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Changes in integrin-positive cells and T cell subpopulations in the peripheral blood and intestine of calves fed soya protein*

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ABSTRACT

In order to examine the relation of known intestinal lesions to changes in T-cell phenotypes and integrin expression, 16 male 10-day-old Holstein calves were divided into two groups. For 28 days of the experiment, eight males were fed NutriMilk in which 50% of the crude protein was soya protein, and eight control animals, with NutriMilk containing only milk casein. The animals fed soya protein showed shorter jejunal villi with a corrugated surface and deeper crypts compared with the control calves. A higher density of CD8+ cells in the intestinal mucosa and a decrease of these cells in peripheral blood were found in calves fed soya protein. The number of CD11b-positive cells was decreased in the peripheral blood of calves fed soya protein. Lower expression of integrin could be related to the appearance of non-mature polymorphonuclear cells. It is not clear if the decrease in CD11b expression on blood cells could also be influenced by milk replacer, i.e. soya protein.

KEY WORDS: calves, soya protein, intestine, immunity, integrin, T cells

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INTRODUCTION

For economic reasons calf rearing is now conducted using substitution of milk protein by milk replacers. In addition to foreign lipids, suckling preruminants are given primarily foreign proteins, such as soya protein. Soya proteins have been shown, however, to reduce growth and feed efficiency in young ruminants when compared with whole milk or casein diets (Drackley et al., 2006). Partial replacement of milk protein by soya protein results in morphological changes of the intestinal structure, including reduced villous circumference and height in the jejunum of kids (Žitňan et al., 2005; Schönhusen et al., 2010).

Small intestine intraepithelial lymphocytes (IEL) represent a unique population of cells. In calves, T lymphocytes, CD4 and CD8 cells, constitute a large population of IEL (Wyatt et al., 1999). IEL are cytolytic (Sydora et al., 1993) and upon activation, express cytokines consistent with a type I (cytotoxic) immune response (Yamamoto et al., 1993; Gelfanov et al., 1995). In addition to T lymphocytes, small intestinal IEL isolated from neonatal calves contain a population of sIgM+ lymphocytes. The reduction of jejunal villi is associated with a dramatic infiltration of the lamina propria by B and T lymphocytes after feeding of preruminant calves with heated soyabean flour (Lallès et al., 1996).

Local inflammatory sites increase the number of phagocytes *via* inflammatory mediators and interleukins (Arnaout, 1990). However, effective neutrophil recruitment to the site of infection requires adhesion molecules from the selectin and β2 integrin families. The most important β2 integrins involved in neutrophil recruitment into inflamed tissue are CD11b/CD18 and CD11c/CD18 (Tizzard, 2009).

The present study was conducted to examine morphological changes of the jejunal villi in relation to the reactivity of peripheral and intraepithelial lymphocytes. Moreover, we evaluated integrin-positive cells in order to find a possible effect of soya protein on the activity of phagocytes in preruminats calves.

MATERIAL AND METHODS

Animals, diets and experimental procedures

Sixteen Holstein bull calves were enrolled onto the study at the age of 10 days. They were born and maintained at the same commercial farm. After birth the calves stayed with their mothers to suck colostrum and milk. At the beginning of the experiment they were randomly assigned to dietary treatment groups of 8 bulls each. The animals were housed in individual boxes in an ambient temperature of

approximately 16°C with continuous access to fresh water. Calves were weighed weekly prior to the morning feeding.

The calves of group I (control) were administered a milk feed mix, NutriMilk (MP), containing milk proteins, and the calves of group II (experimental) were given NutriMilk (SP), containing plant proteins, i.e. 50% of soya proteins in the milk drink. The milk feed mixes were diluted at a ratio of 125 g of mix per litre of water. The producer of the mix used in this experiment was Biofaktory, Ivanka pri Dunaji (Slovakia). The chemical composition and components of the mix are given in Table 1. Both groups of calves were fed three times per day only the milk drink; the amount was gradually increased according to their age from 6 l in week 2 of age to 10 l in week 6 of age (an increase by 1 l every week).

