Leukocytes in bovine virgin mammary gland: flow cytometry imaging during development and resolution of induced influx

Z. SLÁDEK[1] D. RYŠÁNEK[2], M. FALDYNA[2]

[1]*Mendel University of Agriculture and Forestry, Brno, Czech Republic*
[2]*Veterinary Research Institute, Brno, Czech Republic*

ABSTRACT: Distribution of leukocyte types present in virgin bovine mammary glands was analysed in dot plots obtained by flow cytometry (FACS) of samples collected from 10 non-pregnant heifers after induction of leukocyte influx. Changes of percentage of leukocyte types during development and resolution of induced influx in comparison with blood leukocyte pattern allow identification of these cell types on FACS dot plot. The positions of mammary gland granulocyte and lymphocyte regions were identical with those of the corresponding peripheral blood cells. Two basic morphologically distinct types occupying separate regions in dot plots were observed in the population of mononuclear phagocytes (MoP): non-vacuolised monocyte-like macrophages (MoMAC) and vacuolised macrophages (MAC). Influx resolution was characterised by a marked shift of the MoMAC region towards that of MAC recognisable in dot plots by a separate region of intermediate MoP forms. The study provides a pattern of dynamics of percentages of mammary gland leukocyte types during influx development and resolution as imaged by FACS.

Keywords: flow cytometry; bovine virgin mammary gland; induced influx

INTRODUCTION

Virgin bovine mammary glands have become objects of many experimental studies focused mostly on the activity of cells of the defence system including neutrophil granulocytes (neutrophils) and mononuclear phagocytes (Wardley *et al*., 1976; Sanchez *et al*., 1988; Quiroga *et al*., 1993; Ryšánek *et al*., 1999, 2001; Sládek and Ryšánek, 1999a,b).

The model of induced cell influx, based on irritation of the mammary gland by intramammary administration of buffered physiological saline (Derbyshire and Berman, 1968; Desiderio and Campbell, 1980; Sanchez *et al*., 1988), glucose (Paape *et al*., 1977), lipopolysaccharide (Wardley *et al*., 1976; Saad and Östensson, 1990; Sládek and Ryšánek, 2001) and/or muramyl dipeptide (Ryšánek *et al*., 2001; Sládek and Ryšánek, 2000a,b, 2001), was widely used in such studies as a methodical tool. The irritation results in inflammatory response of the mammary gland characterised by migration and subsequent accumulation of neutrophils in the cavity system. The influx resolves by deactivation and physical elimination of neutrophils within 3 to 4 days. Most of the cells die by apoptosis (Sládek and Ryšánek, 2001).

This model was also used within our recent studies of neutrophil apoptosis in the course of resolution of acute mammary gland damage (Sládek and Ryšánek, 2000a,b, 2001). Morphological and functional characteristics of neutrophils were studied above all by light and electron microscopic techniques. Flow cytometry appears currently to be a very suitable method for activity studies in cells of the bovine mammary gland defence system.

Flow cytometry was used earlier in studies of differentiation of bovine peripheral blood leukocytes (Hageltorn and Saad, 1986; Jain *et al*., 1991), mammary gland leukocytes of lactating dairy cows (Hageltorn and Saad, 1986; Östensson *et al*., 1988; Redelman *et al*., 1988; Emanuelson and Wever, 1989) and in endotoxin-induced bovine mastitis (Saad and Östensson, 1990; Miller *et al*., 1993; Paape *et al*., 1996).

The leukocyte population of virgin bovine mammary glands differs from that of lactating or dried off glands primarily in total cell counts and percentages and, what is very important, also in morphological characteristics of the individual cell types (Sládek and Ryšánek, 1999a,b).

Therefore, as an introduction to subsequent activity studies, we analysed flow cytometric dot plots imaging

Supported by the Ministry of Agriculture of the Czech Republic (Project MZE MO3-99-01) and the Ministry of Education, Youth and Sports of the Czech Republic (Project MSM No. 432100001).

the distribution of the individual leukocyte types present in virgin bovine mammary glands during development and resolution of induced influx.

MATERIAL AND METHODS

Animals

The experiments were carried out in 10 clinically normal Holstein × Bohemian Red Pied crossbred heifers aged 15 to 18 months. The heifers were free of intramammary infections as proven by bacteriological examination of all collected cell suspensions. Five heifers were used as blood cell donors and another five as mammary gland cell donors using the induced influx model. The heifers were housed in a stanchion barn and fed a standard ration consisting of hay and concentrates with mineral supplements.

Separation of blood leukocytes

The whole-blood lysis technique was used to separate leukocytes from peripheral blood samples. Fifty microlitres of blood collected from the jugular vein was mixed with 3 ml of a haemolytic solution (8.26 g of NH_4Cl, 1 g of $KHCO_3$ and 0.037 g of Na_4EDTA per 1 litre of distilled water). The suspension was centrifuged, supernatant was removed and sediment was resuspended in 3 ml of the washing solution (1g NaN_3 and 1.84 g of Na_4EDTA per 1 litre of PBS). The cells were ready for flow cytometry after another centrifugation, supernatant removal, and resuspension in the washing solution.

