Immunohistochemical localization of adhesion molecules (CD62 and CD18) in the mammary gland of dairy cows

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ABSTRACT: Localization of the L-selectin (CD62L) and β2-integrin (CD18) bearing cells in different tissues of the bovine mammary gland was examined. Five dairy cows of Holstein-Friesian breed in the middle of their second and third lactation cycle were used in the study. Blood, milk and udder tissue samples were collected from each cow to estimate the milk somatic cell count (SCC) and bacteriological infection of the mammary gland. The expression of CD62L and CD18 on blood cells, milk cells and parenchymal tissues of udder, Fürstenberg's rosette and the transverse section of the central part of the teat was tested. In the mammary gland quarters the value of SCC in milk secretion was also reflected in the presence of $CD18^+$ and $CD62L^+$ leukocytes in mammary tissues. In the quarters where SCC was higher than 10^5 a frequent incidence of L-selectin and β2-integrin cells was observed in the parenchyma and Fürstenberg's rosette region, while in the quarters with low SCC, none or only a few reactive cells were found. In the mammary parenchymal tissue CD18 positive cells were present in both the epithelial and the connective tissue. In Fürstenberg's rosette the cells were concentrated in the connective collagenous and loose tissue. None or only scattered L-selectin (CD62L) and β2-integrin (CD18) bearing cells were identified in the transverse section from the central part of the teat. When we compared the L-selectin and β2-integrin expression, the study revealed the down-regulation of L-selectin on the cells of mammary tissue.

Keywords: cattle; L-selectin; β2-integrin; mastitis

Adhesion molecules such as selectins and integrins expressed on the surface of leukocytes represent essential components of immunity because they are involved in the interaction of the immunocompetent cells and in the communication of these cells with the intercellular matrix. Selectins are membrane-bound lectins that initiate the adhesion of leukocytes to endothelial cells, platelets, or other leukocytes on the vessel wall. An initial slowing of leukocytes mediated by selectins is followed by firm adhesion of leukocytes to the vascular endothelium and transmigration of leukocytes into the site of inflammation. This process is mediated by other adhesion molecules including β2-integrins (Ebnet et al., 1996; Czarnik et al., 2007).

The mammary gland is protected by various defence mechanisms. There is some production of immunoglobulins secreted by plasma cells that are present in the tissue of the mammary gland (Lee et al., 1992). The migration of leukocytes from the blood into the mammary gland is an essential element of resistance to infections. Circulating leukocyte populations are a primary source of cells that transmigrate into the mammary gland of

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healthy and infected animals. The manner of the neutrophil traverse of the secretory epithelia is not quite clear yet. However, adhesion molecules are likely to be one group of molecules that regulate the entry of leukocytes into the mammary gland (Van Kampen et al., 1999). Variations in the expression of integrins and selectins may indicate the important role of these molecules in regulating the movement of leukocytes into the mammary gland. The adhesion molecules on blood leukocytes are also present on somatic cells of milk. During the transendothelial migration of lymphocytes and neutrophils into the mammary gland the expression of L-selectin is down-regulated in contrast to CD18 which is up-regulated (Persson-Waller and Colditz, 1998). Milk is not the only element of the udder defence as leukocytes are also present in mammary gland tissues. However, the distribution of the L-selectin (CD62L) or β2-integrin (CD18) bearing cells in bovine mammary gland tissues has not been studied yet. Therefore, the purpose of this study was to investigate the distribution of CD18 and CD62L positive cells in three different parts of the mammary gland of dairy cows (parenchyma, Fürstenberg's rosette and teat).

MATERIAL AND METHODS

Animals, blood, milk and tissue samples

Five crossbred Slovak Pied × Holstein-Frisian cows aged 5–6 years and in the middle lactation stage were selected for this study. Blood and milk samples were collected from each animal. Postmortem, the mammary gland parenchymal tissue was dissected from the central region of the upper body of the mammary gland and from the region surrounding the mammary gland cistern. Further tissue samples were taken from the region of Fürstenberg's rosette and from the transverse section in the central part of teat immediately after slaughter. The NK-mastitis test was used to select the cows with infected and uninfected udder quarters. The samples were cut into cubes smaller than 1 cm^3.

The tissues were quickly frozen in liquid nitrogen and stored at –70°C until a histochemical analysis. The blood from all animals and the milk from all selected quarters were examined. To determine "the incidence of bovine mastitis", the milk samples were cultured for bacteriology (cultivation on GTK agar at the temperature of 30 ± 1°C during 72 ± 3 hours) and the estimation of SCC was performed with Fossomatic 90 (Foss Electric, Hillerod, Denmark).