Table 1. Chemical composition and components of milk feed mixtures for calves

Item	NutriMilk MP[1]	NutriMilk SP[2]
Dry matter, g/kg	968	964
Crude protein, g/kg of DM	220	220
Crude fat, g/kg of DM	150	150
Crude fibre, g/kg of DM	3	6
Ash, g/kg of DM	80	88
ME, MJ/kg of DM	17.87	17.75

[1]NutriMilk MP - dried whey, dried buttermilk, full cream dried milk, coconut and palm oil, dextrose, vitamin-mineral premix; [2]NutriMilk SP - dried whey, wheat gluten, soya protein concentrate (Hamlet Protein A/S, Horsens, DK), coconut and palm oil, dextrose, vitamin-mineral premix

During the experiment blood was sampled from the jugular vein of the calves into tubes containing disodium-EDTA (1.5 mg·ml^{-1} blood). Blood was sampled for the first time at the age of 7 days before the beginning of the experiment (sampling 0), and then twice in two-week intervals during the experiment (samplings 1 and 2). On day 28 of the experiment the calves were sacrificed and samples of the individual parts of the intestinal tract (duodenum, jejunum and ileum) were collected.

Analytical procedures

White blood cell (WBC) count. Leukocyte counts were determined using a routine laboratory method with a Bürker chamber and Türk solution (475 µl solution plus 25 µl blood). Differential cell counts of 100 cells per slide were done by light microscopy at 1000 x magnification using blood smears and after staining the cells with Hemacolor kit (Merck, Germany). Numbers (actual count) of different types of WBC were determined as follows: total leukocyte count x proportion of differential cells counted (%) /100.

Flow cytometry of blood. The indirect immunofluorescence method and whole blood for phenotyping of lymphocyte subpopulations were used. Primary unlabelled mouse anti-bovine monoclonal antibodies (donated by Illrad, Kenya) are summarized in Table 2. The secondary antibody, a polyclonal goat anti-mouse FITC conjugated immunoglobulin $F(ab')_2$ fragment (Dako, Denmark), was used for staining lymphocytes for indirect immunofluorescence at a working dilution of 1:50 with phosphate-buffered saline and 0.1% sodium azide (PBS+NaN_3).

Table 2. Primary unlabelled mouse-anti-bovine monoclonal antibodies used in the experiment

MoAbs	Specificity	Isotype	Dilution
IL-A30	IgM	IgG1	1:2500
IL-A12	CD4	IgG2	1:2000
IL-A51	CD8	IgG1	1:1000
IL-A42	CD2 (pan T)	IgG	1:1000
IL-A29	BoWC1(TCRγ/δ)	IgG2b	1:1000
IL-A46	CD11c	IgM	1:1000
Il-A130	CD11b	IgG2a	1:1000

Whole blood (100 µl) was incubated with a working dilution of unlabelled primary antibody for 15 min at room temperature in the dark. Erythrocytes were lysed using a lysing solution (8.3 g NH_4Cl, 1 g $KHCO_3$, 200 mM EDTA dissolved in 1 l distilled water) for 10 min at room temperature in the dark. After lysis the samples were centrifuged at 145 *g* for 5 min, and washed twice in PBS. The pellets were mixed with 25 µl of secondary antibody and incubated 30 min at +4°C. After staining the cells were washed once in PBS and resuspended in 0.2 ml of PBS containing 0.1% paraformaldehyde. For each cell suspension, cell population acquisition and analysis were carried out using a flow cytometer with a 15mV argon laser (498 nm, FACScan, Becton Dickinson, Germany). For acquisition a live gate was drawn around the mononuclear cell population containing the lymphocytes. Fluorescence data were collected on at least 10 000 lymphocytes and analysed using the Becton Dickinson Cell Quest (Germany). For each marker, the relative percentage of fluorescent positive cells within this gate was recorded by dot-plot analysis. The numbers of lymphocytes in each subpopulation were computed as follows: actual lymphocyte counts x relative size of subpopulation (%)/100.

Immunohistochemistry. Examination of intestinal CD3 positive cells was done with polyclonal rabbit anti-human T cell CD3 antibody (at a dilution of 1:300, Dako, Denmark) on paraffin sections described by Tomková et al. (2002). Frozen sections were used for evaluation of ileal CD8+ cells by mouse anti-bovine monoclonal antibody (Table 2). The pieces of intestine (1 cm) were put into PBS, held on crushed ice, frozen and cut in a Cryocut E (Reichert, Germany) at -24°C.