Influx induction

All four glands of five heifers were washed with 20 ml of PBS, pH 7.4, to obtain control samples of cell populations. Cell influx was then induced by intramammary administration of PBS (10 ml) and mammary lavages (ML) were done as described by Sládek and Ryšánek (2001). The first sample was collected from the left forequarter at 24 h after the treatment with PBS (ML24). The subsequent samples were collected at 24-h intervals from the remaining quarters in the following order: left hindquarter (ML 48) → right forequarter (ML72) → right hindquarter (ML96).

Cell processing

No bacterial growth was detectable in any of the collected cell suspensions by inoculation of blood agar plates (5% washed sheep erythrocytes) and aerobic incubation at 37°C for 24 h. Total cell counts were determined haemocytometrically. The trypan blue dye exclusion test demonstrated viability in at least 97% of cells in each ML. The cell suspensions were centrifuged at 4°C and 200 × g for 10 min. One millilitre of the supernatant was withdrawn and retained to be used for resuspension of the pellet and the remaining supernatant was decanted.

Light microscopy

Two smears of each processed cell suspension were prepared and stained panoptically by the Papanicolau method (Bessis, 1973). Differential cell counts were determined in ML by enumeration of at least 200 cells as described earlier (Sládek and Ryšánek, 1999a). The preparations were viewed in the JENAMED 250 CF microscope (Carl Zeiss, Jena, Germany). The images were digitalised using the colour camera 3-CCD HV-C20 (Hitachi, Denshi, Japan) and analysed using the supportive software LUCIA G (Laboratory Imaging, Prague, Czech Republic).

Flow cytometry (FACS)

The cells of ML samples were resuspended in the washing solution supplemented with 10% of heat-inactivated porcine serum to obtain the cell concentration of 1×10^6/ml. After 20 minutes, 50 µl of the cell suspension was incubated with 50 µl of monoclonal antibody (VPM 65, murine anti-sheep CD14, Serotec Ltd., U.K., dilution 1 : 20) in a 12 × 75 mm tube at 4°C for 15 min for distinguishing of large lymphocytes from MoP by CD14 expression (Wright *et al.*, 1989). Then, the cells were washed with the washing solution, centrifuged and the sedimented cells were incubated with the secondary antibody (fluorescein isothiocyanate-labelled swine anti-mouse, Sevac, Prague, Czech Republic, dilution 1 : 360) at 4°C for 20 min. Finally, the cells were washed again as described above and resuspended in the washing solution.

The FACS Calibur flow cytometer (Becton Dickinson, Mountain View, California, USA) and the CELLQuest™ software were used. At least 20 000 events were read in each run. Gating of separate leukocyte populations in the peripheral blood and in ML was based on forward-scatter (FSC) and side-scatter (SSC) light characteristics. The distribution of blood leukocytes in dot plots was used for the identification of mammary gland leukocyte types as described by Hageltorn and Saad (1986).

Statistics

The significance of differences between means for differential counts of leukocytes detected by light microscopy and FACS was determined by Student's *t*-test using the software STAT Plus (Matoušková *et al.*, 1992).

RESULTS

Influx model

Intramammary administration of PBS resulted in migration into and subsequent accumulation of leukocytes in the cavity system of the mammary gland. As can be seen in Figure 1, the total cell count peaked at 52.6 ± 17.9 × 10^6/ml 24 h after influx induction. ML48 was characterised by a decrease in total cell count which was indicative of beginning influx resolution. Continuing resolution could be observed in ML72 and total cell count in ML 96 approached the value found in control samples.

Cytology of virgin mammary glands

The following three leukocyte types were identified by light microscopy in the cavity system of virgin mammary glands during induced influx and its resolution: mononuclear phagocytes (MoP), lymphocytes (LYM), and neutrophils (Figures 2a to d).

MoP were represented by two morphologically distinct subtypes. The first one was characterised by rounded to oval, moderately elongated, kidney-shaped nucleus with moderate chromatin condensation, size of 12 to 15 mm, and finely granulated cytoplasm without signs of vacuolisation (Figure 2a, c). Owing to its morphological similarity with circulating monocytes, i.e. cells recently immigrated from the blood circulation and surrounding tissues, this cell type was designated as monocyte-like macrophages (MoMAC). The second MoP subtype were cells 18 to 38 μm in size characterised by irregularly shaped, markedly delineated condensed chromatin-containing nuclei and abundant vacuolisation of cytoplasm (Figure 2b). These cells with foamy appearance, which predominated in the cell population of the mammary gland before influx induction, were designated as macrophages (MAC). Moreover, the population of MoP included cells that could not be unambiguously classified with any of the two subtypes. Most of them showed morphological characteristics of MoMAC and size of MAC. Moreover, the cytoplasm of most of them contained phagocytised apoptotic neutrophils (data not shown here). The cells were designated as intermediate MoP.

LYM were spherical small to medium-sized cells (6 to 12 μm) with compact spherical basophilic nuclei containing condensed chromatin (Figure 2c).

Neutrophils were medium-sized cells (7 to 10 μm) with lobular polymorphous nuclei, pink cytoplasm containing neutral granules, and pseudopodia (Figure 2d).

Identification of mammary gland leukocytes in dot plots during induced influx development and resolution

Dot plots of blood leukocytes were used as a reference pattern for the identification of leukocytes isolated from virgin bovine mammary glands. Three distinct regions can be seen in Figure 3a showing a dot plot of blood leukocytes. The position of the region occupied by LYM was in the middle of and close to X (FSC) and Y (SSC) axes. The region of monocytes was located to the right from (FSC) and moderately above (SSC) LYM, and the region of granulocytes to the left from (FSC) and above (SSC) LYM.

