Effects of dexamethasone on lymphoid tissue in the gut and thymus of neonatal calves fed with colostrum or milk replacer

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ABSTRACT: An increased susceptibility to disease in neonatal calves may be attributable to high glucocorticoid levels that influence immune reactions. We tested whether dexamethasone (DEXA) administration influences the proliferation, apoptosis, and number of B- and T-lymphocytes in Peyer’s patches (PP) and thymus in calves fed colostrum (C) or a milk-derived formula. All calves were subcutaneously administered bovine colostrum-derived immunoglobulin G and fed chicken-egg derived immunoglobulins that protected against rotavirus and pathogenic Escherichia coli. The DEXA (30 μg/kg of BW daily) was injected for 4 d into groups fed colostrum on the first 3 d (CD+ and FD−) and those fed the formula that contained nutrients in amounts as in colostrum but no immunoglobulin G (FD+). Groups CD− and FD− were fed the same as the other two groups, but did not receive DEXA. Immunohistochemical methods were used to evaluate cell proliferation rates (by labeling of 5-bromo-2′-deoxyuridine), apoptosis rates (by terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling). Numbers of T- and B-lymphocytes were determined with antibodies specific for CD3 and CD79 surface proteins. There were significant effects (P < 0.05) of DEXA treatment (decrease of cell proliferation rates in follicles of PP and thymus, increase of apoptotic rate in follicles of PP and thymus, decrease of B-lymphocyte numbers in follicles of PP, increase of B-lymphocyte numbers in domes of PP, increase of T-lymphocyte numbers in follicles of PP, and a decrease of intraepithelial T-lymphocyte numbers). There were significant effects (P < 0.05) of C feeding (decrease of cell proliferation rates in follicles of PP and of B-lymphocyte numbers in interfollicular areas, domes, and follicular-associated epithelium of PP, and an increase of cell proliferation rate in the thymus). A DEXA × feeding interaction (P < 0.001) was found for cell proliferation rate in the thymus. In conclusion, DEXA treatment decreased cell proliferation rates in follicles of PP and thymus and enhanced apoptotic rates in follicles of PP. Colostrum feeding decreased cell proliferation rates, likely of B-lymphocytes, in follicles of PP and numbers of B-lymphocytes in domes, follicular-associated epithelium, and interfollicular areas of PP and enhanced cell proliferation rates and selectively modified DEXA effects in the thymus.

Key Words: Apoptosis, Calves, Cell Growth, Lymphocytes, Peyer Patches, Thymus

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Introduction

Neonates have to respond to new environmental conditions and to nutrients ingested with colostrum. Colostrum contains, besides immunoglobulins (Ig), lactoferrin, hormones, growth factors, cytokines, and leukocytes with immunomodulatory activities (Riedel-Caspari and Schmidt, 1990; Sordillo et al., 1991; Blum and Baumrucker, 2002). Neonatal calves are capable...
of mounting an immune response, but unless adequate immunological assistance is provided in the form of colostral IgG, morbidity and mortality rates due to infections increase (Barrington and Parish, 2001).

Immunosuppression due to stress, reflected by an enhanced glucocorticoid status, can increase the susceptibility to infectious diseases (Concordet and Ferry, 1993; Anderson et al., 1999). The glucocorticoid status in neonatal calves is high (Hadorn et al., 1997), particularly if not fed colostrum (Hammon and Blum, 1998). However, glucocorticoids in neonatal mammals also play an important role in the development of the structure and function of organs such as the lung, the gastrointestinal tract (Sangild et al., 2000), or endocrine systems (Sauter et al., 2003). In response to glucocorticoids in the thymus and lymph nodes, cortical lymphocytes are destroyed, leading to cortical atrophy (Roy and Walsh, 1992).

To our knowledge, nothing is known regarding responses of gut-associated lymphoid tissues (GALT) to glucocorticoids in neonatal calves. The primary objective of the present study was to determine if the number of B- and T-lymphocytes and proliferation and apoptosis in the GALT (especially in Peyer’s patches, PP) and thymus may be affected by high levels of glucocorticoids, using dexamethasone (DEXA) treatment to simulate the effects of high levels of glucocorticoids that might occur in neonatal calves. The second objective was to determine if any noted differences were modulated by feeding the experimental groups colostrum or milk-based formulas containing no Ig.

Material 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, Switzerland), followed the actual law of animal protection, and were supervised by the Federal Veterinary Office (Liebefeld-Berne, Switzerland).

Twenty-eight male calves (11 Holstein Friesian, 12 Simmental × Red Holstein, one Red Holstein × Limousin, and four Brown Swiss) were studied. They were born at the Federal Research Station for Animal Production (Posieux, Switzerland) or at neighboring farms. Calves were born spontaneously and were separated immediately after birth from their dams and held on straw in boxes for 5 d.