Isolation of leukocytes from milk and blood

200 ml of milk was mixed with an equal volume of phosphate-buffered saline (PBS) (pH 7.2) containing 20% of acid citrate dextrose and 20mM EDTA (Park et al., 1992). The mixture was filtered through a nylon net of 50 μm porosity and centrifuged at 400 × g for 30 min at 15°C. The supernatant was discarded and cell pellets were washed several times and used in an indirect immunofluorescence test to examine the CD18 and CD62L positive cells. Blood leukocytes were isolated after haemolysis of erythrocytes with a lysing solution (0.15M NH_4Cl). Smears prepared from blood and mammary cells for a differential analysis were stained by Papenheim's panoptic method. At least 100 cells were identified on the basis of morphological and standard staining characteristics.

Monoclonal antibodies

Hybridoma cell lines producing monoclonal antibodies IVA-35 (CD18) and IVA-94 (CD62L) were obtained after the immunisation of BALB/c mice with bovine peripheral blood leukocytes using standard procedures for the fusion of SP2/0 with splenocytes of immunised mice, selection and cloning of hybridomas (Dušinský et al., 1988). The specificity of both antibodies was verified in tests performed at the 3rd Workshop on Ruminant Leukocyte Antigens (Naessens and Hopkins, 1996). The reactivity of IVA-35 and IVA-94 was analysed on various leukocyte populations and cell lines with a flow cytometer. Generally, both antibodies recognised the target antigen on 95–100% of bovine blood granulocytes, monocytes and lymphocytes. However, the reaction of IVA-94 on lymphocytes was more restricted (70% positive cells).

Immunohistochemical staining and morphometric analysis of tissue sections

For histochemical studies, a series of cryostat sections 5–15 μm thick was prepared from each

sample with Cryocut 1 800 (LEICA). The sections were fixed for 5 min in a cold ethanol-acetone mixture (1:1), air-dried, treated with 0.6% H_2O_2 in PBS and then stained using an indirect immunoperoxidase test. The sections were incubated with the first antibody (IVA-35 and IVA-94) for 45 min at 20°C. The slides were washed with PBS and then incubated for 45 min with peroxidase-conjugated porcine anti-mouse Ig (SEVAC Praha) diluted 1:50 in PBS containing 5% of normal bovine serum. After repeated washing in PBS, the sections were incubated in 0.06% (w/v) diaminobenzidine tetrachloride (Sigma Chemicals, St. Louis, MO) in PBS containing 0.05% (v/v) H_2O_2 for 10 min at room temperature. After washing, the slides were slightly contra-stained with Harris's haematoxylin.

The preparations were evaluated subjectively and quantitatively by a morphometric method. The data were acquired by means of a digital camera using the NIKON and Image Analysis System. Tissue and cell count was carried out so as to evaluate 15 fields per section; one field depicts a rectangle of 353 600 μm^2 at 350× magnification using a monitor micrometer of 221 squares (Nikon) according to the point counting technique by the System for Image Processing and Analysis – Lucia 4.60. The relative volume of glandular parenchyma, connective tissue, loose tissue and also the relative volume of epithelium and alveolar lumen and ducts were determined. The mean and standard deviation (SD) were calculated and the differences between the various tissues of udders were tested by Student's *t*-test.

Indirect immunofluorescence assay

An indirect immunofluorescence assay described by Boucheix et al. (1983) was used to test the binding of IVA-35 and IVA-94 to blood or milk leukocytes. Briefly, the separated cells were washed three times with PBS with 1% bovine serum albumin and 0.1% sodium azide. The cell suspensions were incubated with monoclonal antibodies in round-bottomed 96-well polystyrene plates for 45 minutes at room temperature. Then the cells were washed 3 times and treated with diluted (1:20–30) fluorescein isothiocyanate (FITC)-conjugated porcine anti-mouse immunoglobulin (SEVAC Praha), for 45 minutes at room temperature. After further washing the cell suspensions were placed on slides and examined under an epifluorescence microscope (Jenalumar).

RESULT AND DISCUSSION

The analysis of milk secretions revealed two infection profiles of mammary glands (Table 1). Milk samples of cows number 2 and 4 showed the highest counts of microbes and further features of inflammation. These two cows had the highest values of SCC and an elevated number of PMN (polymorphonuclear) leukocytes in their milk. Cows number 1, 3 and 5 were quite free of infection and mastitis. SCC per ml of milk were markedly lower than 10^5 cells and the predominant type of cells were macrophages and lymphocytes.

