Immunohistochemical Studies on Ontogeny of Bovine Lymphoid Tissues

Seishi ISHINO, Koichi KADOTA, Yutaka MATSUBARA, Hiroo AGAWA¹⁾, and Nozomi MATSUI²⁾

National Institute of Animal Health, Tsukuba, Ibaraki 305, ¹⁾Higashihiroshima Livestock Hygiene Center, Saijo-gojomachi, Higashihiroshima, Hiroshima 724, and ²⁾Kihoku Livestock Hygiene Center, Sonobe, Wakayama 640, Japan

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ABSTRACT. Developing lymphoid tissues of bovine fetuses ranging from 70 to 270 days of fetal age were examined by histological and immunohistochemical procedures. In the peripheral blood, surface membrane immunoglobulin bearing cells (B-lymphocytes) and sheep red blood cell rosette forming cells (T-lymphocytes) had already appeared by 70 days of fetal age. In the lymph nodes intracytoplasmic IgM positive cells appeared at 90 days of fetal age. The cells positive for IgG appeared at 150 days of fetal age and IgA positive cells appeared at 180 days of fetal age. The spleen contained intracytoplasmic immunoglobulin positive cells at almost the same time as those in the lymph nodes. In the ileocecal region, IgM positive cells and IgG positive cells were present at 180 days of fetal age and IgA positive cells were present at 210 days of fetal age. The tonsils contained IgM positive cells and IgG positive cells at 240 days of fetal age. In the thymus, terminal deoxynucleotidyl transferase positive cells appeared at 90 days of fetal age.—**KEY WORDS:** bovine fetus, immunoglobulin, lymphoid tissue, ontogeny, TdT.

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The immune system has important roles for the host defence system. The ontogeny of the immune system has been studied in the fetus of man [1, 4, 6, 7, 10] and several animals [9, 11, 14, 15]. Over the last several years, monoclonal antibodies that recognize lymphocyte antigen have been prepared in ruminants. They have been classified using cluster of differentiation (CD) as described in man and evaluated in their specificity [8]. Although detailed studies on the development of the ovine lymphoid system have been done [9, 16], there are few reports on the morphological development of the lymphoid tissues in bovine fetuses. The subpopulation of lymphocytes [21] or IgM and IgG containing cells have been studied in bovine fetuses [20]. The recent immunoperoxidase procedures will hopefully provide more information on the morphological ontogeny of bovine lymphoid tissues. To understand the ontogenic development of bovine lymphoid tissues, we examined the subpopulations of lymphocytes in the peripheral blood and morphology of the lymphoid tissues at different stages of gestation.

MATERIALS AND METHODS

Bovine fetuses: Twenty one Holstein fetuses (12 males and 9 females) ranging from 70 to 270 days of fetal age were obtained from a local abattoir. The specimens were grouped according to fetal age which was estimated by crown to rump measurements [17].

Hematological examinations: The numbers of red blood cells and white blood cells were counted in the blood collected by a cardiac puncture. The percentage of differential white blood cells was enumerated on blood smears stained with Giemsa's solution.

Histological examinations: At necropsy, the pre-scapular and prefemoral lymph nodes, spleen, tonsils, thymus and ileocecal region of the intestine were sampled. They were fixed in 10% buffered formalin and processed by routine paraffin wax sectioning methods. Sections were stained with hematoxylin and eosin or silver impregnation.

Subpopulation of peripheral lymphocytes: Mononuclear cells were separated from heparinized blood by a Lymphoprep procedure (Nyegaard & Co., Norway). The mononuclear cell fraction contained not only lymphocytes but also nuclear red blood cells, which could not be differentiated morphologically from lymphocytes in the cell suspension, especially in younger fetuses. An E-rosette formation (RF) test using sheep red blood cells (SRBC) for demonstration of T-lymphocytes and a fluorescent antibody method using anti-bovine IgM rabbit serum (Fc specific, Nordic Immunological Lab. Netherland) for detection of surface membrane immunoglobulin (sIg) of B-lymphocytes were carried out as described by Senogles *et al.* [21].

Immunohistochemical examinations: The paraffin wax sections from the lymphoid tissues of bovine fetuses and a 3-month-old calf, as a control, were

examined immunohistochemically. After inactivation of endogenous peroxidase by 3% hydrogen peroxide, the sections were incubated in a rabbit serum of anti-bovine IgM (μ -chain specific, Miles Lab., U.S.A.), IgG (Fc specific, EY Lab., U.S.A.), IgA (α -chain specific, Miles Lab., U.S.A.), anti-terminal deoxynucleotidyl transferase (TdT, Life Sciences Inc., U.S.A.) or a monoclonal antibody of anti-S-100 protein α -subunit (S-100 α , Japan Immuno-Research Lab., Japan). Then the sections were treated with a biotin streptavidin system (BioGenex Lab., U.S.A.).