Mononuclear phagocytes

MoP of control mammary glands occupied almost the whole right part of the dot plot (Figure 3b). Owing to

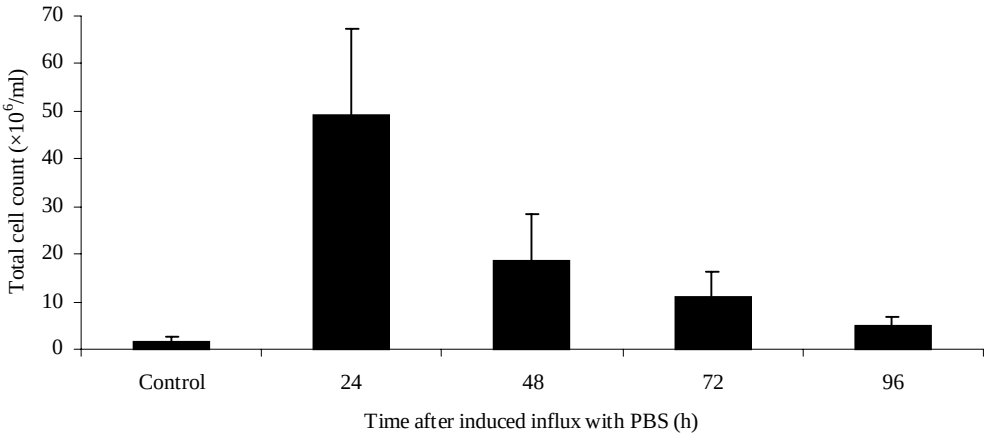

Figure 1. Cell counts in mammary lavages obtained at 0, 24 (ML24), 48 (ML48), 72 (ML72), and 96 hours (ML96) after influx induction with PBS (means ± SD for five heifers)

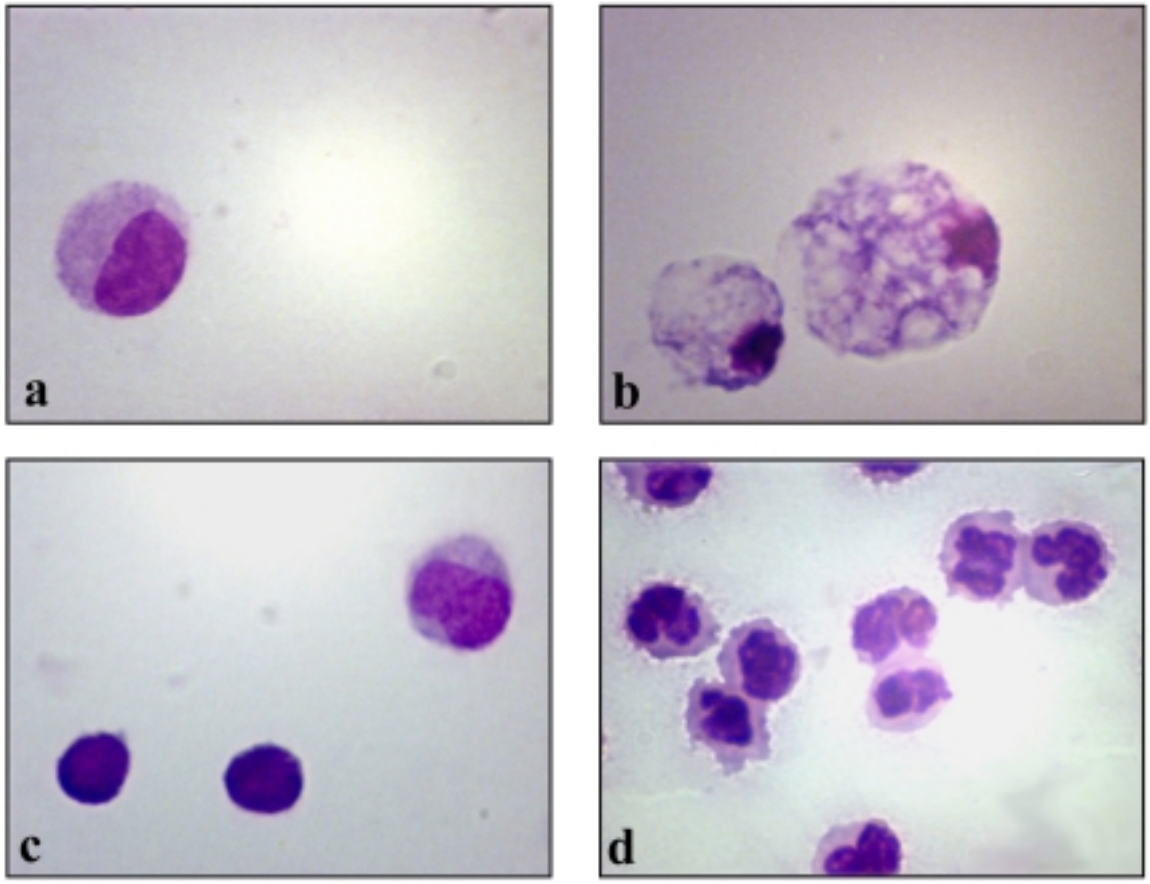

Figure 2. Light microscopic patterns of leukocyte types isolated from virgin bovine mammary glands. Non-vacuolised MoMAC (a), two vacuolised MAC (b), non-vacuolised MoMAC (upper right), two lymphocytes (c), and neutrophils (d)

morphological similarity, MoMAC concentrated in the same region as blood monocytes, i.e. to the right from (FSC) and above (SSC) LYM. MAC formed a relatively large region occupying the whole upper right quadrant of the dot plot. No counterpart to this region in the dot plot of control samples was observed in dot plots of blood leukocytes. Owing to the presence of intermediary MoP types, the transition between the MoMAC and MAC regions in dot plots of control mammary gland samples was rather continuous than sharp (Figure 3b).

