Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in Peyer's Patches of the Ileum, in Thymus and in Lymph nodes of Preterm Calves, and in Full-Term Calves at Birth and on Day 5 of Life

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ABSTRACT

Peyer's patches, thymus, and lymph nodes contain the majority of lymphocytes. We have studied proliferation rates, apoptosis rates, and numbers of B- and T-lymphocytes in Peyer's patches in ileum, thymus, and mesenterial and prescapular lymph nodes (LM and LP) in unfed preterm calves (GrP; born 13 d before expected normal term after dams were injected with prostaglandin F_{2α} and glucocorticoids) and normal-term calves (GrF) immediately after birth and on d 5 of life after feeding colostrum for 4 d (GrC). Immunohistochemical methods in conjunction with incorporation of 5-bromo-2'-deoxyuridine or terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling were used to evaluate cell proliferation rates and apoptosis rates, respectively. The number of T- and B-lymphocytes was determined with monoclonal antibodies directed against CD3 and CD79, respectively. In GrF compared with GrP, there were higher numbers of proliferating and apoptotic cells in LM and LP, of B-lymphocytes in paracortex and follicles of LM and LP, and of proliferating cells in cortex and medulla of thymus. In thymus cortex and medulla, numbers of proliferating cells were higher in GrC than in GrF. Apoptotic rates were generally smaller at all sites of Peyer's patches in GrC than in GrF, and proliferation rates increased from GrP to GrF in intrafollicular areas and from GrF to GrC in all tissues. Numbers of T-lymphocytes in Peyer's patches were higher in GrF than in GrP, but lower in GrC than in GrF, except in the domes. Numbers of B-lymphocytes did not change in Peyer's patches despite high prolifera-

tion and low apoptotic rates, suggesting that they leave Peyer's patches during the first days of life. In conclusion, proliferation and apoptosis rates and numbers of B- and T-lymphocytes in Peyer's patches in ileum, thymus, and LM and LP exhibited different developmental changes and were affected by feeding.

(**Key words:** lymphoid tissue, cell proliferation, apoptosis, neonatal calf)

Abbreviation key: BrdU = 5-bromo-2'-deoxyuridine, CD = cluster of differentiation, FAE = follicle-associated epithelium, GrP = group born preterm, GrF = group born full term, GrC = group born full term and fed colostrum for 3 d and milk replacer for 1 d, IEL = intraepithelial lymphocytes, LM = mesenterial lymph node, LP = prescapular lymph node, TUNEL = terminal deoxynucleotidyl transferase (TdT)-mediated X-dUTP nick end labeling.

INTRODUCTION

Mortality and morbidity rates in young animals, including calves, are particularly high in the neonatal period and are especially elevated if born preterm (Flemming et al., 2001). Perinatal losses are partly due to insufficient maturity and function of various organ systems, especially of the lung (Zaremba et al., 1997), of the gastrointestinal tract (Sangild et al., 2000), and of the immune system (Banks and McGuire, 1989; Barrington et al., 2001). The situation in neonatal calves is complex because they need passive immunoprotection by the ingestion of immunoglobulins with colostrum (Barrington et al., 2001), thereby reducing the need for active immunity and thus delaying the postnatal activation of lymphoid organs (Banks and McGuire, 1989). In view of the great losses due to infections in neonatal calves in general and of preterm calves in particular (Sangild, 2000), more information is required on developmental changes and function of lymphoid tissues in the perinatal period. Gut-associated

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lymphoid tissue in Peyer's patches, lymph nodes, and thymus contain the majority of lymphocytes (Reynolds, 1997). To the best of our knowledge, no quantitative studies have been published on cell proliferation and apoptosis rates and on B- and T-lymphocyte numbers in these lymphoid tissues in calves born prematurely compared with calves born after normal lengths of pregnancy and compared with the end of the neonatal period.

The development of monoclonal antibodies that discriminate between different functional cell types has made it possible to define leukocyte differentiation antigens and categorize them according to the cluster of differentiation (CD). Monoclonal antibodies are widely used in flow cytometry and immunohistochemistry to detect lymphocyte populations based on these markers in cell cultures and tissues, but less information is known on their use in formalin-fixed bovine paraffin-embedded tissue.

The aim of this study was to obtain information on proliferation rates, apoptotic rates, and especially on the number of B- and T-lymphocytes in lymphatic organs of preterm and full-term calves immediately after birth and of full-term calves at the age of 5 d, after being fed colostrum for 3 d followed by milk replacer for 1 d.