RESULTS

Findings of peripheral blood: The results of hematological and immunological examinations of the peripheral blood are presented in Table 1. The lymphocytes were only 3.1% of white blood cells, which contained a large number of nuclear red blood cells, at 70 days of fetal age. They increased to 51.5% by 270 days of fetal age. The sIg bearing cells were 2.1% of the mononuclear cells at 70 days of fetal age and increased to 30% at the terminal stage of gestation. Whereas the RF cells were 0.6% at 70 days of fetal age and adhered to 3 to 7 SRBC, they increased to 5.4% by 270 days of fetal age and adhered to 3 to more than 10 SRBC.

Macroscopical findings: The prescapular and pre-femoral lymph nodes were less than 1 mm in longitudinal length at 70 days of fetal age and developed to 30 mm in length at 270 days of fetal age. The spleen was 4.5 mm in length and had a dark red leaflet-like appearance at 70 days of fetal age and increased to 180 mm in length by 270 days of fetal age. The Peyer's patches in the ileocecal region were not observed through all the fetal ages. In the tonsillar region, small crypts were formed on the oral mucosa at more than 180 days of fetal age. The cervical thymus was already identified as a thin thread-like tissue, 12 mm in length, at 70 days of fetal age. The cervical and thoracic thymus were 265 mm and 110 mm in length, respectively, at 270 days of fetal age.

Microscopical findings: Lymph nodes: The lymph nodes consisted of spindle cells resembling mesenchymal cells without any indication of cortex and medulla at 70 days of fetal age (Fig. 1). They contained a few hematopoietic cells in an admixture of primitive lymphocytes having dark round nuclei and basophilic cytoplasm. Primitive lymphocytes rapidly filled the cortical and paracortical regions between 90 and 120 days of fetal age. In the fetuses more than 150 days old, the cortical structure was distinct with relatively mature lymphocytes. A few lymphocytes were present in the medulla. Follicular

Table 1. Hematological examinations and number of animals having intracytoplasmic immunoglobulin positive cells in lymphoid tissues

Fetal age (days)	Number of fetus examined	Hematological examination of blood					Animal with Intracytoplasmic immunoglobulin positive cells														
		RBC ^{a)}	WBC ^{b)}	Lym ^{c)}	sIg ^{d)}	RF ^{e)}	Lymph nodes			Spleen			Ileocecal region			Tonsils			Thymus		
		10 ⁴ / μ l	10 ³ / μ l	%	%	%	M ^{f)}	G ^{g)}	A ^{h)}	M	G	A	M	G	A	M	G	A	M	G	A
70	2	NE ⁱ⁾	NE	3.1 (± 0.21) ^{j)}	2.1 (± 1.27)	0.6 (± 0.56)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
90	3	233 (± 7.2)	14.2 (± 0.57)	5.8 (± 0.52)	3.5 (± 1.33)	2.3 (± 0.55)	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0
120	3	389 (± 73.1)	14.4 (± 0.35)	6.5 (± 1.38)	5.0 (± 1.00)	3.9 (± 1.01)	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0
150	2	515 (± 96.8)	12.1 (± 2.89)	14.1 (± 4.50)	9.0 (± 1.41)	6.7 (± 4.66)	2	1	0	2	1	0	0	0	0	0	0	0	0	0	0
180	5	550 (± 55.5)	10.6 (± 0.59)	34.3 (± 10.34)	8.4 (± 3.15)	5.1 (± 3.06)	5	3	1	5	3	1	1	1	0	0	0	0	0	0	0
210	3	522 (± 120.1)	9.5 (± 1.89)	45.3 (± 7.45)	17.5 (± 6.59)	3.1 (± 0.49)	3	3	0	3	3	1	3	2	1	0	0	0	0	0	0
240	2	559 (± 19.7)	8.8 (± 1.55)	51.0 (± 19.09)	16.3 (± 2.12)	5.5 (± 4.90)	2	2	1	2	2	1	1	1	1	2	1	0	1	1	1
270	1	583	8.4	51.5	30.0	5.4	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0

a) Average count of red blood cells. b) Average count of white blood cells including nuclear red blood cells. c) Average percent of lymphocytes in white blood cells. d) Average percent of surface membrane immunoglobulin positive cells in mononuclear cell fraction separated by a Lymphoprep procedure. e) Average percent of rosette forming cell in mononuclear cell fraction separated by a Lymphoprep procedure. f) IgM. g) IgG. h) IgA. i) Not examined. j) Figures in parenthesis are values in standard deviation.