The 28 calves were allotted in a 2 × 2 factorial arrangement fed colostrum combined with DEXA (CD+), or without DEXA (CD−) administration or a formula combined with DEXA (FD++) or without DEXA administration (FD−). Each group consisted of seven calves. Calves were primarily grouped with the goal to obtain similar BW and breed distributions in all four groups. Calves of CD− and CD+ received pooled colostrum obtained from milkings 1, 3, and 5 (d 1, 2, and 3 after parturition, respectively) on the first 3 d of life. Calves of FD− and FD+ were fed three different milk-based formulas on d 1, 2, and 3 that contained similar amounts of nutrients as colostrum milkings 1, 3, and 5, respectively. Calves of all groups received a milk replacer on d 4. Amounts of colostrum and formula fed were 6% of BW on d 1, 8% of BW on d 2, and 10% of BW from d 3 on. Calves were fed by bottle twice daily. To ensure that all calves received the same amounts of formula or colostrum, calves with reduced appetite were tube-fed their planned amounts of formula or colostrum. Calves received their first meal at 3.1 ± 0.04 h (FD−), 2.2 ± 0.02 h (FD++), 2.4 ± 0.03 h (CD−), and 3.4 ± 0.04 h (CD+) after birth, respectively. The following feedings were at 8, 24, and 32 h after the first feeding. From d 3 on, calves were fed daily at 0800 and at 1600. Additionally, calves of FD− and CD+ were injected i.m. with DEXA (15 μg/kg of BW; DEXA TAD, Lohmann Animal Health, Cuxhaven, Germany) twice daily at feeding times from d 1 to d 4.

Colostrum was from cows of the Federal Research Station for Animal Production (Posieux, Switzerland). Cows were milked twice daily and the colostrum of milkings 1, 3, and 5 was stored separately in plastic bottles at −20°C. Separate pools for milkings 1, 3, and 5 were prepared at the beginning of the study. Each of these pools was stored in multiple aliquots in plastic bottles at −20°C. Before feeding, colostrum was warmed up to 40°C, and then fed immediately. Three formulas were created that contained nutrients (protein, fat, lactose) in amounts comparable to those of milkings 1, 3, and 5 of colostrum after parturition, but contained almost no biological active substances, such as Ig, hormones, and growth factors, and were fed on d 1, 2, and 3 after birth, respectively. Formulas were produced by UFA AG (Sursee, Switzerland) and consisted of calcium-caseinate (Emmi Milch AG, Lucerne, Switzerland), lactalbumin 90 (Emmi Milch AG), double cream (Institut Agricole de l’Etat de Fribourg, Grangeneuve, Switzerland), and a vitamin and mineral premix (Provim S.A., Cossonay-Gare, Switzerland). The three formulas were dissolved by adding water and stored in plastic bottles at −20°C until used. Before feeding, the bottles were warmed up to 40°C and then fed immediately. The MR (UFA 200 Natura, without antibiotics; UFA AG) was prepared as 100 g/L of solution. Contents of different colostrum milkings, formulas, and MR are shown in Table 1.

To protect against infections, all calves were subcutaneously injected 2 g of a bovine colostral Ig preparation (Gammaserin; Gräub AG, Berne, Switzerland) before first feed intake. Additionally, all calves were fed chicken egg-derived Ig containing high antibody titters against rotavirus and pathogenic Escherichia coli type K 99 (Globigen 88; kindly donated by Lohmann Animal Health, Cuxhaven, Germany). Amounts fed were 10 (d 1), 8 (d 2), 6 (d 3), and 4 g (d 4) per meal. On days 1, 2, and 3, all calves were subcutaneously injected with...
antibiotics (25 mg of Enrofloxac in per 10 kg of BW, Baytril 5%, purchased from Bayer AG, Leverkusen, Germany). To avoid different treatment protocols, drugs were given to both formula- and colostrum-fed calves.

**Blood Samples and Analyses**

Blood samples were taken from the jugular vein with evacuated tubes without anticoagulants to measure IgG in serum on d 1, 2, and 4 at 0, 1, 2, 4, and 8 h after the first, third, and seventh feeding and on d 5, respectively. Tubes were centrifuged at 1,000 g, 20 min, and supernatants were stored at –20°C. Serum IgG concentrations were determined by ELISA according to Erhard et al. (1995).

**Analysis of Colostrum, Formula, and Milk Replacer**

Samples of formula 1, 2, and 3, and samples of the individual pools of colostrum of milkings 1, 3, and 5 were lyophilized to determine DM, CP (by the Kjeldahl method), crude fat (by the Berntrop method), and ash (after combustion at 550°C) using standard procedures. Contents of nitrogen-free extracts and GE (based on energy equivalents of 36.6, 17.0, and 24.2 MJ/kg of fat, nitrogen-free extracts, and CP, respectively) were calculated. The producer gave information on contents of MR. Concentrations of insulin and IGF-I in formula and colostrum were analyzed as described by Hammon and Blum (1997, 1998). The IgG concentrations were determined by ELISA according to Erhard et al. (1995).