MATERIALS AND METHODS

Animals, Husbandry, Feeding, and Experimental Procedures

The experimental procedures were approved by the Cantonal Committee for the Permission of Animal Experimentation (Granges-Paccot, Canton of Fribourg, Switzerland), followed the actual law of animal protection, and were supervised by the Federal Veterinary Office (Berne-Liebefeld, Switzerland).

We have studied 21 calves (14 Holstein, Brown Swiss, Simmental, Red Holstein $\times$ Limousin) that were assigned to three groups. All calves were single-born and were separated from their dams immediately after birth. Calves of the group born full term (**GrF**) ($n = 7$) were spontaneously born after normal lengths of pregnancy. In the group born preterm and fed colostrum (**GrP**) ($n = 7$), parturition was induced by injection of 500 μ g of prostaglandin $F_{2\alpha}$ (Estrumate; Essex Pharma GmbH, Friesoythe, Germany) and 5 mg of glucocorticoids (Flumethason; Veterinaria AG, Zürich, Switzerland) 12 d before the expected date of parturition. Calves of GrP and GrF were euthanized immediately after birth, with intravenously administered pentobarbital (Eutha 77, 80 mg/kg BW; Berne Biotech, Berne, Switzerland). Calves of the group born full term and

fed colostrum (**GrC**) ($n = 7$) were born spontaneously and received colostrum from milkings 1, 3, and 5 of lactation on d 1, 2, and 3, respectively. On d 4, calves received a milk replacer. Fed amounts of colostrum were 6% of BW on d 1, 8% of BW on d 2 and 10% of BW from d 3 on. Colostrum was obtained from cows of the Federal Research Station for Animal Production (Posieux, Switzerland). Cows were milked twice daily, and the colostrum of milkings on d 1, 3, and 5 was stored separately in plastic bottles at -20°C . Individual pools of milkings on d 1, 3, and 5 were prepared at the beginning of the study and then stored again in plastic bottles at -20°C until used. Before feeding, colostrum was warmed up to 40°C and fed immediately. Calves of GrC were euthanized on d 5.

After calves were euthanized, thymus, preascapular (**LP**), and mesenteric lymph nodes (**LM**) and ileum were removed. A 4×4 -cm tissue sample of the ileum, tacked onto a piece of cork and tissue samples of thymus, LP, and LM were transferred into a paraformaldehyde (40 g/L) solution of PBS. After 24 h, three 1-mm thick cross sections were cut and embedded in a paraffin block. Seven longitudinal 5- μ m thick cuts were made of intestinal samples, lymph nodes, and thymus.

Evaluation of Cell Proliferation

Calves were intravenously injected with 500 mg of 5-bromo-2'-deoxyuridine (**BrdU**; Roche Diagnostics, Rotkreuz, Switzerland), dissolved in 20 ml of saline, at 60 min before euthanasia. Slides were stained using a mouse monoclonal anti-BrdU antibody (# 1 170 376, Roche Diagnostics) for the detection of BrdU incorporation into the DNA. The BrdU incorporation was visualized using biotinylated goat anti-mouse Ig (DAKO, Glostrup, Denmark), StreptABComplex/AP (DAKO) and Fast Red TR/Naphtol AS-Mix (Sigma, St. Louis, MO).

Evaluation of Apoptosis

Apoptotic cells were visualized using a terminal deoxynucleotidyl transferase (TdT)-mediated X-dUTP nick end labeling (**TUNEL**)-assay. After dewaxing, rehydration, and treatment with proteinase K (10 μ g/ml; Roche Diagnostics) for 10 min at 37°C , the slides were incubated with the TUNEL reaction mixture (Roche Diagnostics) for 1 h at 37°C . Terminal deoxynucleotidyl transferase from calf thymus (Roche Diagnostics) was used to label DNA strand breaks of apoptotic cells, and fluorescein-12-2'-deoxy-uridine-5'-triphosphate (Roche Diagnostics) to visualize the labeled cells. Afterwards, the slides were counterstained with Hoechst reagent (DAPI, D9542, Sigma) to label DNA and thus the cell

nucleus and mounted for fluorescent microscopy with Fluorescent Mounting Medium (Fluorescent Mounting Medium; DAKO).

Evaluation of T-Lymphocytes

After dewaxing, rehydration, and treatment with 3% H₂O₂ in methanol (Merck, Darmstadt, Germany), the slides were placed in a 0.1% trypsin solution (TRYPSIN 250; Difco Laboratories, Detroit, MI) for 6 min at 37°C, followed by incubation with anti-human CD3 antibody (code no. H 0068; DAKO) for 1 h at 37°C. Then the slides were washed in PBS (pH 7.4), incubated with biotinylated link anti-mouse and anti-rabbit Ig (DAKO LSAB2 System, Peroxidase; DAKO) and streptavidin HRP (DAKO LSAB2 System, Peroxidase; DAKO) each for 20 min at 37°C, and washed in PBS. T-Lymphocytes were visualized using AEC Substrate (AEC Substrat Chromogen; DAKO) and counterstained with Mayers Hematoxylin (Merck), rinsed with tap water, and treated with water containing ammonia (1%). Finally, sections were mounted with Faramount mounting medium (DAKO Faramount aqueous mounting medium; DAKO).

