl):61s–71s.
- 5) Chen D, Hoshi H, Tanaka K and Murakami G. Postnatal development of lymphoid follicles in rat Peyer's patches, with special reference to increased follicle number. *Arch Histol Cytol* 1995; **58**:335–343.
- 6) Conway W. Cyclic changes in lymphatic nodules. *Anat Rec* 1937; **69**:487–513.
- 7) Crabb PA, Basin H, Eyssen H and Hermans JF. The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. *Int Arch Allergy and Appl Immunol* 1968; **34**:362–376.
- 8) Dijkstra CD, Tilburg NJ and Dpp EA. Ontogenetic aspects of immune complex trapping in the spleen and popliteal lymph nodes of the rat. *Cell Tissue Res* 1982; **223**:545–552.
- 9) Dijkstra CD, TE Velde AA and VAN Rooijen N. Localization of horseradish peroxidase (HRP)-anti-HRP complexes in cryostat sections: Influence of endotoxin on trapping of immune complexes in the spleen of the rat. *Cell Tissue Res* 1983; **232**:1–7.
- 10) Eikelenboom P, Lvenbach MGE, VAN DEN Brink HR and Streefkerk JG. Development of T and B cell areas in peripheral lymphoid organs of the rat. *Anat Rec* 1979; **194**:523–538.
- 11) Gerdes J and Stein H. Complement (C3) receptors on dendritic cells of normal and malignant lymphoid tissue. *Clin Exp Immunol* 1982; **48**:348–352.
- 12) Gray D. Recruitment of virgin B cells into an immune response is restricted to activation outside lymphoid follicles. *Immunology* 1988; **65**:73–79.
- 13) Gray D, Kosco M and Stockinger B. Novel pathways of antigen presentation for the maintenance of memory. *Int Immunol* 1991; **3**:141–148.
- 14) Groscurth P. Non-lymphatic cells in the lymph node cortex of the mouse. II. Postnatal development of the interdigitating cells and the dendritic reticular cells. *Pathol Res Pract* 1980; **169**:235–254.
- 15) Gutman GA and Weissman L. Lymphoid tissue architecture. *Immunology* 1972; **23**:465–479.
- 16) Hanson LA, Ahlstedt S, Andersson B, Carisson B, Fllstrm SP, Mellander L, Possas O, Soderstrom T and Eden CS. Protective factors in milk and the development of the immune system. *Pediatr* 1985; **75**(suppl):172–176.
- 17) Horie K and Hoshi H. Induction of lymph follicle formation with several mitogens and adjuvants in the mouse popliteal lymph node. *Histol Histopathol* 1989; **4**:17–25.
- 18) Horie K, Nagata H, Aizawa S and Hoshi H. An immunohistochemical study on the development of antigen trapping capacity in reactively induced lymphoid follicles in mouse popliteal lymph nodes. *Acta Anat Nippon* 1997; **72**:358.
- 19) Horie K, Chen D and Hoshi H. Development of immune complex trapping: experimental study of lymphoid follicles and germinal centres newly induced by exogenous stimulants in mouse popliteal lymph nodes. *Histol Histopathol* 1999; **14**:11–21.
- 20) Hoshi H, Kamiya K, Takemoto T and Aijima H. Formation of lymph follicles and germinal centers in draining lymph nodes after local injection of phytohemagglutinin and lipopolysaccharide in mice. *Arch Histol Jap* 1984; **47**:505–518.
- 21) Hoshi H, Kamiya K, Nagata H, Yoshida K and Aijima H. Formation of lymph follicles in draining lymph nodes after local injection of various antigenic substances in mice. *Arch Histol Jap* 1986; **49**:25–37.
- 22) Hoshi H, Nagata H and Horie K. Induction of lymph follicle formation with endotoxin lipopolysaccharide in the draining lymph node of athymic nude mice. *Pathol Res Pract* 1989; **184**:60–68.
- 23) Hoshi H, Nagata H, Aijima J and Horie K. Functional compartmentalization of the lymph node architecture and afferent lymphatic vessels. In: (ed. by) Ohtani O, Kato S and Uchino S: *The Lymphatic Vessels: Morphology, Function, Development*. (p 45–52) Nishimura, Niigata, 1997. (In Japanese).
- 24) Hoshi H, Horie K, Tanaka K, Nagata H and Aizawa S. Patterns of age-dependent changes in the numbers of lymph follicles and germinal centres in somatic and mesenteric lymph nodes in growing C57B1/6 mice. *J Anat* 2001; **198**:189–205.
- 25) Imai Y, Yamakawa M and Kasajima T. The lymphocyte-dendritic cell system. *Histol Histopathol* 1998; **13**:502–544.
- 26) Klaus GGB, Humphrey JH, Kunkl A and Dongworth DS. The follicular dendritic cells: its role in antigen presentation in the generation of immunological memory. *Immunol Rev* 1980; **53**:3–28.