The labelling of blood and milk cells with monoclonal antibodies IVA-35 and IVA-94 was tested on the whole leukocyte population. The informative values measured in blood and milk just before killing the animals are given in Table 2. Considerable proportions (percentage) of immunofluorescence labelled leukocytes were found in the blood and milk secretion of all animals independently of the infection stage of the udder. Both the blood and milk leukocytes contained a lower percentage of L-selectin positive cells compared to β2-integrin bearing cells.

Table 1 Bacterial infection, NK mastitis test, SCC and leukogram of the milk of experimental cows

Cow No.	Mammary gland quarter	Incidence of bacterial CPM	NK values	SCC × 10^3	PMN (%)	Lymphocytes (%)	Macrophages (%)
1	right fore	1 800	–	92	35	17	48
2	right fore	5 100	++	1 320	85	4	11
3	right fore	2 300	–	11	34	21	45
4	left fore	8 500	+	854	81	7	12
5	right fore	570	±	26	31	23	46

Table 2 Expression of CD18 and CD62L on bovine blood and milk leukocytes tested by an indirect immunofluorescence test

Cow No.	Percentage of positive			
	CD18		CD62L	
	blood	milk	blood	milk
1	100	98	85	72
2	98	95	82	65
3	97	92	87	45
4	96	90	83	57
5	100	89	80	52

There was a coincidence between SCC in milk and the presence of CD18 and CD62L positive leukocytes in the udder tissues. Integrin and selectin bearing cells were rarely present (only a few scattered cells) in the mammary gland tissues of cows number 1, 3 and 5, showing low SCC in their milk. On the other hand, these cells were frequently observed in the udder tissues of cows number 2 and 4, whose milk contained elevated SCC. Similarly like Hodgkinson et al. (2007) we were not able to detect L-selectin positive cells in the udder tissue of healthy cows in the different physiological stages of mammary gland. In the mammary tissue, integrin- and selectin-bearing cells were concentrated in the region of parenchyma and Fürstenberg's rosette. None or only few scattered cells were found in the sections from the central part of the teat (Table 3). In the mammary parenchymal tissue CD18 positive cells were present in both the epithelial and connective tissue region. In the epithelial region CD18 leukocytes were most frequently located in close proximity to the mammary epithelium among epithelial cells (Figure 1a) and in the lumen of alveoli. Fewer leukocytes were reactive with CD62L monoclonal antibody in the same part of the mammary gland. Cells with lower intensity of staining were found mainly in the interalveolar connective loose tissue (Figure 1b). A high concentration of CD18 leukocytes was found in the region of Fürstenberg's rosette. In this region, the presence of β_2 integrin positive cells was restricted to the interalveolar and interlobular connective tissue (Figure 1c). Similarly to the parenchyma, a lower number of L-selectin than β_2-integrin positive cells with lower intensity of staining was observed in the connective tissue of Fürstenberg's rosette (Figure 1d). Scattered β_2-integrin positive cells (Figure 1e) and L-selectin positive cells (Figure 1f) were also detected on the section from the central part of teat.

The relative frequency of the selectin and integrin positive cells was analysed morphometrically in three parts of the udder in the tissue of two cows (number 2 and 4) which showed an elevated value of somatic cells in their milk and mammary gland tissue (Table 4). A lower number of selectin positive cells was found in all three regions of the udder under study, although the difference between selectin and integrin positive cells in the parenchyma was not significant. A significantly lower percentage of both adhesion molecule positive cells was found in the teat compared with parenchyma and Fürstenberg's rosette ($P < 0.001$).

In this study the mammary tissue localisation of the L-selectin and β_2-integrin bearing leukocytes was analysed in the cows with apparent signs of mastitis (elevated SCC value, bacterial infection) and in the cows free of inflammation. Remarkable differences were found in the expression of L-selectin and β_2-integrin on tissue leukocytes in the three mammary gland regions of the cows with mastitis. L-selectin was expressed at lower intensity on much fewer cells than was β_2-integrin. The down-regulation of L-selectin could be explained as a result of the role of the CD62L antigen in the rolling of leukocytes along endothelial cells in the microvasculature. After L-selectin mediates the slow rolling of leukocytes on the vascular endothelium, proteases induce the shedding of L-selectin from the leukocyte surface (Radi et al., 2001).