Evaluation of B-Lymphocytes

After dewaxing, rehydration treatment with 3% H₂O₂ in methanol (Merck) and washing in distilled water, the slides were placed in a microwave-resistant dish and fully covered with 0.1% sodium citrate. The dish was put in a microwave-resistant box, which was filled with distilled water and was pierced with holes and microwaved for 6 min at 750 W, always ensuring that the slides remained completely covered with liquid. After a short break, the dish was microwaved another 6 min at 750 W, and afterwards slides were allowed to stand for 30 min before they were removed and rinsed in PBS (pH 7.4) for 5 min. Then the slides were incubated with monoclonal mouse anti-human CD79 α -marker (Clone HM 57; DAKO) for 120 min at 37°C. Slides were incubated with biotinylated link anti-mouse and anti-rabbit Ig (DAKO LSAB2 System, Peroxidase; DAKO) and Streptavidin HRP (DAKO LSAB2 System, Peroxidase; DAKO) each for 30 min at 37°C. B-Lymphocytes were visualized using DAB substrate (DAKO) for 10 min. Then slides were rinsed in distilled water, counterstained in Mayer's Hematoxylin (Merck), rinsed in tap water, and treated with water containing ammonia (1%). Finally, sections were mounted with Faramount mounting medium (DAKO Faramount mounting medium; DAKO).

Evaluation of Lymphocytes Subpopulations and Monocytes/Macrophages

Monoclonal antibodies against lymphocyte surface markers CD1 (TH 97A), CD4 (ILA 11A, CACT 138A, CACT 178A, CACT 187A), CD8 (CACT 80C, CACT 88C, CACT 168, 7C2B), CD25 (CACT 116A, LCTB 2A, LCTB 32A, GB 112A), $\gamma\delta$ T cells (CACT 61A, GB 21A, CACT 148A), WC1 positive $\gamma\delta$ cells (GB45A), and against a combination of monocytes plus macrophages (MM 29A) and activation markers (CACT 206A, CACT 77A, CACT 7A), were tested on a bovine lymph node that was embedded in O. C. T. (Miles, Elkart, IN) and snap-frozen in liquid nitrogen. The results were compared with formalin-fixed bovine paraffin-embedded lymph nodes. From snap-frozen tissues, 5- to 10- μ m thick sections were made and fixed in acetone. Sections were treated with 100% ethanol (Merck). Then the sections were allowed to dry for 20 min at room temperature, followed by incubation in PBS. Nonspecific binding was blocked with 5% BSA. Afterwards, the slides were rinsed in PBS and incubated for 60 min with the monoclonal antibodies (15 μ g/ml) at room temperature, followed by incubation with biotinylated link anti-mouse and anti-rabbit Ig (DAKO LSAB2 System, Peroxidase; DAKO) and Streptavidin HRP (DAKO LSAB2 System, Peroxidase; DAKO). Then the slides were reacted with DAB substrate (DAKO) for 10 min and were then mounted with Faramount mounting medium (DAKO Faramount mounting medium; DAKO). The monoclonal antibodies were also tested on tissue sections pretreated by enzymatic digestion with 0.1% proteinase (Roche Diagnostics GmbH) or 0.1% trypsin (TRYPSIN 250; Difco) or by microwave irradiation, as described in the protocol for CD79 staining, or a combination of microwave irradiation and 0.1% trypsin.

Microscopic Analyses

Slides for analyses of cell proliferation and enumeration of B- and T-lymphocytes were examined by light microscopy, whereas slides for detection of apoptotic cells were examined by fluorescent microscopy. Pictures were taken from all slides with a digital camera (Axio Cam HR with the software Axio Vision v3.1; Carl Zeiss Vision GmbH, Munich-Hallbergmoos, Germany). Pictures were evaluated on a computer using a graphic program (Corel Draw 9, Version 9.337, Corel Corporation, Ottawa, Ontario, Canada).