Influx induction and resolution were characterised by marked shifts in proportions of the two MoP subtypes (Figures 3 to 5). Only MoMAC, LYM and neutrophil regions, but no MAC region were recognisable in dot plots of ML24. Hence, they closely resembled the dot plot of blood leukocytes (Figure 4a).

In the dot plot of ML24, the position of the MoMAC region was identical with that of monocytes in the dot plot of blood leukocytes (Figure 4a). The higher density which can be seen closely above the region of MoMAC of ML48

Table 1. Differential cells count during induced influx detected in light microscopy and in flow cytometry

	Control		24 hours		48 hours		72 hours		96 hours	
	LM	FACS	LM	FACS	LM	FACS	LM	FACS	LM	FACS
Neutrophile	2.7 ± 1.3	3.9 ± 1.4	86.9 ± 6.2	82.3 ± 11.3	69.8 ± 10.3	60.8 ± 8.8	17.4 ± 5.3	15.5 ± 1.8	6.6 ± 4.2	8.4 ± 1.5
MoMAC	15.5 ± 4.7	13.9 ± 3.4	8.7 ± 2.4	8.4 ± 3.7	8.8 ± 5.6	10.6 ± 2.7	21.5 ± 7.8	28.7 ± 5.1	22.8 ± 6.7	20.7 ± 5.7
MAC	48.4 ± 9.1	43.5 ± 4.5	0 ± 0	1.9* ± 0.6	11.8 ± 6.1	8.4 ± 3.5	36.4 ± 9.2	29.3 ± 7.4	41.2 ±8.6	40.4 ± 7.7
LYM	32.8 ± 9.9	38.7 ± 3.7	4.4 ± 2.1	7.4 ± 4.9	8.6 ± 3.9	11.5 ± 2.8	23.9 ± 4.5	26.5 ± 3.8	29.4 ± 8.2	30.4 ± 5.6

*$P < 0.05$; flow cytometry versus light microscopy

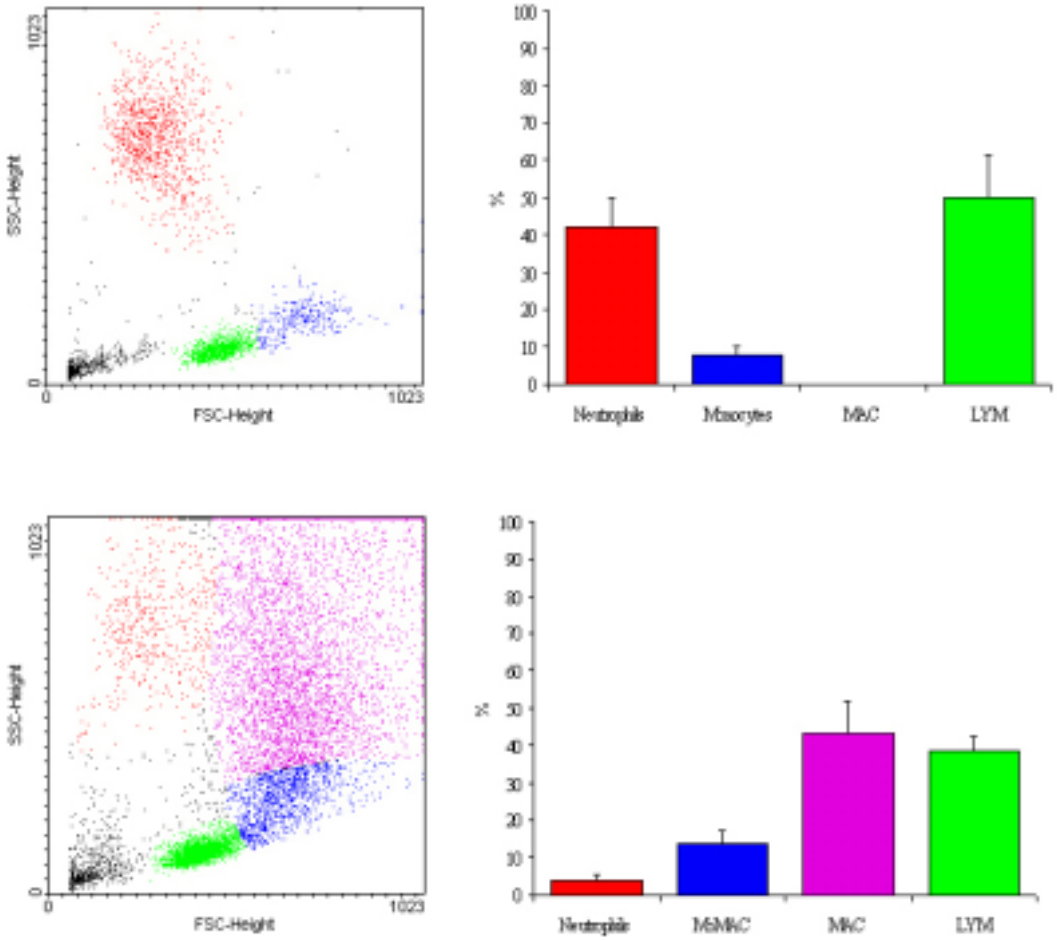

Figure 3. Region distribution in dot plots of blood leukocytes (a) and intact virgin mammary gland leukocytes (b): neutrophil region (red), monocyte/MoMAC region (blue), MAC region (violet), and lymphocyte region (green). The plot to the right shows differential counts of the cells