**Table 1. Composition of colostrum milkings, formula, and milk replacer (as-fed basis)**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
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<tr>
<td></td>
<td>Colostrum</td>
<td>Formula</td>
<td>Colostrum</td>
<td>Formula</td>
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<td></td>
<td>Milking 1</td>
<td>Day 1</td>
<td>Milking 3</td>
<td>Day 2</td>
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<td>Dry matter, g/kg</td>
<td>240</td>
<td>235</td>
<td>265</td>
<td>236</td>
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<td>Gross energy, MJ/kg</td>
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<td>6.0</td>
<td>4.1</td>
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<td>Crude protein, g/kg</td>
<td>133</td>
<td>127</td>
<td>77</td>
<td>68</td>
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<tr>
<td>Crude lipids, g/kg</td>
<td>64</td>
<td>66</td>
<td>43</td>
<td>45</td>
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<tr>
<td>Nitrogen free extract, g/kg</td>
<td>25</td>
<td>30</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td>Crude ash, g/kg</td>
<td>18</td>
<td>13</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>IgG, g/L</td>
<td>38.2</td>
<td>0.01</td>
<td>12.6</td>
<td>0.01</td>
</tr>
<tr>
<td>IGF-I, μg/L</td>
<td>255</td>
<td>n.m.</td>
<td>155</td>
<td>n.m.</td>
</tr>
<tr>
<td>Insulin, μL/L</td>
<td>16.1</td>
<td>1.2</td>
<td>5.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*The formula (per kilogram) was composed of calcium-caseinate (86, 208, and 282 g on d 1, 2 and 3, respectively), lactalbumin (576, 245, and 80 g on d 1, 2 and 3, respectively), milk fat (267, 262, and 353 g of double cream on d 1, 2, and 3, respectively), lactose (57, 271, and 271 g on d 1, 2, and 3, respectively), and a mineral premix (per kg) containing calcium (186 g), magnesium (224 g), sodium (31 g), phosphorus (92 g), iron (12 g), manganese (1.6 g), zinc (7.8 mg), iodine (0.03 mg), cobalt (0.02 mg), and selenium (0.02 mg).

**Analysis of Cell Proliferation, Apoptosis, and Number of B- and T-lymphocytes**

After calves were euthanized on d 5 of life using pentobarbital (Eutha 77; 80 mg/kg of BW; Berne Biotech, Berne, Switzerland), mid-ileum and thymus were removed and pieces (2 × 2 cm) from both organs were cut, pinned on a piece of cork, and transferred to formaldehyde solution. After 24 h, three 1-mm-thick cross sections were cut and embedded in a paraffin block. Seven longitudinal 5-μm-thick cuts were made of each intestinal sample and thymus. Histomorphometrical methods were used to determine areas of at least 10 follicles of PP and thymus. To study cell proliferation 500 mg of 5-bromo-2′-deoxyuridine (BrdU; Roche Diagnostics GmbH, Rotkreuz, Switzerland), dissolved in 20 mL of saline, was intravenously injected 60 min before euthanasia. The BrdU is incorporated into nuclear DNA during mitosis in the S-phase of all cells (Yanai et al., 1996). Slides were stained using a monoclonal anti-BrdU antibody (1:1 70 376, Roche Diagnostics) for the detection of BrdU incorporation into DNA. The BrdU incorporation was visualized using a biotinylated goat anti-mouse Ig (DAKO, Glostrup, Denmark), Strept-ABComplex/AP (DAKO), and Fast Red TR/Naphtol AS-MIX (Sigma, St. Louis, MO).

To study apoptosis, cells were visualized using a Terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling (TUNEL) assay, which detects nuclear DNA strand breaks (Afford and Randhawa, 2000). After dewaxing, rehydrating, and treatment with proteinase K (10 μg/mL; Roche Diagnostics) for 10 min at
37°C, 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 labeled cells. Then the slides were counterstained with Hoechst reagent (DAPI, D9542, Sigma) to label DNA and thus, the cell nucleus, and mounted for fluorescence microscopy.

To study T-lymphocytes, cells were visualized by using an anti-human CD3 marker (code No. H 0068; DAKO) because no anti-bovine CD3 markers were available. After sections were deparaffinized, rehydrated, and treated with 3% H2O2 in methanol (Merck, Darmstadt, Germany), the slides were exposed to 0.1% trypsin (trypsin 250; Difco Laboratories, Detroit, MI) for 6 min at 37°C to retrieve the antigen and were then incubated with anti-human CD3 marker (diluted 1:50) for 1 h at 37°C. The slides were then washed in PBS (pH 7.4) and incubated with a biotinylated link anti-mouse and anti-rabbit Ig and streptavidin horseradish peroxidase (LSAB2 System, peroxidase, DAKO) each for 20 min at 37°C. The slides were then washed in PBS, and positive cells were visualized using AEC substrate (AEC Substrate Chromogen; DAKO), counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), rinsed with tap water, and treated with ammonia (1%) water. Finally, sections were mounted with Faramount mounting medium (Faramount aqueous mounting medium; DAKO).

To study B-lymphocytes, an anti-human CD79 marker (Clone HM57; DAKO) was used because no anti-bovine CD79 markers were available. 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 placed in a microwave-resistant box with holes and filled with distilled water. The box was placed in the microwave oven for 2×5 min at 750 W, and afterwards was allowed to stand for 30 min before being removed and rinsed in PBS (pH 7.4) for 5 min. The sections were then incubated with monoclonal mouse anti-human CD79 (diluted 1:50) for 120 min at 37°C. Then the slides were incubated with biotinylated anti-mouse and anti-rabbit Ig and streptavidin horseradish peroxidase (LSAB2 System, peroxidase, DAKO) each for 30 min at 37°C. Afterwards, the positive cells were visualized using DAB substrate (DAKO) for 5 to 10 min. The slides were counterstained in Mayer's hematoxylin (Merck), rinsed in tap water, and treated with ammonia water (1%). Finally, sections were mounted with Faramount mounting medium (Faramount mounting medium; DAKO).