Fig. 1. Prescapular lymph node, 70 days of fetal age. The parenchyma consists of spindle cells resembling mesenchymal cells with a few primitive lymphocytes and hematopoietic cells. HE. $\times 40$.

Fig. 2. Spleen, 70 days of fetal age. It consists of spindle cells occasionally with a few primitive lymphocytes and hematopoietic cells. HE. $\times 100$.

Fig. 3. Ileocecal region, 210 days of fetal age. Follicular structures are formed. HE. $\times 100$.

Fig. 4. Thymus, 70 days of fetal age. Thymic structure is lobulated with numerous thymocytes. HE. $\times 40$.

structures appeared at 180 days of fetal age. After 210 days of fetal age, the lymph nodes showed a similar appearance to those of neonatal calves.

Spleen: The spleen was composed of spindle cells occasionally with a few hematopoietic cells and primitive lymphocytes at 70 days of fetal age (Fig. 2). The white pulp was partially differentiated into laminar tissue around the central arteries and contained a small number of primitive lymphocytes between 90 to 120 days of fetal age. This structure developed into distinct white pulp and contained

numerous lymphocytes after 150 days of fetal age but follicular structures were not identified in all the age groups.

Tonsils: Lymphocytes were not detected until 150 days of fetal age. A small number of lymphocytes infiltrated around the submucosal blood vessels at 150 days of fetal age. In the several fetuses aged more than 180 days, epithelial crypts grew down into the submucosa with moderate lymphocytic infiltration. There was no evidence of follicular structures even in a 270-day-old fetus.

Fig. 5. IgM positive cells (arrow) in prescapular lymph node, 90 days of fetal age. Biotin-streptavidin (B-SA) method. $\times 1,000$.

Fig. 6. IgG positive cells (arrow) in prescapular lymph node, 150 days of fetal age. B-SA method. $\times 1,000$.

Fig. 7. IgA positive cells (arrow) in prescapular lymph node, 180 days of fetal age. B-SA method. $\times 1,000$.

Fig. 8. Terminal deoxynucleotidyl transferase positive cells (arrows) in cortex of thymus, 270 days of fetal age. B-SA method. $\times 1,000$.

Ileocecal region: Peyer's patches were first identified as mild infiltration of lymphocytes around blood vessels in the lamina propria at 120 days of fetal age. Distinct lymphoid aggregations were present at 150 days of fetal age. Follicular structures were first observed in 2 cases at 180 days of fetal age and increased in number with fetal growth (Fig. 3).

Thymus: At 70 days of fetal age, the thymic structure was somewhat lobulated and the cortical region contained numerous medium sized cells having dark nuclei and scanty basophilic cytoplasm,

which were similar to primitive lymphocytes (Fig. 4). The medullary region was narrow and contained a small number of lymphocytes and Hassall's corpuscles. The lymphocytes increased in number and corticomedullary differentiation became more distinct between 90 and 120 days of fetal age. After 150 days of fetal age, the thymus was deeply lobulated and similar to the structure in new born calves.

Immunohistochemical findings: The youngest fetal age at which the lymph nodes contained IgM cells (Fig. 5) was 90 days (Table 1), a few positive cells

being scattered in the cortex at this stage. IgM positive cells were then markedly increased in number in the cortex and medulla by 150 days of fetal age. Subsequently, IgG positive cells were detected at 150 days of fetal age (Fig. 6) and increased in number parallel to fetal growth. The cells positive for IgA were present at 180 days of fetal age (Fig. 7). They were very few in number until the terminal stage of gestation. The cell types positive for intracytoplasmic immunoglobulin (cIg) were relatively large lymphocytes in younger fetuses by 150 days of fetal age and medium to large sized lymphocytes in older fetuses over 180 days of fetal age. The spleen had the same tendency for the appearance of IgM, IgG or IgA containing cells as those of the lymph nodes. The tonsils had IgM or IgG containing cells at 240 days of fetal age. In the Peyer's patches of the ileocecal region, IgM or IgG containing cells appeared at 180 days of fetal age and IgA containing cells appeared at 210 days of fetal age. In the thymus a few lymphocytes positive for each class of the immunoglobulins were mixed among numerous lymphocytes at 240 days of fetal age.

Although follicular dendritic reticulum cells (FDC) positive for S-100 α were observed in follicles of the lymph nodes of a 3-month-old calf, these cells were not detected in follicular structures of the lymph nodes and Peyer's patches of the ileocecal region in bovine fetuses.