indicated an increase in the number of the intermediate MoP type (Figure 4b). The influx resolution was characterised by increasing density in the MAC regions of ML48 through ML96 and a shift of the MoP region to the upper right quadrant of the dot plot. The distribution of MoP in the dot plot of ML96 closely resembled that of the control sample (Figure 5b). The changes in the proportions of the two MoP subtypes corresponded with differential counts determined by light microscopy (Table 1).

Lymphocytes

The LYM region in dot plots of virgin mammary gland samples was in the same position as its counterpart of blood samples, i.e. in the middle of (FSC) and close to (SSC) the X axis (Figures 3 to 5). Unlike the MoP region, influx induction and resolution were accompanied only by dot density change reflecting varying percentages of LYM at the individual time points, while the position of the LYM region remained constant.

Neutrophils

The position of the neutrophil region of control mammary gland samples was identical with that of blood granulocytes (Figures 3a, b), i.e. to the left from (FSC) and above (SSC) the LYM region. Its density was low because of the low percentage of neutrophils in normal mammary glands (Figure 3). The initial phase of influx (ML24) was characterised by a sharp increase in density of the region, which occupied almost the whole left upper quadrant of the dot plot (Figure 4a). The density decreased during the subsequent phase of influx resolution and dot plot of ML96 was very similar to that of the control sample. These dynamics fully corresponded with those of neutrophil counts determined by light microscopy (Table 1).

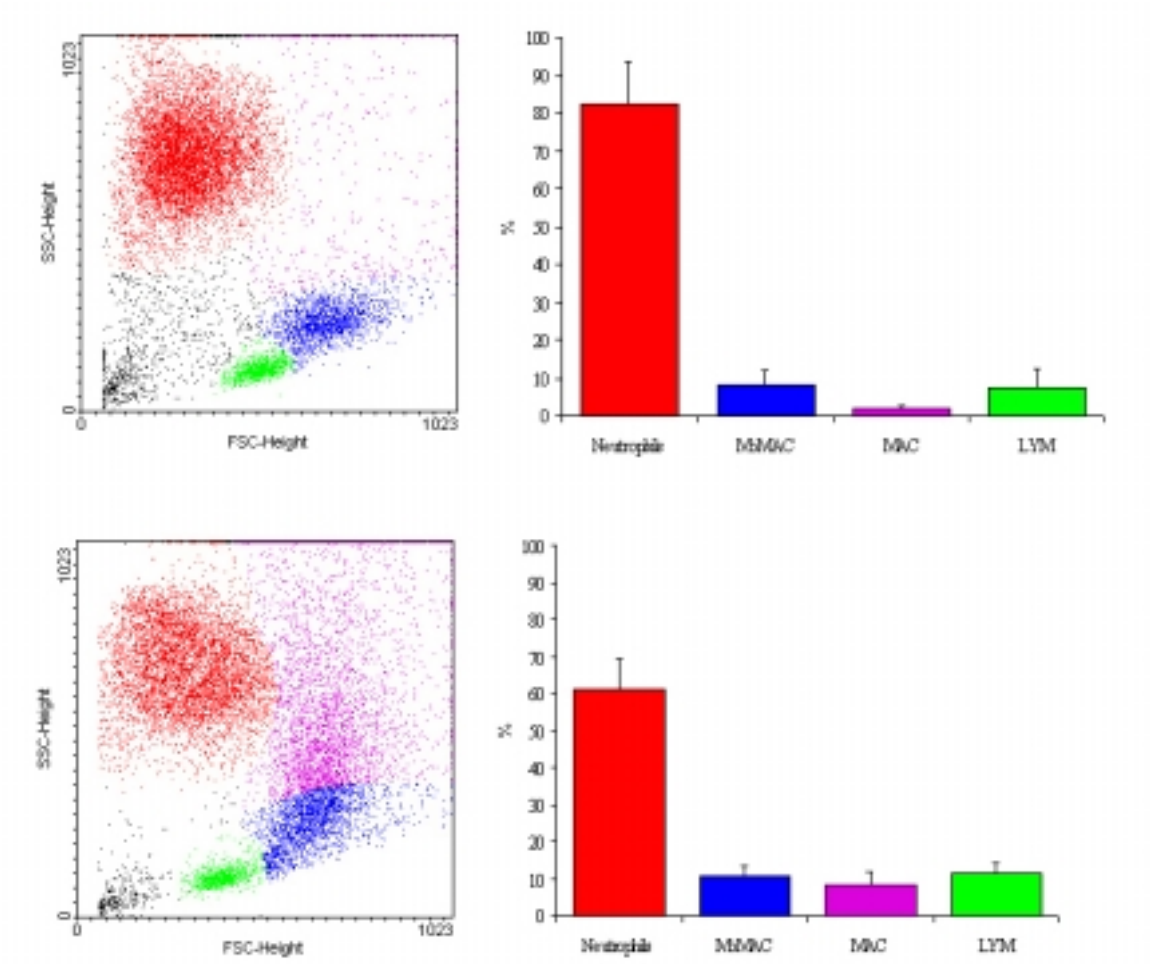

Figure 4. Region distribution in dot plots of virgin mammary gland leukocytes at 24 hours (a) and 48 hours (b) after influx induction. For further details see legend of Figure 3

Data on differential leukocyte counts during development and resolution of induced influx obtained by light microscopy and FACS are shown in Table 1.