The two antibodies detecting CD3 and CD79 cell membrane markers on T- and B-lymphocytes worked appropriately in bovine formalin-fixed and paraffin-embedded tissues. The general distribution of B- and T-lymphocytes was similar to that of lymphoid tissues of humans using the same antibodies (Jones et al., 1993) and was similar to that of calves when other antibodies were used (Halleraker et al., 1990; Hein et al., 1989).

Microscopical Analysis

Slides for analysis 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 fluorescence microscopy. Pictures were taken from all slides with a digital camera (Axio Cam HR with the Axio Vision version 3.1 software; Carl Zeiss Vision GmbH, Munich-Hallbergmoos, Germany). Pictures were evaluated on a computer using a graphic program (Corel Draw 9, Version 9.337, Corel Corp., Ottawa, Ontario, Canada). In PP, at least five different follicles, four interfollicular areas, and three domes were evaluated from each calf.

In PP, a vertical axis was drawn through each follicle, interfollicular area, and dome (Figure 1). Three horizontal lines through each follicle (localized at 25, 50, and 75% on the vertical axis through the follicle), two horizontal lines through each interfollicular area (localized at 33 and 66% on the vertical axis of the interfollicular area), and three domes were evaluated from each calf.

Figure 1. Schema of small intestinal structure with major emphasis on lymphoid tissues (Peyer’s patches and intraepithelial lymphocytes). Arrows (→) 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. Interfoll. area = interfollicular area, IEL = intraepithelial lymphocytes, FAE = follicle-associated epithelium.
ular area), and three 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. Follicles of PP were separated into peripheral and central regions. Peripheral zones were defined to be within about 15% of the edge of follicles, whereas central zones were defined as about 70% of the area of follicles. The B-lymphocytes were counted along a line drawn through the follicle-associated epithelium (FAE) and intraepithelial lymphocytes (IEL) were counted along a line drawn through the ileal 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.

**Statistical Analysis**

Serum IgG concentrations, areas of PP follicles, cell proliferation, and the number of apoptotic cells, as well as the number of T- and B-lymphocytes, are shown as means ± SEM. The IgG values were evaluated using the RANDOM and REPEATED methods of the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). The DEXA treatment, feeding, and time were fixed effects and the individual calves were random effects. Treatment, feeding, and time differences were localized by Bonferroni t-test and taken as significant if \( P < 0.05 \). Different distributions of BrdU-labeled and apoptotic cells, as well as numbers of B- and T-lymphocytes in various segments of the ileum and thymus and effects of DEXA treatment and feeding, as well as DEXA × feeding interactions on labeled cells, were analyzed by the PROC GLM procedure of SAS. When the \( F \)-test was significant \( (P < 0.05) \), differences were identified by Bonferroni \( t \)-test and taken as significant if \( P < 0.05 \). For correlations of BrdU- and TUNEL-labeled cells with B- and T-lymphocytes, the PROC CORR procedure of SAS was used.

**Results**

**Blood Serum IgG Concentrations**

Concentrations of IgG on d 1 in the colostrum-fed groups increased similarly \( (P < 0.001) \) after first feed intake from 0.45 ± 0.24 g/L to peak values of 7.6 ± 1.2 g/L on d 2 and remained elevated 7.2 ± 0.8 g/L up to d 5 of life. Concentrations did not change in formula-fed calves during the experimental period and were much lower \( (P < 0.001) \) than in colostrum-fed calves (mean IgG concentration for formula-fed calves on d 1 was 0.5 ± 0.2 g/L and on d 4 was 0.9 ± 0.4 g/L).

**Histomorphometry of Follicles of Peyer’s Patches**

Areas of the follicles in CD− and FD− were similar \( (0.14 ± 0.1 \text{ mm}^2) \). The areas of follicles in CD+ and FD+ were 0.04 ± 0.004 mm². Areas of follicles in DEXA-treated groups were smaller \( (P < 0.001) \) than in untreated groups.

**Histochemical Measurements in the Ileum**

The number of proliferating (BrdU-labeled) cells in PP of all groups was higher \( (P < 0.001) \) in follicles than in interfollicular areas and domes (Table 2). Within follicles, the number of proliferating cells was higher \( (P < 0.05) \) in peripheral than in central regions (peripheral and central regions contained 71.2 and 28.8% in CD−, 72.8 and 27.2% in CD+, 65.1 and 34.9% in FD−, and 73.2 and 26.8% in FD+, respectively). The DEXA treatment and colostrum intake decreased \( (P < 0.001) \) the number of BrdU-labeled cells in follicles.