In Peyer's patches, at least five different follicles, interfollicular areas, and domes were evaluated from each calf. A vertical axis was drawn through each follicle, interfollicular area, and dome. Three horizontal lines through each follicle (localized at 25, 50, and 75%

Figure 1. Schema of small intestinal structure with major emphasis on lymphoid tissues (Peyer's patches and intraepithelial lymphocytes). Arrows ($\rightarrow$) show the sites where the numbers of proliferating cells (based on incorporation of 5-bromo-2'-deoxyuridine), apoptotic cells (evaluated with TUNEL-assay; Terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling), T-lymphocytes (recognized by anti-human-CD3-marker) and B-lymphocytes (recognized by anti-human-CD79-marker) were evaluated. Interfollicular area = interfollicular area, IEL = intraepithelial lymphocytes, FAE = follicle-associated epithelium.

on the vertical axis through the follicle), horizontal lines through each interfollicular area (localized at 33 and 66% on the vertical axis of the interfollicular area), and horizontal lines through each dome (localized at 25, 50, and 75% on the vertical axis through the domes) were drawn, and positive cells were counted that were crossed by the horizontal lines (Figure 1). Positive cells per unit length (mm) were calculated, thus resulting in number of positive cells/mm. Follicles of Peyer's patches were separated into peripheral and central regions. Peripheral zones were defined to be within about 15% of the edge of follicles, whereas central zones contained correspondingly about 70% of the area of follicles. B-Lymphocytes were counted along a line drawn through the follicle-associated epithelium (FAE). Intraepithelial (T)-lymphocytes (IEL) were counted along a line drawn through the epithelia.

The thymus was divided into cortex and medulla, and five different sections for each calf were evaluated. Five to 25 lines were drawn through the different sections of the medulla, and 10 lines were drawn through the different sections of the cortex. Positive cells per unit length (mm) were calculated, thus resulting in number

of positive cells/mm. The T-lymphocytes in the thymus could not be enumerated due to the excessive cell density.

In LP and LM, the B-lymphocytes for each calf were based on data from pictures of each lymph node. Lymphocytes were counted in each picture along 10 lines in the paracortex and lines at 25, 50, and 75% of the area of the follicle. The TUNEL- and BrdU-labeled cells were measured into five pictures of each lymph node and by counting the positive cells on 10 lines drawn in each picture. In the BrdU and TdT 3' end labeling assays, we could not divide the LM and LP into paracortex and follicles. Lymph nodes of one calf in group GrF could not be measured due to technical problems. Positive cells per unit length (mm) were calculated, thus resulting in number of positive cells/mm.

Statistical Analyses

Cell proliferation and apoptotic values as well as the number of T- and B-lymphocytes were expressed as means $\pm$ SEM. The numbers of BrdU labeled and apoptotic cells as well as numbers of B- and T-lymphocytes in various segments of Peyer's patches, thymus, LP, and LM for each group and differences between groups (GrP vs. GrF and GrF vs. GrC) were analyzed by the PROC GLM procedure (SAS, 1995). When the *F* test was significant ($P < 0.05$), differences were localized by Bonferroni *t* test ($P < 0.05$).

RESULTS

Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in the Ileum

The BrdU-labeled cells differed in distribution and concentration within Peyer's patches (follicles $>$ interfollicular areas $\geq$ domes; $P < 0.05$) (Table 1). In the peripheral area of follicles, the number of BrdU-labeled cells was 2.6 ± 0.2 times higher ($P < 0.001$) than in the central area in all groups (data not shown). The number of BrdU-labeled cells in interfollicular areas was higher ($P < 0.05$) in GrF than in GrP, and higher in follicles of Peyer's patches ($P < 0.001$), in interfollicular areas ($P < 0.01$), and in domes ($P < 0.001$) in GrC than in GrF.

The apoptotic cells differed in distribution and concentration within Peyer's patches (domes $>$ interfollicular areas in GrP; domes $>$ interfollicular areas and follicles in GrF; follicles $>$ domes in GrC; $P < 0.05$). The number of apoptotic cells in central and peripheral areas of follicles was similar (data not shown). The numbers of apoptotic cells in interfollicular areas were higher ($P < 0.05$) in GrF than in GrP, and higher in

Table 1. Cell proliferation, apoptosis, and B- and T-lymphocytes in the ileum of preterm (GrP) and full-term (GrF) calves at birth and in full-term colostrum-fed calves on d 5 of life (GrC).¹ Values are means $\pm$ SEM, n = 7 per group.