DISCUSSION

Changes in the distribution of leukocyte types collected from virgin bovine mammary glands during the development and resolution of induced influx were analysed by flow cytometry. This study is a continuation of earlier experiments in which leukocyte types were identified in the same model by light and electron microscopy (Wardley *et al*., 1976; Sládek and Ryšánek, 1999a, b).

Induced cell influx into the bovine mammary gland consists of the initial and the resolution phases. The initial phase (development) is a response of the mammary gland to irritation caused by intramammary treatment with PBS which results in marked migration and accumulation of cells, predominantly neutrophils, in the cavity system of the mammary gland. The period of accumulation is limited, however, because neutrophils are cells with a short life span which are liable to apoptosis and subsequent phagocytosis by MoP with 1 to 2 days (Sládek and Ryšánek, 2000b, 2001). Apoptosis starts the resolution phase characterised by marked decreases in total count and percentage of neutrophils 48 and 72 h after influx induction, respectively. The decreasing percentage of neutrophils during the resolution phase is accompanied by an increase in percentages of MoP and LYM.

Hageltorn and Saad (1986) adapted the flow cytometric technique to quantify physiological and biochemical characteristics of bovine milk somatic cells using leukocyte distribution in dot plots of bovine blood as a reference pattern for the identification of milk leukocyte regions. Thus, these authors were the first to publish basic information on the distribution of milk neutrophils, LYM and MoP derived from flow cytometry dot plots.

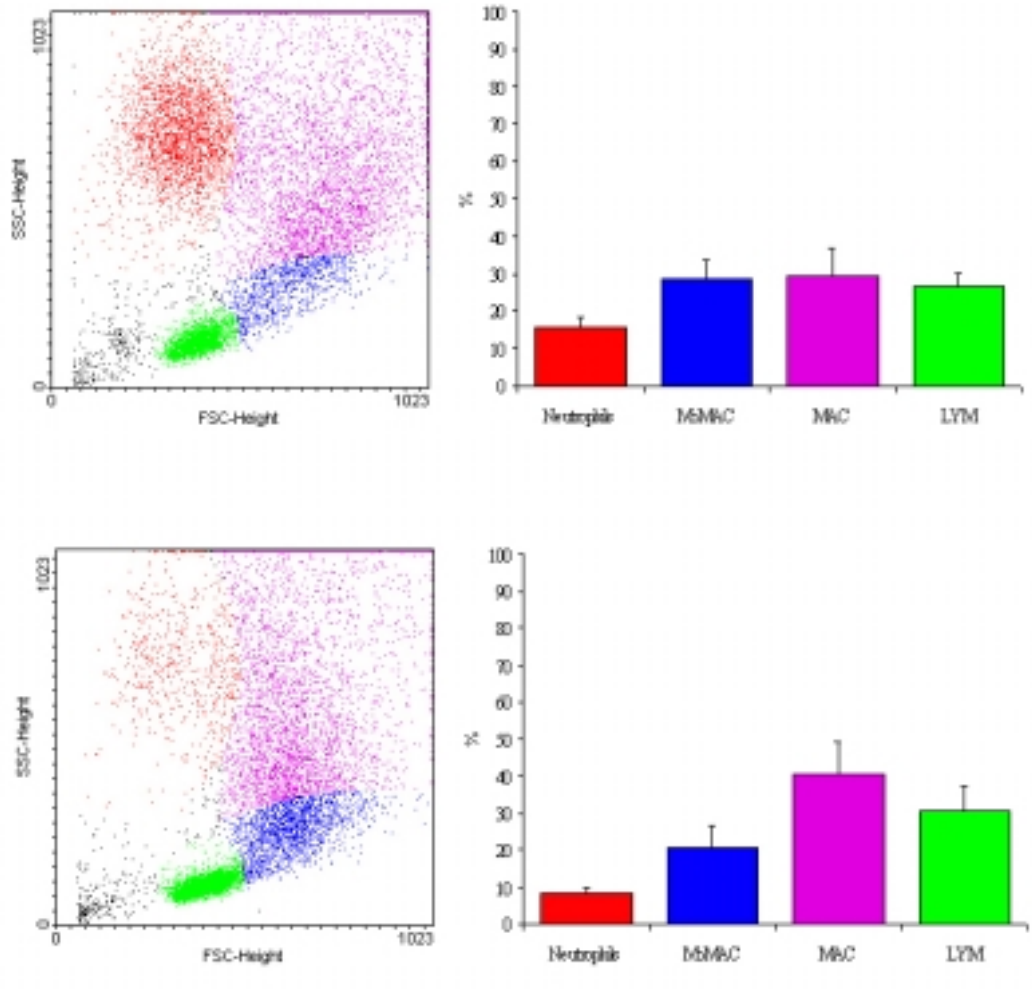

Figure 5. Region distribution in dot plots of virgin mammary gland leukocytes at 72 hours (a) and 96 hours (b) after influx induction. For further details see legend of Figure 3

The same technique was subsequently used for the determination of differential counts of somatic cells in normal bovine milk (Östensson *et al*., 1988), milk of cows with sponatenous mastitis (Redelman *et al*., 1988; Emanuelson and Wever, 1989), and milk of cows with endotoxin-induced mastitis (Saad and Östensson, 1990; Miller *et al*., 1993; Paape *et al*., 1996), as well as bovine blood leukocytes (Jain *et al*., 1991; Miller *et al*., 1993; Paape *et al*., 1996).