The number of apoptotic (TdT-end labeled) cells in CD+ was higher \( (P < 0.05) \) in follicles and interfollicular areas than in domes of PP. The DEXA treatment increased \( (P < 0.05) \) the number of apoptotic cells in follicles, but tended to decrease \( (P < 0.10) \) the number of apoptotic cells in interfollicular areas of colostrum-fed calves.

The apoptosis:proliferation (TUNEL to BrdU) ratios in CD− were higher \( (P < 0.05) \) in domes and interfollicular areas than in follicles \( (2.13, 0.96, \text{ and } 0.64, \text{ respectively}) \), higher \( (P < 0.05) \) in domes and follicles than in interfollicular areas \( (2.35, 2.35, \text{ and } 0.70 \text{ respectively}) \) in CD+, and higher \( (P < 0.05) \) in domes than in follicles and interfollicular areas \( (3.50, 0.61, \text{ and } 0.65, \text{ respectively}) \) in formula-fed calves. The TUNEL:BrdU ratio in follicles of PP increased \( (P < 0.001) \) by DEXA treatment compared with non-DEXA treatment \( (1.58 \text{ and } 0.49, \text{ respectively}) \) and by colostrum feeding compared with formula feeding \( (1.08 \text{ and } 0.61, \text{ respectively}) \). There was also a DEXA × feeding interaction \( (P < 0.05) \), and the TUNEL:BrdU ratio in DEXA-treated calves was higher \( (P < 0.05) \) in colostrum- vs. formula-fed calves \( (2.35 \text{ and } 1.15, \text{ respectively}) \).

The number of T-lymphocytes in CD− and FD− was higher \( (P < 0.001) \) in interfollicular areas than in villus epithelium, follicles and domes, and the number of T-lymphocytes was higher \( (P < 0.001) \) in CD+ in interfollicular areas than in villus epithelium, follicles, and domes, and was higher \( (P < 0.001) \) in FD+ in interfollicular areas than in villus epithelium, follicles, and domes. Within follicles, the number of T-lymphocytes in CD−, CD+, and in FD+ was higher \( (P < 0.05) \) in peripheral vs. central regions (peripheral and central regions contained 80.1 and 19.9% in CD−, 67.7 and 32.3% in CD+, 63.1 and 36.9% in FD−, and 78.4 and 21.6% T-lymphocytes in FD+, respectively). The DEXA treatment increased \( (P < 0.01) \) the number of T-lymphocytes in follicles, but decreased \( (P < 0.05) \) the number of IEL. Colostrum feeding tended to decrease T-lymphocyte numbers in domes \( (P < 0.10) \), especially in DEXA-treated calves.
The number of B-lymphocytes in CD− was higher (P < 0.01) in follicles than in domes, interfollicular areas, and FAE (P < 0.001) of PP, was higher (P < 0.001) in follicles than domes, interfollicular areas, and FAE in CD+, was higher (P < 0.001) in follicles and domes than in interfollicular areas and FAE in CD−, and was higher (P < 0.001) in domes than in follicles, interfollicular areas, and FAE in FD+. The number of B-lymphocytes within follicles was higher (P < 0.05) in peripheral vs. central regions (peripheral and central regions contained 43.7 and 56.3% in CD−, 43.6 and 56.4% in CD+, 44.8 and 55.2% in FD−, and 41.0 and 59.0% B-lymphocytes in FD+, respectively). The DEXA treatment decreased (P < 0.001) the number of B-lymphocytes in follicles but increased (P < 0.01) the number of B-lymphocytes in domes and tended to increase (P < 0.1) the number of B-lymphocytes in FAE of formula-fed calves. The numbers of B-lymphocytes in interfollicular areas, domes, and FAE were lower (P < 0.001, P < 0.05, and P < 0.001, respectively) in Colostrum- vs. formula-fed calves. The DEXA effects in the ileum are also demonstrated on Figure 2.

A positive correlation (r = 0.55, P < 0.01) was calculated for cell proliferation rates and the number of B-lymphocytes in follicles of PP. A negative correlation (r = −0.46, P < 0.01) was calculated for cell proliferation rates and the number of T-lymphocytes in follicles of PP.

### Histochemical Measurements in the Thymus

The number of BrdU-labeled cells in CD− and FD− was 3.5 times higher (P < 0.001) and in CD+ and FD+ was 2.5 (P < 0.001) times higher in the cortex than in the medulla (Table 3). The DEXA treatment decreased (P < 0.001) the number of BrdU-labeled cells in the cortex and medulla, and DEXA effects were more pronounced in formula-fed calves. The number of BrdU-labeled cells in the cortex and medulla was higher (P < 0.001) in Colostrum- vs. formula-fed calves. Because the medulla:cortex ratios of BrdU-labeled cells were increased (P < 0.001) by DEXA treatment compared with non-DEXA-treated calves (0.39 and 0.29, respectively), the depression of proliferative cells by DEXA was greater in the cortex than in the medulla. There was also a DEXA × feeding interaction because the medulla:cortex ratios after DEXA treatment were lower (P < 0.001) in Colostrum- vs. formula-fed calves (0.38 and 0.42, respectively). The number of apoptotic cells was increased (P < 0.001) by DEXA treatment. For technical reasons, we could not differentiate between cortex and medulla (i.e., the great number of T-lymphocytes in the thymus did not allow quantitative evaluations).