	Experimental groups			Group differences (<i>P</i> -values)	
	GrP	GrF	GrC	GrP vs. GrF	GrF vs. GrC
BrdU ¹ -labeled cells/mm					
Follicles	11.1 $\pm$ 0.7 ^a	11.7 $\pm$ 0.8 ^a	20.1 $\pm$ 1.4 ^a	NS ⁷	***
Interfollicular areas	1.0 $\pm$ 0.2 ^b	2.1 $\pm$ 0.3 ^b	10.9 $\pm$ 1.6 ^b	**	***
Domes	0.7 $\pm$ 0.2 ^b	0.9 $\pm$ 0.1 ^b	3.7 $\pm$ 0.8 ^c	NS	**
TdT 3' end-labeled cells ² /mm					
Follicles	18.7 $\pm$ 1.2 ^{ab}	16.4 $\pm$ 1.4 ^b	12.9 $\pm$ 0.9 ^a	NS	**
Interfollicular areas	12.3 $\pm$ 0.9 ^b	19.3 $\pm$ 2.6 ^b	10.5 $\pm$ 0.6 ^{ab}	*	**
Domes	22.5 $\pm$ 3.4 ^a	27.4 $\pm$ 1.9 ^a	7.9 $\pm$ 1.9 ^b	NS	***
B-lymphocytes ³ /mm					
Follicles	84.6 $\pm$ 8.5 ^a	94.9 $\pm$ 12.6 ^a	104.3 $\pm$ 8.6 ^a	NS	NS
Interfollicular areas	33.9 $\pm$ 3.2 ^b	34.3 $\pm$ 3.3 ^b	33.8 $\pm$ 2.6 ^c	NS	NS
Domes	62.9 $\pm$ 7.2 ^a	66.2 $\pm$ 5.0 ^a	67.1 $\pm$ 6.1 ^b	NS	NS
FAE ⁴	8.8 $\pm$ 1.1 ^c	12.5 $\pm$ 1.8 ^b	13.3 $\pm$ 1.8 ^c	NS	NS
T-lymphocytes ⁵ /mm					
Follicles	2.6 $\pm$ 0.7 ^c	7.0 $\pm$ 1.2 ^c	0.8 $\pm$ 0.1 ^c	**	***
Interfollicular areas	41.0 $\pm$ 1.3 ^a	47.0 $\pm$ 0.6 ^a	35.6 $\pm$ 2.2 ^a	*	***
Domes	1.7 $\pm$ 0.5 ^c	2.9 $\pm$ 0.9 ^c	2.9 $\pm$ 0.7 ^c	NS	NS
IEL ⁶	12.7 $\pm$ 1.6 ^b	29.8 $\pm$ 2.7 ^b	20.5 $\pm$ 2.0 ^b	***	*

^{a,b,c}Within groups and within parameters, means with different superscripts are significantly different ($P < 0.05$) between different localizations.

¹Enumeration of BrdU (= 5-bromo-2'-deoxyuridine)-labeled cells served to evaluate proliferation rates.

²Enumeration of TdT 3' end-labeled cells served to evaluate apoptosis rates.

³B-Lymphocytes were labeled using a CD79-specific antibody.

⁴Follicle-associated epithelium.

⁵T-Lymphocytes were labeled using a CD3-specific antibody.

⁶Intraepithelial lymphocytes.

⁷NS = not significant ($P < 0.1$).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

follicles ($P < 0.01$), in interfollicular areas ($P < 0.01$), and in domes ($P < 0.001$) in GrF than in GrC.

The B-lymphocytes differed in distribution and concentration within Peyer's patches (follicles and domes > interfollicular areas $\geq$ FAE in GrP; follicles and domes > interfollicular areas and FAE in GrF; follicles > domes > interfollicular areas and FAE in GrC; $P < 0.05$). The number of B-lymphocytes in peripheral areas of Peyer's patches was 1.3 times higher ($P < 0.01$) in GrP, similar in GrF, and 1.3 times higher ($P < 0.05$) in GrC than in central areas (data not shown). The number of B-lymphocytes in GrP compared with GrF and in GrF compared with GrC was similar.

Differences were also noted in the distribution and concentration of T-lymphocytes within Peyer's patches and IEL (interfollicular areas > IEL > follicles and domes). In the peripheral area of follicles, the number of T-lymphocytes was 5.2 ± 0.4 times higher ($P < 0.05$) than in the central area (data not shown). Numbers of T-lymphocytes were higher in follicles of Peyer's patches ($P < 0.01$), interfollicular areas ($P < 0.05$), and

within villus epithelia ($P < 0.001$) in GrF than in GrP, but lower in follicles of Peyer's patches ($P < 0.001$), in interfollicular areas ($P < 0.001$) and within villus epithelia ($P < 0.05$) in GrC than in GrF.

Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in Lymph Nodes

The numbers of BrdU-labeled cells in LM and LP were higher ($P < 0.001$) in GrF than in GrP. The numbers of apoptotic cells were higher ($P < 0.001$) in LP and higher ($P < 0.01$) in LM in GrF than in GrP (Table 2).