Frozen 7 μm sections fixed in cold acetone and rinsed in PBS were incubated with unlabelled primary monoclonal antibody for 1 h. A biotin-streptavidin amplified peroxidase detection system (Biogenex, USA) was used to detect a positive reaction. All incubations were done at room temperature; between the two consecutive 1 h incubations the sections were rinsed three times with PBS; it was also used instead of monoclonal antibodies as a negative control. The specific colour reaction was developed for 5 min with 3.5 mmol·l^{-1} 3,3′-diaminobenzidine (Sigma, Germany), and 30 ppm hydrogen peroxide in 200 mmol·l^{-1} Tris/HCl (pH 7.6). The sections were counterstained with haematoxylin and mounted into Entellan (Merck, Germany).

Intestinal morphometry. Intestinal tissue (1 cm^2) from the duodenum, jejunum and ileum was fixed in 4% neutral formaldehyde solution. After being rinsed in water, the samples were dehydrated in a graded series of ethanol (30, 50, 70, 90% and absolute ethanol), cleared in benzene, saturated with and embedded in paraffin. Sections of 5 μm thickness (10 slices of each sample) were stained with haematoxylin/eosin and observed under a light microscope. The height of 30 villi and depth of 30 crypts were determined by the computer-operated *Image C* picture analysis system (Imtronic GmbH, Berlin, Germany) and the IMES (interactive measurement) analysis programme, by using a colour video camera (SONY 3 CCD, Sony Electronics Ltd., Tokyo, Japan) and a light microscope (Axiolab, Carl Zeiss AG, Jena, Germany).

Scanning electron microscopy. Tissue samples of 0.5 cm^2 were cut from the jejunum and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.2). After washing in this buffer they were postfixed in 1% osmic oxide and dehydrated through a graded series of ethanol, placed in acetone and, finally, critical-point dried in liquid CO_2. Samples were then coated with gold at a thickness of 20 nm at 4°C. Thus prepared samples were examined using a JOEL JEM-100 CX II electron microscope (Joel Ltd., Akishima, Japan) with an ASID-4D high resolution scanning system at an accelerating voltage of 15 kV.

Statistical analyses

Statistical analyses were carried out using Microsoft Excel and the unpaired Student's *t*-test. The results are expressed as mean values ± SD. Mean differences were considered significant if $P<0.05$.

RESULTS

The animals were clinically healthy and daily body weight gain (milk protein 784±73 g; soya protein 763±63 g) was not different between the dietary groups.

Small intestine morphometry and morphology of the jejunum. Villi height was lower ($P<0.05$) in preruminant calves fed soya protein than in those fed milk protein. By contrast, the depth of the crypts was higher ($P<0.05$) in calves fed soya protein (Table 3). Light microscopy did not show alteration of the mucosa in the small intestine of either group. Scanning electron microscopy did, however, reveal a corrugated surface of the villi in the jejunum and a tendency to blend in the experimental group fed soya protein. The villi of this group were shorter and less uniform (Figure 1A). The villi of the jejunum were finger-shaped, uniform, and higher in control animals (Figure 1B).

Table 3. Height of villi and depth of crypts in the small intestine of calves fed milk protein (NutriMilk MP) and soya protein (NutriMilk SP)

Small intestine parts	NutriMilk MP	NutriMilk SP	NutriMilk MP	NutriMilk SP
	sampling			
	height of villi, μm		depth of crypts, μm	
Duodenum	663 ± 36	586 ± 43	242 ± 18	279 ± 19
Jejunum	735 ± 42[a]	591 ± 38[b]	226 ± 16[a]	287 ± 17[b]
Ileum	578 ± 31	532 ± 27	281 ± 22	315 ± 24

values are means ± SD; specific superscripts in row - significant differences [ab] $P<0.05$