### Table 2. Cell proliferation, apoptosis and T- and B-lymphocytes in the ileum of neonatal calves fed formula or colostrum and treated with dexamethasone (DEXA; FD+, CD+) or without DEXA (FD−, CD−)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experimental groups</th>
<th>ANOVA (P-values)</th>
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<tbody>
<tr>
<td></td>
<td>CD−</td>
<td>CD+</td>
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<tr>
<td>BrdU-labeled cells/mm×</td>
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<tr>
<td>Follcles of PP</td>
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<td>Interfollicular area of PP</td>
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<td>Dome of PP</td>
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<td>TdT3′-end labeled cells/mm×</td>
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<td>Follcles of PP</td>
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<td>Domes of PP</td>
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<td>T-lymphocytes/mm</td>
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<tr>
<td>Interfollicular areas of PP</td>
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<td>38.4</td>
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<tr>
<td>Domes of PP</td>
<td>2.9</td>
<td>2.6</td>
</tr>
<tr>
<td>IEL</td>
<td>20.5</td>
<td>8.8</td>
</tr>
<tr>
<td>B-lymphocytes/mm</td>
<td>104.3</td>
<td>62.7</td>
</tr>
<tr>
<td>Follcles of PP</td>
<td>33.8</td>
<td>33.5</td>
</tr>
<tr>
<td>Interfollicular areas of PP</td>
<td>67.1</td>
<td>81.5</td>
</tr>
<tr>
<td>Dome of PP</td>
<td>13.3</td>
<td>13.2</td>
</tr>
</tbody>
</table>

aValues are means with pooled SEM, n = 7 per group.
bCounting of 5-bromo-2′-deoxyuridine (BrdU)-labeled cells allowed for evaluation of the proliferation rates in PP.
cPeyer’s patches.
dCounting of TdT3′-end labeled cells allowed to evaluate the apoptotic rates in PP.
eIntraepithelial lymphocytes.
fFollicle-associated epithelium.

The number of B-lymphocytes was higher (P < 0.01) in follicles than in domes, interfollicular areas, and FAE (P < 0.001) of PP, was higher (P < 0.001) in follicles than domes, interfollicular areas, and FAE in CD+, was higher (P < 0.001) in follicles and domes than in interfollicular areas and FAE in CD−, and was higher (P < 0.001) in domes than in follicles, interfollicular areas, and FAE in FD+. The number of B-lymphocytes within follicles was higher (P < 0.05) in peripheral vs. central regions (peripheral and central regions contained 43.7 and 56.3% in CD−, 43.6 and 56.4% in CD+, 44.8 and 55.2% in FD−, and 41.0 and 59.0% B-lymphocytes in FD+, respectively). The DEXA treatment decreased (P < 0.001) the number of B-lymphocytes in follicles but increased (P < 0.01) the number of B-lymphocytes in domes and tended to increase (P < 0.1) the number of B-lymphocytes in FAE of formula-fed calves. The numbers of B-lymphocytes in interfollicular areas, domes, and FAE were lower (P < 0.001, P < 0.05, and P < 0.001, respectively) in Colostrum- vs. formula-fed calves. The DEXA effects in the ileum are also demonstrated on Figure 2.
Discussion

The immune system of the gastrointestinal tract comprises three main compartments with distinct functions: the GALT, the intestinal epithelium, and the mucosal lamina propria (Boll et al., 1995). In the GALT, the PP are the main component, and in calves, are especially present in the ileum (Brandtzaeg et al., 1999). Ileal PP play a major role in the development of primary B-cells in the calf and in other species (Gerber et al., 1986; Motyka and Reynolds, 1991; Yasuda et al., 2002) and are a primary lymphoid organ (Reynolds and Morris, 1983; Landsverk, 1984). The peripheral areas of the follicles in the ileal PP of calves are mainly composed of proliferating B-lymphocytes, whereas the central areas consist mainly of nondividing B-lymphocytes (Yasuda et al., 2002), as shown in sheep (Reynolds, 1987) and in the present study. Furthermore, there are different stages of B-lymphocyte development from the follicle to the dome region (Hein et al., 1989). In the present study, most proliferating cells and B-lymphocytes were in the periphery of follicles and there was a significant correlation between proliferation rates and the number of B-lymphocytes.

About 5% of ileal follicular B-lymphocytes of lambs enter the metaphase each hour, which is about 10 times greater than the proliferation rate of T-lymphocytes in the thymus (Reynolds et al., 1987). There must be regulatory mechanisms that prevent excessive cell proliferation (i.e., cell production), and thus, tissue mass must be balanced by cell immigration into or emigration out of tissues (Reynolds, 1986) or by changes in apoptotic rates. Apoptotic rates of B-lymphocytes are much more evident in ileal PP than are apoptotic rates of T-lymphocytes in the thymus (Motyka and Reynolds, 1991). Thus, up to 95% of immature B-lymphocytes produced in follicles undergo apoptosis (Motyka et al., 1995; Griebel et al., 1996).