The numbers of B-lymphocytes in the paracortex and in the follicles of LM and LP were higher ($P < 0.001$) in GrF than in GrP.

The number of B-lymphocytes was 6.4 ± 0.6 times higher ($P < 0.001$) in follicles of LP and 6.3 ± 1.1 times higher ($P < 0.001$) in follicles of LM compared with the paracortex in all groups.

The great numbers of T-lymphocytes in lymph nodes did not allow quantitative evaluations to be made. We

Table 2. Cell proliferation, apoptosis, and B- and T-lymphocytes in prescapular and in mesenterial lymphnode (LM, LP) of preterm (GrP) and full-term (GrF) calves at birth and in full-term colostrum-fed calves on d 5 of life (GrC). Values are means $\pm$ SEM, $n = 7$ per group.

	Experimental groups			Group differences (<i>P</i> -values)	
	GrP	GrF	GrC	GrP vs. GrF	GrF vs. GrC
BrdU-labeled cells/mm					
Prescapular lymph node	1.2 $\pm$ 0.01	2.3 $\pm$ 0.01	ND ²	***	ND
Mesenterial lymph node	1.0 $\pm$ 0.01	1.8 $\pm$ 0.01	ND	***	ND
TdT 3'end labeled cells/mm					
Prescapular lymph node	0.3 $\pm$ 0.04	0.7 $\pm$ 0.10	ND	**	ND
Mesenterial lymph node	0.3 $\pm$ 0.03	0.7 $\pm$ 0.01	ND	***	ND
B-lymphocytes/mm					
Prescapular lymph node					
Paracortex	16.1 $\pm$ 0.3 ^b	24.2 $\pm$ 0.2 ^b	ND	***	ND
Follicles	93.9 $\pm$ 8.7 ^a	169.9 $\pm$ 6.1 ^a	ND	***	ND
Mesenterial lymph node					
Paracortex	15.8 $\pm$ 0.2 ^b	23.6 $\pm$ 0.1 ^b	ND	***	ND
Follicles	82.6 $\pm$ 3.4 ^a	173.9 $\pm$ 6.9 ^a	ND	***	ND
T-lymphocytes/mm					
Prescapular lymph node	NM ¹	NM	NM	ND	ND
Mesenterial lymph node	NM	NM	NM	ND	ND

^{a,b}Within groups and within parameters, means with different superscripts are significantly different ($P < 0.05$) between different localizations.

¹NM = not measurable.

²ND = Not done; NS = not significant ($P > 0.1$).

** $P < 0.01$.

*** $P < 0.001$.

could not evaluate the lymph nodes of GrC due to technical problems.

Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in the Thymus

The numbers of BrdU-labeled cells were 4.1 ± 1.0 times higher ($P < 0.001$) in the cortex than in the medulla in all groups (Table 3). The numbers of BrdU-labeled cells in cortex and medulla were higher ($P < 0.001$) in GrF than in GrP and higher in cortex ($P < 0.1$) and medulla ($P < 0.001$) in GrC than in GrF. The ratio of BrdU-labeled cells of medulla to cortex was higher ($P < 0.001$) in GrC than in GrF. B-Lymphocytes were not detectable. The great numbers of T-lymphocytes in the thymus did not allow quantitative evaluations.

Evaluation of Lymphocyte Subpopulations and Monocytes/Macrophages

The monoclonal antibodies worked well in O. C. T.-fixed and snap-frozen tissue of the lymph node, but none worked on formalin-fixed bovine paraffin-embedded lymph nodes.

DISCUSSION

General Aspects

The two antibodies used were suitable for detection of CD3 and CD79 surface markers on T- and B-lymphocytes in bovine formalin-fixed and paraffin-embedded tissues, and the distribution of B- and T-cells in bovine lymphoid tissues was similar as in humans using the same antibodies (Jones et al., 1993) and as in calves using other antibodies (Hein et al., 1989; Halleraker et al., 1990). Other antibodies tested in this study would have allowed the evaluation of other types of lymphocytes than B- and T-lymphocytes in snap-frozen tissues, but not if formalin-fixed. Unfortunately, only formalin-fixed tissue was available.