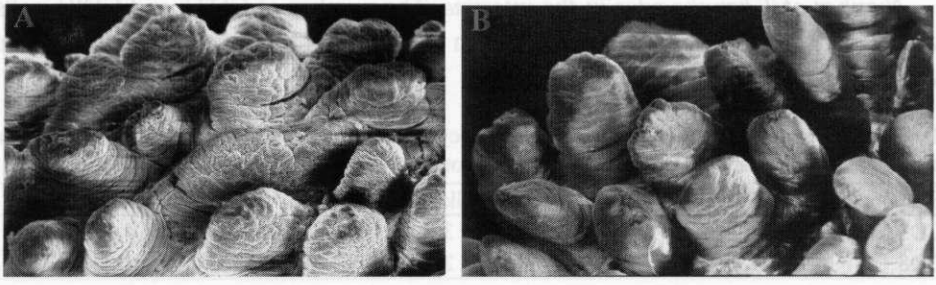

Figure 1. Scanning electron microscopy (250 x) of jejunal intestinal villi in calves fed soya protein (A - NutriMilk SP) and milk protein (B - NutriMilk MP)

Peripheral blood cells - counts and immunophenotyping. Values of peripheral white blood cells were not changed in either group (Table 4). Phenotyping of lymphocytes by flow cytometry (Table 5) did, however, show a decrease of CD8+ cells ($P<0.05$) in both samplings in calves fed soya protein compared with control animals. Similarly, the number of cells bearing CD11b was lower ($P<0.05$)

in the first sampling in the calves fed soya protein than in those fed milk protein. A similar trend ($P<0.08$) was observed in the second sampling.

Table 4. Numbers of peripheral white blood cells ($G \cdot l^{-1}$-$1 \cdot 10^9 \cdot l^{-1}$; average ± SD) in calves fed milk protein (NutriMilk MP) and soya protein (NutriMilk SP)

Cells	NutriMilk MP			NutriMilk SP		
	sampling[1]					
	0	1	2	0	1	2
Leukocytes	8.5 ± 3.5	9.3 ± 2.6	9.2 ± 1.4	7.5 ± 2.4	8.7 ± 1.8	9.3 ± 2.5
Lymphocytes	5.2 ± 2.3	6.6 ± 2.3	6.5 ± 1.4	4.9 ± 1.5	5.6 ± 1.3	5.9 ± 2.1
Neutrophils	3.1 ± 1.3	2.5 ± 1.0	2.7 ± 1.7	2.4 ± 0.9	2.8 ± 1.3	2.7 ± 1.1
Monocytes	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

[1] 0 - at 7 d before start of study; 1 - at 14 d and 2 - at 14 d after start of study

Table 5. Absolute counts of lymphocyte subpopulations (actual counts, $G \cdot l^{-1}$-$1 \cdot 10^9 \cdot l^{-1}$; means ± SD) in calves fed milk protein (NutriMilk MP) and soya protein (NutriMilk SP)

Subpopulations	NutriMilk MP			NutriMilk SP		
	sampling[1]					
	0	1	2	0	1	2
CD2	2.81 ± 1.26	3.07 ± 1.17	2.93 ± 0.88	2.66 ± 0.69	2.41 ± 0.78	2.80 ± 1.14
CD4	2.78 ± 1.41	2.88 ± 1.04	2.89 ± 0.48	2.56 ± 0.68	2.33 ± 0.64	2.62 ± 1.06
CD8	0.87 ± 0.38	0.96 ± 0.32[a]	0.95 ± 0.29[a]	0.66 ± 0.19	0.60 ± 0.18[b]	0.64 ± 0.21[b]
IgM	0.94 ± 0.46	2.58 ± 1.06	2.45 ± 0.99	0.97 ± 0.44	2.32 ± 0.66	2.15 ± 1.00
BoWC1	1.08 ± 0.52	1.19 ± 0.56	1.24 ± 0.53	1.17 ± 0.55	1.38 ± 0.62	1.20 ± 0.55
CD11b	ND	0.85 ± 0.22[a]	0.93 ± 0.35	ND	0.61 ± 0.05[b]	0.65 ± 0.08
CD11c	ND	0.83 ± 0.26	0.49 ± 0.15	ND	0.64 ± 0.19	0.44 ± 0.18

specific superscripts in row = significant differences [ab] $P<0.05$; expalantion of sampling see Table 4

Number of T lymphocytes in the intestine. The number of CD3+ and CD8+ cells was higher ($P<0.05$) in the villi of the ileum in the calves fed soya protein than in the villi of the control ileum (Table 6).