The apoptosis:proliferation (i.e., TUNEL:BrdU) ratios were lower in follicles than in the domes of PP in CD− and FD−. Under the assumption that the methods used reflect equal kinetics of cell proliferation and apoptosis, the number of cells in follicles should increase. Maintenance of stable cell numbers in follicles may be possible if fewer cells immigrate into or more cells emigrate out of follicles, or if there is an increased number of cells that are directed towards apoptosis. On the other hand, in the domes of CD− and FD−, the apoptosis:proliferation ratios were markedly higher, suggesting that more cells may die than proliferate. Maintenance of a stable cell number in domes may, in this situation, be possible by enhanced immigration of cells from other lymphoid tissues into or reduced emigration from domes.

The number of T-lymphocytes in the ileal PP follicles is very low (Hein et al., 1989). The T-lymphocytes in the ileum are mainly found in the interfollicular areas of PP (Pospischil, 1989) and as IEL (Willard, 1992; Wy-att et al., 1999). This was also the case in the present study.

The thymus gland is a primary lymphoid organ in which bone marrow-derived T-lymphocyte precursors undergo maturation, eventually followed by migration of selected thymocytes to peripheral lymphoid organs such as spleen, lymph nodes, PP, and tonsils. The fact that there were more proliferating cells in the cortex than in the medulla of the thymus in 5-d-old calves in the present study indicates that more immature T-lymphocytes were present in the cortex, in accordance with Savino and Dardenne (2000).

The administration of DEXA caused a remarkable reduction of the size of PP follicles. This reduction was likely due to reduced proliferation rates and increased apoptotic rates of B-lymphocytes in PP follicles by DEXA. Reduced numbers of B-lymphocytes in follicles by DEXA treatment, as in the present study, were also seen in sheep and rabbits (Roy and Walsh, 1992; Griebel et al., 1996). In pigs, DEXA suppressed the proliferation of B-lymphocytes and Ig production (Rogers et al., 2001) and reduced circulating Ig levels (Roy and Walsh, 1992). However, serum IgG concentrations were not affected by DEXA in the present colostrum-fed calves because plasma IgG levels were mainly the result of intestinally absorbed colostral IgG. The immature B-lymphocytes in PP may be particularly susceptible to apoptosis induced by glucocorticoids. Glucocorticoid effects on B-lymphocytes may be mediated by other cells, such as stromal cells (Halleraker et al., 1990; Nicander et al., 1991; Griebel et al., 1993). In contrast to follicles, DEXA decreased the apoptotic rates in interfollicular areas of PP, but only in colostrum-fed calves.

Treatment with DEXA increased the number of B-lymphocytes in domes. This increase was not associated with higher proliferation rates and reduced apoptotic rates because the proliferation and apoptotic rates in the domes did not change with DEXA treatment. Therefore, the decrease of B-lymphocyte numbers in follicles may be due not only to the loss of B-lymphocytes, but may also be due to the movement of these cells toward the domes. The preemigrant B-lymphocyte population is located in crescent-shaped regions between follicles and domes called the corona (Hein et al., 1989). The dome area contains many small lymph vessels that enable B-lymphocytes to leave follicles and to reach the mesenteric lymph nodes (Rothkött er et al., 1999). Another explanation could be that B-lymphocytes from other lymphoid tissues immigrate into the domes. In addition, DEXA increased the number of B-lymphocytes in FAE, but only in formula-fed calves.

In the present study, the number of T-lymphocytes in the follicles after DEXA treatment increased. The simultaneous reduction in follicle size was probably one reason for the greater number of T-lymphocytes. Interestingly, the number of intestinal IEL decreased in the DEXA-treated groups. In mice, glucocorticoids induce apoptosis of intestinal IEL (Muroasaki et al.,
Figure 2. Panel A shows B-lymphocytes (CD79), dark stained, in ileum of a calf fed colostrums, and panel B shows B-lymphocytes in a calf fed colostrum and treated with dexamethasone. In panel A, B-lymphocytes are mainly seen in the follicles and domes. In panel B, the follicles are smaller and B-lymphocytes are mainly seen in the domes. 1 = lamina muscularis externa, 2 = submucosa, 3 = follicle, 4 = interfollicular area, 5 = dome, 6 = crypt, 7 = villus, 8 = lamina propria, 9 = follicle-associate epithelium, 10 = gut lumen.