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Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in the Ileum

The proliferation rate of lymphocytes in Peyer's patches in premature calves increases up to birth (Sangild et al., 2000), similar to our study during late fetal development in interfollicular areas and after birth in interfollicular areas, in domes and in follicles of Peyer's patches. Therefore, the proliferation in interfollicular areas of Peyer's patches starts when only small amounts of antigens are ingested with amniotic fluid. In the colostrum-fed group, the rise in proliferating cells in all areas of Peyer's patches was likely the result of stimulation by ingested antigens. A peptide from sheep and bovine colostrum enhances mitosis of B-lymphocytes (Julius et al., 1988), and there may be many other factors that stimulate cell proliferation in response to colostrum intake. Removal of B-lymphocytes from the ileal Peyer's patches causes a dramatic reduction in the

Table 3. Cell proliferation, apoptosis, and B- and T-lymphocytes in the thymus of preterm (GrP) and full-term (GrF) calves at birth and in full-term colostrum-fed calves on d 5 of life (GrC). Values are means $\pm$ SEM, $n = 7$ per group.

	Experimental groups			Group differences (<i>P</i> -values)	
	GrP	GrF	GrC	GrP vs. GrF	GrF vs. GrC
BrdU-labeled cells/mm					
Cortex	4.0 $\pm$ 0.01 ^a	4.6 $\pm$ 1.5 ^a	5.4 $\pm$ 0.1 ^a	***	†
Medulla	0.8 $\pm$ 0.1 ^b	1.0 $\pm$ 0.1 ^b	1.7 $\pm$ 0.09 ^b	***	***
TdT 3'-end-labeled cells/mm	0.9 $\pm$ 0.04	0.9 $\pm$ 0.1	1.0 $\pm$ 0.1	NS ²	NS
B-lymphocytes/mm	NM	NM ¹	NM	ND ³	ND
T-lymphocytes/mm	NC ⁴	NC	NC	ND	ND

^{a,b}Within groups and within parameters, means with different superscripts are significantly different ($P < 0.05$) between different localizations.

¹NM = not measurable.

²NS = not significant ($P > 0.1$).

³ND = Not done.

⁴Not countable because of too high density.

† $P < 0.1$.

*** $P < 0.001$.

proliferation rate in Peyer's patches (Reynolds, 1997), suggesting that mainly B-lymphocytes are proliferating. It cannot be excluded that other cells, such as stromal cells, which can stimulate B-cell proliferation, are also proliferating (Borghesi, 1997). B-Lymphocytes are known to emigrate from Peyer's patches to other lymphoid tissues, but only to a small extent (Reynolds, 1986, 1997), suggesting that most B-lymphocytes will undergo natural cell death (Andersen et al., 1999). Cell proliferation in our study was much higher in follicles than in other areas of Peyer's patches, in thymus or lymph nodes in preterm calves, in full-term calves, and after birth. In sheep, proliferating cells were also more common in Peyer's patches than at other sites of the body (Reynolds, 1986).

The growth processes in the Peyer's patches must be under rigid control to exclude excessive tissue expansion, i.e., lymphocyte production must be balanced either by cell emigration or cell death. We found an increase of apoptotic cells in the interfollicular areas in Peyer's patches during late fetal development, suggesting that a high proliferation rate as seen in follicles of Peyer's patches may be linked to an enhanced apoptotic rate in interfollicular areas. Apoptosis of B-cells in ileal Peyer's patches is an important mechanism in the selection of nonreactive B-lymphocytes (Motyka et al., 1991; Hartley et al., 1993; Reynolds et al., 1986, 1997). Interestingly, in colostrum-fed calves we found a smaller number of apoptotic cells in follicles, interfollicular areas, and domes of Peyer's patches than in full-term calves at birth, suggesting that colostrum intake reduces apoptosis in Peyer's patches as in humans (Playford et al., 2000). Colostral effects may be medi-

ated by various bioactive factors, which are present in high concentrations in bovine colostrum (Blum and Baumrucker, 2002) and by stromal cells that protect lymphocytes from apoptosis (Borghesi et al., 1997). It may be speculated that proliferating lymphocytes in the colostrum-fed group, which are protected from cell death, emigrate from the Peyer's patches because the number of B-lymphocytes in Peyer's patches in the colostrum-fed group did not rise. Apoptosis was much more evident in Peyer's patches than in the thymus or lymph nodes. Because proliferation rates, at least in follicles, were also much higher in Peyer's patches than in lymph nodes and thymus, cell turnover rates in Peyer's patches were very high.

The ileal Peyer's patches in calves is a primary lymphoid organ that produces immature B-lymphocytes (Press et al., 2001; Yasuda et al., 2002). The peripheral area of follicles in the ileum of calves is composed of proliferating B-lymphocytes, whereas the central area consists of nondividing B-lymphocytes (Yasuda et al., 2002), which is supported by our studies and by studies in sheep (Reynolds, 1987).