A set of sections was prepared from the central part of the teat. The majority of sections contained no CD62L and CD18 positive cells or only a small number of these cells. Actually, a section with a large number of scattered cells bearing CD18 or

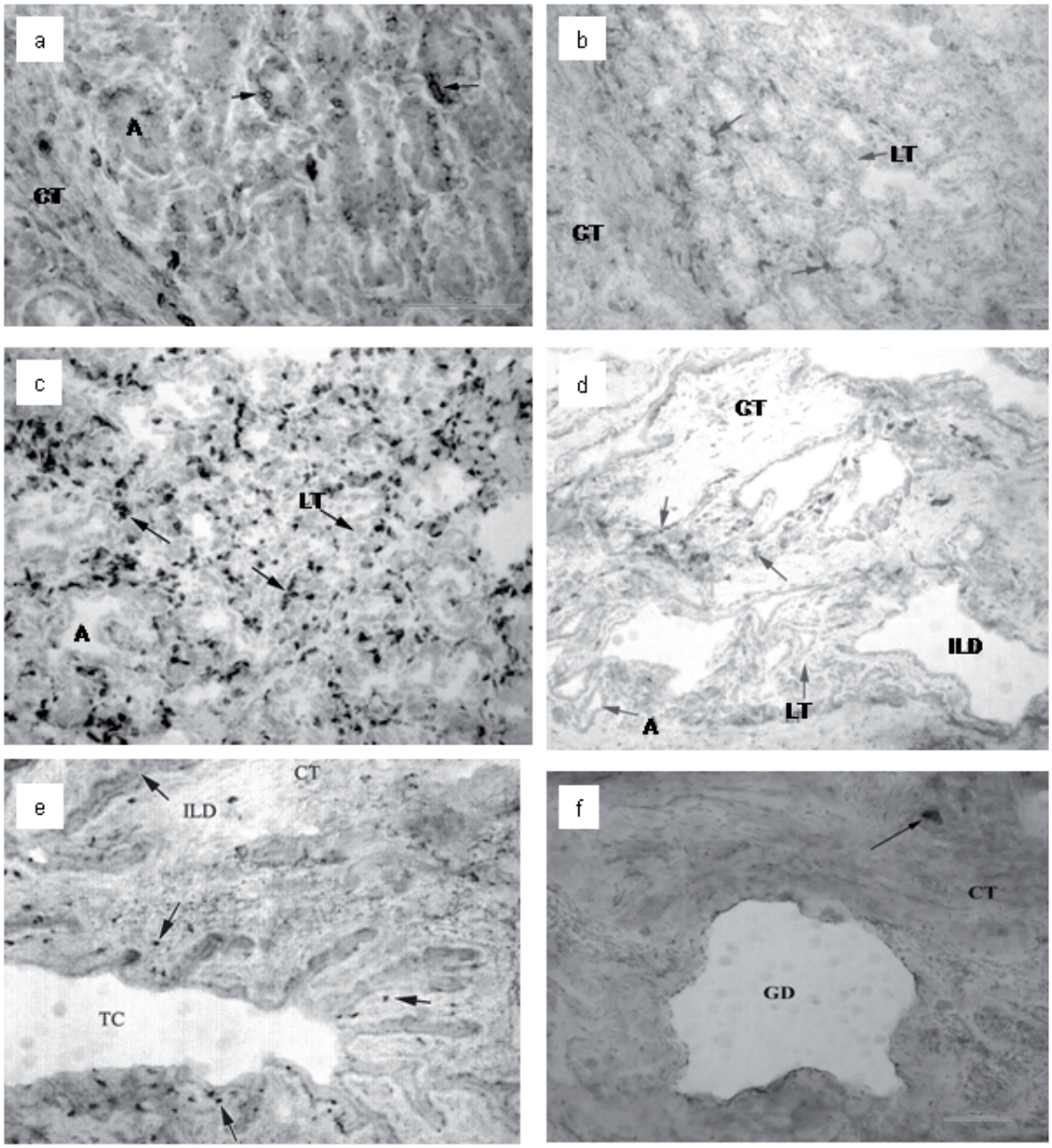

Figure 1.

(a) Immunoperoxidase reaction of monoclonal antibody IVA-35 (CD18) with leukocytes between epithelial cells in mammary parenchyma alveoli (arrows – reactions of IVA-35, A – alveolus, CT – collagenous connective tissue)

(b) Lower intensity of staining of CD62L bearing cells in the interalveolar connective loose tissue of mammary gland parenchyma with monoclonal antibody IVA-94 (arrows – reactions of IVA-94, LT – loose connective tissue, CT – collagenous connective tissue)

(c) Intensive immunoperoxidase reaction of IVA-35 in the connective tissue of Fürstenberg's rosette (arrows – reactions of IVA-35, A – alveolus, LT – loose connective tissue)