In our experiments, we used the same technique of leukocyte identification in dot plots of FACS as described by Hageltorn and Saad (1986). Like milk leukocytes in cow's milk, leukocytes of virgin bovine mammary glands migrate from the blood. It could therefore be expected that their regions in dot plots would occupy the same positions as those of blood leukocytes. Actually, this presumption was wrong, because leukocytes present in virgin mammary glands differ from blood leukocytes in morphological characteristics. This applies particularly to MoP.

The MoP population, which predominated over other leukocyte types in control virgin mammary glands, consisted of two morphologically distinct subtypes: MoMAC and MAC. MoMAC were morphologically identical with blood monocytes and therefore the position of their region in dot plots of virgin mammary gland leukocytes was identical with that of the region of blood monocytes. However, a separate MoMAC region could be observed only in dot plots of ML24 that were collected at a time when the MoP population consisted exclusively of MoMAC. As resident cells were washed out before the intramammary treatment with PBS, it is apparent that the MoMAC in ML24 were recent immigrants from the blood and surrounding tissues. The same conclusion was formulated by Hageltorn and Saad (1986). However, in dot plots of ML48 through ML96, the region of MoMAC continuously extended towards the upper right quadrant, which was empty in dot plots of blood leukocytes. We assume that this change resulted from intensive scaveng-

ing activity of MoMAC. Such assumption is supported by results of our recent studies (Sládek and Ryšánek, 2000b, 2001) in which an increase in the proportion of myeloperoxidase-positive MoP was observed in the period of influx resolution in virgin bovine mammary glands. Intensive phagocytosis of apoptotic neutrophils by myeloperoxidase-positive MoP peaked between 48 h and 72 h after influx induction. Due to phagocytosis, MoP increased in size and rounded out; this morphological change resulted in the extension of their region towards the upper right corner of dot plot, which was occupied by MAC, giving rise to intermediate types situated between the MoMAC and MAC regions without clear demarcation. These findings can explain the absence of the MAC region from dot plots of ML24, merging of the MoMAC and MAC regions, and increasing density of the MAC region in dot plots of ML72 and ML96, and are also relevant to studies of activities of MoP subpopulations during influx induction and resolution in which their specific positions in dot plots must be considered. It must be borne in mind that the intermediary MoP subtypes are highly active cells involved in the process of clearing of apoptotic neutrophils. This fact was not mentioned by Hageltorn and Saad (1986).

The position of the LYM region in dot plots of virgin mammary gland leukocytes was identical with that of the region of blood LYM. No change in morphological characteristics was observed during influx induction and resolution and the position of the region in dot plots remained unchanged. As stated by Hageltorn and Saad (1986), large LYM could not be distinguished from monocytes by light scatter parameters in dot plots of blood leukocytes. Now the two types of mononuclear leukocytes can be differentiated by specific surface receptors, such as CD14 shared by blood monocytes and mammary gland MoP (Paape *et al*., 1996), but not expressed in LYM (Wright *et al*., 1989).

The region of neutrophils occupied almost the whole upper left quadrant of dot plots of virgin mammary gland leukocytes. This observation is fully consistent with the data published by Hageltorn and Saad (1986). Like in LYM, the position of this region remained relatively constant during influx induction and resolution. Abnormalities in neutrophils observed in our recent studies using also influx induction by intramammary administration of PBS as a methodical tool (Sládek and Ryšánek, 1999a, b; Sládek and Ryšánek, 2000a), included apoptosis and necrosis which differed from each other in morphological characteristics. It can therefore be expected that apoptotic and necrotic neutrophils will be imaged as separate neutrophil subregions in FACS, because this is one of specific manifestations of cells undergoing either type of cell death (Darzynkiewicz *et al*., 1992; Vermes *et al*., 2000). This fact should be considered in future FACS studies of neutrophils. Detailed studies will be necessary to assess correctly differences in positions of regions in dot plots of morphologically altered neutrophils collected from bovine mammary glands .

Results of analyses of dot plots of virgin mammary gland leukocytes collected during influx induction and resolution can be summarised as follows: In the initial phase, MoP occupied a single region corresponding to MoMAC. Influx resolution was characterised by extension of the MoMAC region towards the MAC region and rise of a fuzzy transitional zone between them occupied by intermediary MoP subtypes. This finding reflects intensive involvement of MoP in scavenging of neutrophils and confirms their important role in the resolution of induced influx. The positions of LYM and neutrophil regions in dot plots of virgin mammary gland and blood leukocytes were identical and remained constant during influx induction and resolution.

REFERENCES

Bessis M. (1973): Living Blood Cells and Their Ultrastructure. Springer-Verlag, New York. 767 pp.