B-Lymphocyte numbers increase before and after birth and even after this period (Griebel et al., 1992; Reynolds, 1997). However, in our study B-lymphocyte numbers in Peyer's patches did not rise from preterm to full-term born calves, suggesting that rapid expansion of B-lymphocytes may begin before this period. In sheep, B-lymphocytes expand during the last third of fetal development and in this period the rapid development of B-lymphocytes switches from spleen to Peyer's patches (Reynolds, 1997). Emigration of B-lymphocytes from the germinal centers to extrafollicular compart-

ments (Brandtzaeg et al., 1999) could be a reason why we could not demonstrate a significant increment in B-lymphocytes numbers in Peyer's patches.

T-Lymphocytes are mainly present in interfollicular areas of Peyer's patches (Pospischil, 1989) and as IEL (Wyatt et al., 1998), whereas their number in ileal follicles is very low (Yasuda et al., 2002). During the last 2 wk of fetal development, T-lymphocyte numbers increased in villus epithelia and in follicles, interfollicular areas of Peyer's patches, but not in domes, suggesting that in domes maximal T-lymphocyte numbers are reached well before normal term. After colostrum feeding, T-lymphocytes numbers decreased in follicles and interfollicular areas. Human and bovine colostrum contain a factor, which has been termed colostrum inhibitory factor, that possibly reduced T-lymphocyte production during the first 5 d of life in our study through suppression of the production of interleukin-2 (Mandalapu et al., 1995; Sambasivarao et al., 1996). The inhibition of T-lymphocyte proliferation might prevent newborn calves from overresponding to environmental antigens to which they are exposed after birth (Mandalapu, 1995).

Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in Lymph Nodes

The LM strongly interacts with antigens resulting from intestinal digestions (Lillehoj and Chung, 1992; Willard, 1992). In this respect, we could not see a difference between LM and LP in the distribution of T- and B-lymphocytes, apoptosis, or proliferation rates. In both types of lymph nodes studied, the number of proliferating cells and B-lymphocytes in follicles and paracortex was greater in GrF than in GrP, demonstrating that B-lymphocytes proliferate up to birth at full term (Barrington and Parish, 2001). Enhanced B-lymphocyte proliferation rate may be followed by enhanced apoptosis of B-lymphocytes (Reynolds, 1997). In accordance, the number of apoptotic cells was higher in GrF than in GrP. Furthermore, B-lymphocyte numbers were higher in the paracortex than in follicles in GrF than in GrP, also demonstrating an age-dependent effect. The high number of T-lymphocytes in lymph nodes did not allow an exact enumeration. In addition, in GrC the LM and LP could not be evaluated due to technical problems.

Cell Proliferation, Apoptosis, and B- and T-Lymphocytes in the Thymus

The thymus gland is a primary lymphoid organ in which bone marrow-derived T-lymphocyte precursors undergo maturation, eventually leading to migration of selected thymocytes to T-lymphocyte-dependent areas

such as spleen, lymph nodes, Peyer's patches, and tonsils (Wilson and Dardenne, 2000). Numbers of proliferating cells in the cortex and medulla were higher in GrF than in GrP. In the thymus, juvenile cells mainly proliferate in the cortex, as in our study, and then move to the medulla (Wilson and Dardenne, 2000). The rise of proliferating cells in the medulla in GrC and perhaps the transfer of cells from the cortex to the medulla lead to the increased proliferation rate and to an increased medulla/cortex ratio of proliferating cells in GrC than GrF.

Only a small number of mature thymocytes leave the thymus (Scollay and Shortman, 1994). The major loss is due to apoptosis of thymocytes that possibly failed positive selection (Surh and Sprent, 1994). Because the apoptotic rate was not rising from preterm to full-term calves and during the first 5 d after birth, positively selected thymocytes during our study possibly migrated out of the thymus to T-lymphocyte-dependent areas of peripheral lymphoid organs (Wilson and Dardenne, 2000).

In conclusion, the different lymphoid sites exhibited marked ontogenetic changes during a short period before and after birth. Apoptotic rates were generally smaller at all sites of Peyer's patches in GrC than in GrF and proliferation rates increased from GrP to GrF and from GrF to GrC in all lymphoid tissues. Numbers of T-lymphocytes in Peyer's patches were higher in GrF than in GrP, but lower in Peyer's patches in GrC than in GrF. Numbers of B-lymphocytes did not change significantly in Peyer's patches despite high proliferation rates and low apoptotic rates, suggesting that many B-lymphocytes leave Peyer's patches during the first days of life. Interestingly, there was a decline of T-lymphocyte numbers and of apoptotic rates in Peyer's patches during colostrum feeding. The implied impact of this study is that the days immediately before parturition are critical to the development of a fully functional immune system. Around this period, an opportunistic window exists in which the potential exists to modulate immune functions, such as by nutrition, and by changes in the endocrine status that could influence the future health of calves.

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