Table 6. Number of CD3 and CD8 positive T lymphocytes in ileum of calves (average from 10 villi per a sample) fed milk protein (NutriMilk MP) and soya protein (NutriMilk SP)

Subpopulations	NutriMilk MP	NutriMilk SP
CD3 + T lymphocytes	48 ± 3	52 ± 4
CD8 + T lymphocytes	31 ± 2[a]	39 ± 3[b]

values are means ± SD; specific superscripts in row = significant differences [ab] $P<0.05$

DISCUSSION

The present observation showing a shortening of the jejunal villi when preruminant calves are fed soya protein is similar to the results of earlier studies. Kelly et al. (1991) and Kelly and Couts (2000) reported decreases in the villi in young pigs subjected to feed restriction, Lallès et al. (1996), in calves fed antigenic heated soyabean flour,

and Schönhusen et al. (2010) in goats fed soya protein. Finally, the integrity of small intestinal tissue is changed by infectious and non-infectious diseases, including the ruminal drinking syndrome (Van Weeren Keverling Buisman et al., 1988). Compromised surface integrity of jejunal villi was also found by Kuhla et al. (2007) in young goats fed a milk diet containing part soya protein. These changes were related to protein turnover, energy metabolism, cytoskeleton assembly (Kuhla et al., 2007), and altered RNA metabolism (Schönhusen et al., 2007).

Increased T cell density in the jejunum of calves fed partial replacement of casein with soya protein was essentially accounted for by CD8+ cells in the epithelium. These results confirm the implication of a CD8+ T cell-mediated cytotoxicity mechanism at the epithelial level. Similar results were demonstrated by Drèau et al. (1995) and Lallès et al. (1996) in pigs and calves fed heated soyabean protein. Inflammatory damaged tissue increased the release of proinflammatory mediators in the blood (Diez-Fraile et al., 2003) with reaction of the systemic immune response.

The decrease of CD11b-positive peripheral blood cells in calves fed 50% soya protein seems to be related to the appearance of immature cells in blood. Indeed, it has been shown that CD11b is expressed in a lower percentage of bone marrow immature PMN compared with the mature pool (Van Merris et al., 2002). This rather small population of PMN staining negative for CD11b might not be able to migrate to the inflammatory tissue. Van Merris et al. (2002) also demonstrated decreased phagocytosis and oxidative burst activity in immature bovine PMN. Our suggestion is supported by the inverse ratio of PMN (day 0 vs 14 of the experiment) in blood of calves fed NutriMilk MP compared with polymorphonuclear cells of calves fed 50% soya protein (NutriMilk SP). Similarly, it has been reported in humans that immature cells express fewer adhesion molecules (Van Eeden et al., 1997). It is questionable if the decrease of CD11b on the peripheral blood cells can be attributed to the feeding of 50% soya protein.

Milk has diverse protective, anti-inflammatory and immunostimulating ingredients that influence the immune system of the offspring locally and systemically (Kelleher and Loennerdal, 2001). Bovine colostrum contains concentrations of viable leukocytes similar to those in peripheral blood and T lymphocytes from the major population of lymphocytes (Liebler-Tenorio et al., 2002).

Soya protein-based diets contain non-nutritive components, e.g., isoflavones (Chen et al., 2005), or a deficit of essential amino acids, such as Thr, Met, Lys (Kanjanapruthipong, 1998). Moreover, milk and colostrum contain the glycoprotein, lactoferin, which has immunomodulation properties (Adlerova et al., 2008).

The decrease of CD11b-positive peripheral blood cells in calves fed 50% soya protein indicates impaired migration of neutrophils to the site of infection and

vulnerability to a number of enteric diseases. Reduced surface expression of CD11/CD18 is also observed in neonatal granulocytes (Arnaout, 1990), and decreases the resistance to mastitis of cows with a specific genotype of the chemokine receptor CXCR2 (Rambeaud and Pighetti, 2005).

CONCLUSIONS

Feeding calves soya protein in the amount of 50% of total dietary crude protein confirmed the decrease of jejunal villi and alteration of their surface structure. The higher density of CD8+ cells in jejunal mucosa points to inflammatory processes in that site and can be the result of increasing migration of CD8+ lymphocytes from peripheral blood. The decrease of CD11b-positive peripheral blood cells can lead to a higher incidence of enteric and other diseases of calves fed 50% soya protein. Whether non-milk protein sources can lead to changes in other cell surface receptors on immunocompetent cells of the preruminant calf requires further investigation.

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