(d) Scattered L-selectin positive cells in the connective tissue of Fürstenberg's rosette (arrows – reactions of IVA-94, A – alveolus, ILD – interlobular duct, LT – loose connective tissue, CT – collagenous connective tissue)

(e) Scattered integrin positive cells in the central part of the teat stained with monoclonal antibody IVA35 (arrows – reactions of IVA-35, TC – part of teat cisternae, ILD – longitudinal section of interlobular duct, CT – collagenous connective tissue)

(f) Scattered L-selectin positive cells in the connective tissue (CT) of the teat. (TC – part of teat cisternae)

Table 3. Expression of CD18 and CD62L on bovine mammary gland tissues tested by an indirect immunoperoxidase test

Cow No.	Parenchyma		Fürstenberg's rosette		Central teat transverse section	
	CD18 distribution in	CD62L distribution in	CD18 distribution in	CD62L distribution in	CD18 distribution in	CD62L distribution in
1	scattered cells in loose connective tissue	infrequent or no cells	scattered cells in interalveolar loose and collagenous connective tissue	scattered cells in interalveolar loose and collagenous connective tissue	infrequent or no cells	infrequent or no cells
2	close proximity to the alveoli epithelial cells (Figure 1a) lumen of the alveoli interalveolar connective collagenous and loose tissues	interalveolar loose tissues surrounding alveoli (Figure 1b)	loose connective tissue and scattered positive cells in connective collagenous tissue (Figure 1c)	connective collagenous tissue (Figure 1d)	infrequent or no cells	infrequent or no cells
3	infrequent or no cells	infrequent or no cells	infrequent or no cells	infrequent or no cells	infrequent or no cells	infrequent or no cells
4	interalveolar connective collagenous and loose tissues	interalveolar connective collagenous and loose tissues	loose connective tissue (Figure 1c)	connective collagenous and loose tissues (Figure 1d)	scattered cells in collagenous connective tissue (Figure 1e)	scattered cells in collagenous connective tissue (Figure 1f)
5	infrequent or no cells	infrequent or no cells	infrequent or no cells	infrequent or no cells	infrequent or no cells	infrequent or no cells

Table 4. Percentage of CD18 and CD62L positive cells and tissues in different parts of the udder (mean ± SD)

Tissue	Stained cells in whole tissue	Alveolar epithelium	Alveolar lumen	Collagenous connective tissue	Loose connective tissue
Parenchyma					
CD18	9.95 ± 1.86	30.32 ± 6.0	28.51 ± 6.61	17.65 ± 5.72	13.57 ± 4.70
CD62L	7.65 ± 1.31	23.35 ± 4.27	19.30 ± 3.91	24.20 ± 6.82	25.50 ± 7.41
Fürstenberg's rosette					
CD18	8.35 ± 2.10	9.86 ± 1.53	11.75 ± 2.45	36.10 ± 4.72	33.94 ± 2.88
CD62L	5.90 ± 1.93***	14.57 ± 2.56	19.00 ± 3.96	30.67 ± 3.20	29.86 ± 3.84
Central teat transverse section					
CD18	2.26 ± 1.28**	18.10 ± 4.80	0.90 ± 0.45	75.57 ± 26.24	3.17 ± 24.96
CD62L	0.50 ± 0.26*	11.31 ± 3.52	7.69 ± 1.61	45.53 ± 14.23	34.97 ± 4.50

*$P < 0.001$ lower from CD18 and CD62L in parenchyma and Fürstenberg's rosette;
$P < 0.001$ lower from CD18 in the central teat transverse section
**$P < 0.001$ lower from CD18 and CD62L in parenchyma and Fürstenberg's rosette
***$P < 0.05$ lower from CD18 Fürstenberg's rosette and parenchyma

CD62L was found only in cow number 4. Nickerson and Pankey (1984) observed a massive neutrophil migration through the teat end tissues of the quarters experimentally infected with Staphylococcus aureus. Similarly, Persson et al. (1992) found an invasion of neutrophils to the teat epithelium after endotoxin infusion into the teat cistern. A weak invasion of β_2-integrin and L-selectin positive cells into the central part of the teat of the studied animals might be due to the fact that their quarters were "naturally infected" while in the previous studies a massive application of infectious agents was performed. Another reason for the lower number of the CD18 and CD62L bearing leukocytes in the teat tissue might be the strong "anatomical immune defence" present in the teat and a weaker level of cellular immunity. The teat canal is lined with keratin, which is crucial for the maintenance of the barrier function of the teat end. The removal of keratin was correlated with increased susceptibility to bacterial invasion and colonization (Capuco et al., 1992).

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