- 27) Kramer DF and Cebra JJ. Early appearance of 'natural' mucosal IgA responses and germinal centres in suckling mice developing in the absence of maternal antibodies. *J Immunol* 1995; **154**:2051–2062.
- 28) Kroese FGM, Wubbena AS, Kuijpers KC and Nieuwenhuis P. The ontogeny of germinal centre forming capacity of neonatal rat spleen. *Immunology* 1987a; **60**:597–602.
- 29) Kroese FGM, Wubbena AS, Seijen HG and Nieuwenhuis P. Germinal centres develop oligoclonally. *Eur J Immunol* 1987b; **17**:1069–1072.
- 30) Liu Y-J, Zhang J, Lane PJL, Chan EYT and MacLennan ICM. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur J Immunol* 1991; **21**:2951–2962.
- 31) Liu Y-J and Appin C. Germinal centre development. *Immunol Rev* 1997; **156**:111–126.
- 32) Mackenzie HM. Transport of maternally derived immunoglobulin across the intestinal epithelium. In: (ed. by) Macdonald TT: *Ontogeny of the Immune System of the Gut*. (p 69–81). CRC Press, Boca Raton, 1991.
- 33) Mandel TE, Phipps RP, Abbot A and Tew JG. The follicular dendritic cells: long term antigen retention during immunity. *Immunol Rev* 1980; **53**:29–59.
- 34) Murakami Y, Nagata H and Hoshi H. Effect of blockage of the afferent lymphatic vessels on the development of popliteal lymph nodes in the rat. *Acta Anat Nipp* 1989; **64**:550–560 (In Japanese).
- 35) Nagata H, Tanaka K, Imazeki T, Adachi N and Hoshi H. Functional compartmentalization of the nodal architecture in the mouse brachial and axillary lymph nodes. *J Lymphol* 1996; **19**:73–84 (In Japanese).
- 36) Nieuwenhuis P, Korese FDM, Opstelten D and Seijen HG. De novo germinal centre formation. *Immunol Rev* 1992;

- 126:77–98.
- 37) Nossal GJV, Abbot A, Mitchell J and Lummus Z. Antigen in immunity. XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles. *J Exp Med* 1968; **127**:277–290.
- 38) Parrott DMV and Macdonald TT. The ontogeny of the mucosal immune system in rodents. In: (ed. by) Macdonald TT: *Ontogeny of the Immune System of the Gut*. (p 69–81). CRC PRESS, Boca Raton, 1990.
- 39) Radoux D, Kinet-Denoël C, Heinen E, Moeremans M, DE Mey J and Simar LJ. Retention of immune complexes by Fc receptors on mouse follicular dendritic cells. *Scand J Immunol* 1985; **21**:345–353.
- 40) Reynes M, Aubert JP, Cohen JHM, Audouin J, Tricottet V, Diebold J and Kazatchkine MD. Human follicular dendritic cells express CR1, CR2, and CR3 complement receptor antigens. *J Immunol* 1985; **135**:2687–2694.
- 41) Sainte-Marie G, Peng FS and Bêlisle C. Overall architecture and pattern of lymph flow in the rat lymph node. *Amer J Anat* 1982; **164**:275–309.
- 42) Sjövall A and Sjövall H. Experimentelle Studien über die Sekundärknötchen in den Kniekehlenlymphknoten des Kaninchens bei *Bacillus phocyaneus*-Infektion. *Virchow Archiv Pathol Anat* 1930; **278**:258–283.
- 43) Suzuki S, Chen D, Tanaka K and Hoshi H. Effects of sublethal X-irradiation on the postnatal development of lymphoid follicles in the popliteal lymph node of the rat. *J Nihon Univ Med Assoc* 1996; **55**:39–48 (In Japanese).
- 44) Tanaka K, Chen D, Negichi S, Aizawa S and Hoshi H. Splenic adherent cells, stimulated in vitro, induce the reactive formation of lymphoid follicles and germinal centres in draining lymph nodes after subcutaneous transfusion into syngeneic mice. *J Anat* 1998; **193**:49–59.
- 45) Tilney NL. Patterns of lymphatic drainage in the adult laboratory rat. *J Anat* 1971; **109**:369–383.
- 46) VAN DEN Berg TK, Yoshida K and Dijkstra CD. Mechanism of immune complex trapping by follicular dendritic cells. *Curr Topic Microbiol Immunol* 1995; **201**:49–67.
- 47) VAN Rees EP, Dopp EA, Dijkstra CD and Sminia T. The postnatal development of cell populations in the rat popliteal lymph node. An immunohistochemical study. *Cell Tissue Res* 1985; **242**:391–398.
- 48) VAN Rees EP, Dijkstra CD and VAN Rooijen N. The early postnatal development of the primary immune response in TNP-KLH-stimulated popliteal lymph node in the rat. *Cell Tissue Res* 1986; **246**:673–677.
- 49) Yoshida K, VAN DEN Berg TK and Dijkstra CD. Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and FcRgII-mediated immune-complex trapping. *Immunology* 1993; **80**:34–39.