Darzynkiewicz Z., Bruno S., Del Bino G., Gorczyca W., Hotz M.A., Lassota P., Traganos F. (1992): Features of apoptotic cells measured by flow cytometry. Cytometry, *13*, 795–808.

Derbyshire J.B., Berman D.T. (1968): Leukocytic responses of the bovine udder to infusion of certain irritants. Am. J. Vet. Res., *29*, 1971–1977.

Desiderio J.V., Campbell S.G. (1980): Bovine mammary gland macrophages: isolation, morphologic features, and cytophilic immunoglobulins. Am. J. Vet. Res., *41*, 1595–1599.

Emanuelson U., Wever P. (1989): Potential of differential somatic cell counts as indicators of mastitis in quarter milk samples from dairy cows. Acta Vet. Scand., *30*, 475–481.

Hageltorn M., Saad M.A. (1986): Flow cytofluorometric characterization of bovine blood and milk leukocytes. Am. J. Vet. Res., *47*, 2012–2016.

Jain N.C., Paape M.J., Miller R.H. (1991): Use of flow cytometry for determination of differential leukocyte counts in bovine blood. Am. J. Vet. Res., *52,* 630–636.

Matoušková O., Chalupa J., Cigler M., Hruška K. (1992): STAT Plus – Users Manual (in Czech). 1st ed. Veterinary Research Institute, Brno. 168 pp.

Miller R.H., Paape M.J., Filep R., Link S. (1993): Flow cytometric analysis of neutrophils in cows' milk. Am. J. Vet. Res., *54*, 1975–1979.

Östensson K., Hageltorn M., Astrom G. (1988): Differential cell counting in fraction-collected milk from dairy cows. Acta Vet. Scand., *29*, 493–500.

Paape M.J., Pearson R.E., Wergin W.P., Guidry A.J. (1977): Enhancement of chemotactic response of polymorphonuclear leukocytes into the mammary gland and isolation from milk. J. Dairy Sci., *60*, 53–62.

Paape M.J., Lilius E.R.M., Wiitanen P.A., Kontio M.P., Miller R.H. (1996): Intramammary defense against infections

induced by *Escherichia coli* in cows. Am. J. Vet. Res., *57*, 477–482.

Quiroga G.H., Sordillo L.M., Atkinson R.W., Nickerson S.C. (1993): Cytologic responses of *Staphylococcus aureus*-infected mammary glands of heifers to interferon gamma and interleukin-2 treatment. Am. J. Vet. Res., *54*, 1894–1900.

Redelman D., Butler S., Robison J., Garner D. (1988): Identification of inflammatory cells in bovine milk by flow cytometry. *Cytometry, 9*, 463–468.

Ryšánek D., Šedivá A., Sládek Z., Babák V. (1999): Intramammary infections of juvenile mammary glands of heifers: absolute and differential somatic cell counts. Vet. Med. – Czech, *44*, 199–203.

Ryšánek D., Babák V., Sládek Z., Toman (2001): Variation among unbred heifers in the activities of their mammary gland and blood polymorphonuclear leukocytes. J. Vet. Med. B., *48*, 31–42.

Saad A.M., Östensson K. (1990): Flow cytometric studies on the alteration of leukocyte populations in blood and milk during endotoxin-induced mastitis in cows. Am. J. Vet. Res., *51*, 1603–1607.

Sanchez L., Aranda P., Perez M.D., Calvo M. (1988): Concentration of lactoferrin and transferrin throughout lactation in cow´s colostrum and milk. Biol. Chem. Hoppe Seyler, *369*, 1005–1008.

Sládek Z., Ryšánek D. (1999a): Morphological characteristic of somatic cells from mammary glands of unbred heifers. Vet. Med. – Czech, *44*, 205–214.

Sládek Z., Ryšánek D. (1999b): Ultrastructure of phagocytes from mammary glands of non-pregnant heifers. Anat. Histol. Embryol., *28*, 291–298.

Sládek Z., Ryšánek D. (2000a): Morphology of apoptosis of polymorphonuclear leukocytes isolated from mammary glands of unbred heifers. Vet. Med. – Czech, *45*, 71–81.

Sládek Z., Ryšánek D. (2000b): Apoptosis of polymorphonuclear leukocytes of the juvenile bovine mammary gland during induced influx. Vet. Res., *3*, 553–563.

Sládek Z., Ryšánek D. (2001): Neutrophil apoptosis during the resolution of bovine mammary gland injury. Res. Vet. Sci., *70*, 41–46.

Vermes I., Haanen C., Reutelingsperger C. (2000): Flow cytometry of apoptotic cell death. J. Immunol. Methods, *243*, 167–190.

Wardley R.C., Rouse B.T., Babiuk L.A. (1976): The mammary gland of the ox: a convenient source for the repeated collection of neutrophils and macrophages. J. Reticuloendothel. Soc., *19*, 29–36.

Wright S.D., Ramos R.A., Ulevitch R.J. (1989): Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. J. Exp. Med., *70*, 1231–1241.

Received: 01–06–18
Accepted after corrections: 01–10–29

Corresponding Author:

MVDr. Zbyšek Sládek, PhD., Mendel University of Agriculture and Forestry, Zemědělská 1, 613 00 Brno, Czech Republic
Tel. +420 5 45 13 31 51, fax +420 5 41 21 11 28, e-mail: sladekz@seznam.cz; rysanek@vri.cz