In the thymus, TdT positive cells first appeared in the cortical region at 90 days of fetal age. They occupied the cortical region after 210 days of fetal age (Fig. 8).

DISCUSSION

The lymphocytes first appear in human peripheral blood between 49 and 56 days of fetal age and increase up to 30% of the white blood cells with predominance of T-lymphocytes until 140 days of fetal age [18]. In bovine fetuses, the lymphocytes appear in the peripheral blood by 45 days of fetal age [19]. The lymphocytes having B- or T-cell marker are identified at 90 days of fetal age. T-lymphocytes increase to 45% of the lymphocytes in spite of only 1% B-lymphocytes in the terminal stage of gestation [21]. The present study clarified that the positive cells in sIg and RF tests were already present at 70 days of fetal age, 20 days earlier than that reported elsewhere [21]. Because

conventional bovine fetuses were used, a high level of sIg bearing cells, which were 30% of the mononuclear cells, at the terminal stage of gestation may be due to some antigenic stimulation. On the other hand, a low level of RF cells may be related to the time after death of the fetuses and also the low viability of cells.

Human lymph nodes and spleen are first seen at 49 days of fetal age [1, 7, 18] and the lymphocytes having B- or T-cell marker are already detectable in the spleen at 98 days of fetal age [13]. In bovine fetuses, the lymph nodes first appear at 60 days of fetal age and IgM containing cells are detected at 70 days of fetal age [20]. The spleen is developed by 55 days of fetal age and IgM and IgG containing cells appear 59 and 145 days of fetal age, respectively [19, 20]. In the present study, immunoglobulin containing cells were not demonstrated in the lymph nodes and spleen at 70 days of fetal age. The fetuses of this age group may not be exposed to antigenic stimulation as described previously [20] and, besides, most of lymphocytes may be too primitive to produce immunoglobulins. The cells containing IgM appeared at 90 days of fetal age, 31 days later than that in a previous report [20]. The cells containing IgG appeared at 150 days, being almost the same stage as that described in the similar report [20]. It was clarified that IgA containing cells appeared at 180 days of fetal age, later than IgM or IgG containing cells.

The tonsils are first identified in 98-day-old human fetuses [5, 7] and follicular structures in these organs develop earlier than in all the other lymphoid tissues [5]. The present fetuses had lymphocyte infiltration in the tonsils after 150 days of fetal age but follicular structures were not formed in all the age groups. The immunoglobulin containing cells appeared at a relatively later stage. Therefore, development of bovine tonsils may be completed after birth.

The Peyer's patches are first detected in 98-day-old human fetuses [7]. The Peyer's patches of sheep, which appear first in the jejunum at 60 days of fetal age, may play a role similar to that of the bursa of Fabricius in birds [16]. In bovine fetuses, the Peyer's patches are recognized first in the jejunum, and develop lastly in the ileum [3]. As the Peyer's patches of the other portions in the intestine were not examined in the present study, we can only speculate that their early development may occur in the jejunum and the development of gut associated lymphoid tissue of bovine fetuses may be similar to

that of ovine fetuses. Infiltration of lymphocytes and appearance of immunoglobulin containing cells in the ileocecal region were later than that in the lymph nodes and spleen. The development of the lymph nodes and spleen may at least be earlier than the ileocecal region and a specific microenvironment for differentiation into immunoglobulin producing cells may be provided.

FDC may be a prerequisite for the immunological maturity of B-lymphocytes in the human fetus [10]. In follicular structures of bovine fetuses, FDC could not be demonstrated by an immunoperoxidase method using anti-S-100 α antibody in spite of demonstration of FDC in the postnatal follicles. FDC in bovine fetuses may be still immature and may have a little amount of S-100 protein which can not be detected by the present method.

In the thymus, CD2 antigen is expressed in the early stage of differentiation of thymocytes. Then the double positive cells for CD4 and CD8 antigens appear in the cortical thymocytes. Some of them are selected and become single positive cells for CD4 or CD8 antigen [12]. On the other hand, TdT is present in cortical thymocytes as well as primitive bone marrow cells but not contained in fetal thymocytes by 140 days in man [2]. The present study showed that TdT positive cells appeared at 90 days of fetal age in spite of accumulation of numerous lymphocytes in the thymus at 70 days of fetal age. This result may show that TdT negative cells first populate the thymus and then TdT positive cells migrate secondarily into this organ as described by Bodger *et al.* [2].

The ontogeny of the lymphoid tissues in the present study will help us to understand the bovine immune system clearly.

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