Cell proliferation rates were decreased and apoptotic rates were increased in the cortex and medulla of the thymus in DEXA-treated calves. The proliferation rate in the cortex was more dramatically affected by DEXA than in the medulla. Glucocorticoids can nearly extinguish thymopoiesis and thereby interrupt the supply of virgin $\alpha\beta$ and $\gamma\delta$ T-lymphocyte to the periphery (Kong et al., 2002). The apoptotic effect of glucocorticoids is particularly notable in immature thymocytes (Kong et al., 2002). Hence, primarily the cortical thymocytes are affected by glucocorticoids (Cohen, 1992). As a result, the proliferation of immature thymocytes was more sensitive to DEXA-induced apoptosis in the thymus of neonatal calves.
Lower proliferation rates and higher apoptosis:proliferation ratios in the follicles of PP and lower numbers of B-lymphocytes in interfollicular areas, domes, and FAE of PP were found more in colostrum-fed calves than in formula-fed calves. The apoptosis:proliferation ratio in DEXA-treated calves increased in colostrum- vs. formula-fed calves in follicles. In addition, the number of T-lymphocytes tended to decrease in colostrum-fed calves in the domes, especially in DEXA-treated calves. These immunosuppressive effects were unexpected. Although colostrum is essential for passive immunity for calves, it also has potent immunomodulatory properties because it can preclude calves from developing an active immune response to certain antigens (Barrington and Parish, 2001). Ingested colostral IgG reduces the uptake of dietary and microbial antigens, and therefore may decrease their contact with the GALT system. Absorbed colostral IgG results in systemic protection. In addition, absorbed colostral Ig are resecreted by mucosal surfaces into the intestinal lumen (Stokes and Bourne, 1989). Furthermore, passively transferred Ig may also suppress neonatal immunity nonspecifically. This is illustrated by the finding that endogenous antibody production starts sooner and antibody levels reach higher peak concentrations in colostrum-deprived vs. colostrum-fed calves (Banks and McGuire, 1989). Antigen-specific inhibition is observed in calves fed colostrum, which contains antigen-specific antibodies. These calves do not develop an antibody response when exposed to that antigen (Barrington and Parish, 2001). Another aspect would be the transfer of colostral leukocytes that modulate immune responses (Riedel-Caspari and Schmidt, 1990). Therefore, the greater number of T- and B-lymphocytes in formula- vs. colostrum-fed calves in the dome area and the higher proliferation rate in the follicles lead to the assumption that the immune system of colostrum-deprived calves starts to work sooner than that of colostrum-fed calves.

In the thymus, proliferation rates were higher in colostrum- vs. formula-fed calves, whereas apoptotic rates were not changed. Compared with lymphoid tissues in the ileum, colostrum intake had opposite (i.e., stimulatory) effects on lymphoid tissue in the thymus. The DEXA × feeding interactions in the thymus were highly significant. Thus, in CD− proliferation rates decreased about 50%, whereas in FD+ the proliferation rates decreased about 75%. Based on cortex to medulla ratios, the proliferation in the cortex was more affected in formula-fed than in colostrum-fed calves. The DEXA effects were not as marked in colostrum-fed calves as in formula-fed calves. Colostrum feeding thus protected the thymus from DEXA effects; however, this effect was not mediated by reduced apoptotic rates in neonatal calves.

In conclusion, colostrum feeding decreased cell proliferation rates in follicles of PP and the number of B-lymphocytes in domes, FAE, and in interfollicular areas of PP, but enhanced cell proliferation in the thymus compared with formula-fed calves, implying that colostrum ingestion has immunomodulatory properties beyond its effects on circulating IgG levels. The DEXA treatment enhanced apoptotic rates and decreased proliferation rates in PP follicles and thymus, decreased the number of IEL, and reduced B-lymphocyte numbers in follicles, but increased B-cell numbers in domes. Thus, DEXA treatment disrupts the normal lymphocyte production pattern in both ileal PP and thymus of neonatal calves. Most impressive were the decrease of IEL numbers, depletion of follicles, the decrease of B-lymphocyte numbers in follicles, and the increase of B-lymphocyte numbers in domes in DEXA-treated calves. Immunomodulatory effects of colostrum feeding on cell proliferation in follicles and on the number of B-lymphocytes in the interfollicular areas, domes, and FAE were marked also.

Implications

The intake of colostrum may decrease the need of neonatal calves to develop an active immune response. In colostrum-deprived calves, this immunological as-

### Table 3. Cell proliferation and apoptosis in the thymus of neonatal calves fed formula or colostrum and treated with dexamethasone (DEXA; FD+, CD−)

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>ANOVA (P-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEXA</td>
</tr>
<tr>
<td>CD− Cortex 5.4 2.1 5.2 1.2 0.05</td>
<td>***</td>
</tr>
<tr>
<td>Medulla 1.8 0.8 1.3 0.5 0.02</td>
<td>***</td>
</tr>
<tr>
<td>TdT 3′-end labeled cells/mm² 1.0 1.7 1.0 1.7 0.7</td>
<td>***</td>
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</tbody>
</table>

aValues are means with pooled SEM, n = 7 per group.
bCounting of 5-bromo-2′-deoxyuridine (BrdU)-labeled cells allowed to evaluate the proliferation rates.
cCounting of TdT3′-end labeled cells allowed to evaluate the apoptotic rates in PP.
NS = not significant (P > 0.1).
*** = P < 0.01.
**** = P < 0.001.
sistance is lacking and the immune system of these calves must therefore start to work sooner after birth. Because the ileal Peyer’s patches in neonatal calves are the primary source of B-lymphocytes for all lymphoid tissues and because the thymus is the primary source of T-lymphocytes, the severely depleted lymphoid tissues in follicles of Peyer’s patches and in the thymus due to dexamethasone treatment may lead to changes in the circulating lymphocyte populations. Thus, due to a high glucocorticoid status mimicked by dexamethasone treatment in the present study, neonatal calves under adverse conditions are expected to become more susceptible to infections.

**Literature Cited**


