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Original article

Analysis of selective mobilization of L-selectin and Mac-1 reservoirs in bovine neutrophils and eosinophils

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Abstract – Following activation of granulocytes, L-selectin (CD62L) is generally shed from the cellular surface, whereas Mac-1 (CD11b/CD18) expression is well known to increase. However, a number of studies in bovines and humans show that the expression of L-selectin may increase as well. This urged us to examine the possible existence of both L-selectin and Mac-1 reservoirs in bovine neutrophil and eosinophil populations through the use of flow cytometry in combination with an optimized method for cell membrane permeabilization. Augmented L-selectin and Mac-1 expression was detected in both granulocyte populations upon saponin treatment. Confocal microscopic studies indicated that both molecules exhibit a different pattern of subcellular localization. Incubation with sialidase revealed the existence of hidden L-selectin epitopes at the cell surface, while no additional Mac-1 epitopes were exposed. Platelet-activating factor stimulation decreased surface and total expression of L-selectin to the same extent in both populations, but solely affected Mac-1 surface expression on eosinophils. Moreover, cytoskeletal actin filaments and microtubules were found to be involved in the regulation of Mac-1 surface expression on bovine neutrophils and eosinophils. In marked contrast, expression of L-selectin was minimally affected by cytoskeleton perturbing agents. The present study indicates that L-selectin and Mac-1 adhesion molecules reside in distinctly located reservoirs in bovine granulocytes and can be selectively mobilized upon *in vitro* stimulation.

bovine granulocyte / adhesion molecule reservoir / platelet-activating factor / cytoskeleton / sialidase

1. INTRODUCTION

Granulocytes play a pivotal role in the surveillance system of the animal against invading microorganisms, parasites, and in

acute inflammation. The capacity of granulocytes to attach to the vessel wall and migrate into the surrounding tissues depends on intercellular adhesion mediated by members of the selectin and β_2 -integrin surface glycoproteins. L-selectin (CD62L)

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mediates the initial transient attachment of circulating granulocytes to the activated endothelium. Surface expression and rapid functional activation of Mac-1 (CD11b/CD18) is essential for subsequent granulocyte migration to the site of inflammation [7]. Following activation, L-selectin is shed from the cell surface by proteolysis, while surface expression of Mac-1 is up-regulated [14, 17].

Despite the generally accepted shedding model it has been recently reported that L-selectin expression can also be augmented. Indeed, increased surface expression of L-selectin has been described in vitro on PAF-stimulated bovine neutrophils [25], interferon- γ -stimulated human eosinophils [20], and on cultured bone marrow cells [4, 24]. Interestingly, an increase in L-selectin surface expression was also observed in vivo shortly after intramammary *Escherichia coli* challenge [6] and on circulating human neutrophils upon trauma injury [3, 18]. Increased expression of Mac-1 on bovine neutrophils has also been reported in vitro following stimulation by inflammatory mediators [5, 11, 19] and in vivo after intramammary *Escherichia coli* infection [8]. However, none of these studies examined the possibility of the existence of mobilizable L-selectin and Mac-1 reservoirs in bovine granulocytes.

In order to assess surface-bound and total detectable pools of L-selectin and Mac-1 of bovine granulocytes we developed a flow cytometric technique. A first approach to determine adhesion molecule localization was conducted by confocal microscopic analysis and sialidase treatment in unstimulated cells. In addition, the stimulated mobilization of both adhesion receptors was evaluated in the presence of platelet-activating factor (PAF). While this potent inflammatory mediator has been shown to down-regulate L-selectin and up-regulate Mac-1 expression in human neutrophils, inconsistent results have been obtained in bovine models [2, 19, 25].

Actin microfilaments and microtubules have been recognized as important cytoskeletal structures involved in transporting granules to the cellular surface during neutrophil activation [12]. Therefore, the effect of cytochalasin D and colchicine on adhesion receptor mobilization was also examined.

2. MATERIALS AND METHODS

2.1. Animals and blood sampling

Experiments were performed on 24 clinically healthy, high yielding dairy cows of the Holstein-Friesian breed during mid lactation (114–188 days after calving) between their 1st and 4th parity. Blood was aseptically collected with pyrogen-free heparinized vacuum tubes (Chromogenix, Milano, Italy) by jugular venipuncture between 08.00 h and 09.00 h.

2.2. Monoclonal antibodies (mAb)

11G10 and 2G8 mAb [28] are both IgG1 isotype antibodies directed to bovine L-selectin. CC126 mAb (ProBio, Margate Kent, UK) is an IgG2b isotype directed to bovine Mac-1. Optimal binding concentration of each mAb was determined by flow cytometric titration. Mouse IgG1 and IgG2b (Dako A/S, Glostrup, DK) were used as non-binding isotype controls. For correct gating of leukocyte populations three additional primary antibodies were used: CH138A mAb (VMRD Inc., Pullman, WA, USA) is an IgM isotype that mainly cross-reacts with bovine neutrophils, Du12-116b mAb is an IgG2a isotype antibody raised against an ovine intracellular antigen expressed in eosinophils but also recognizing bovine eosinophils (Dr. W.R. Hein, personal communication), and VPM65 mAb (Serotec, Oxford, UK) is an IgG1 isotype antibody raised against ovine CD14 and predominantly cross-reacting with bovine monocytes. Primary antibodies were of mouse origin and were

detected with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma-Aldrich, Bornem, Belgium) secondary antibodies.

2.3. Immunofluorescence staining of cell surface antigens

Blood aliquots of 100 μ L were incubated in polystyrene round-bottom tubes (Becton Dickinson, San José, CA, USA) for 30 min at 37 °C with 50 μ L of either anti-bovine L-selectin 11G10 or 2G8 mAb or anti-bovine Mac-1 CC126 mAb at saturating concentrations. Control samples were incubated with 50 μ L of cross-matched isotypes in RPMI 1640 (Gibco Brl., Scotland, UK), 1% bovine albumin fraction V (Merck KG&A, Darmstadt, Germany) and 0.2% NaN₃. Red blood cells were lysed with 300 μ L of a cold sterile buffered solution (pH 7.4) containing 21.47 mmol/L Trizma[®] Base (Sigma-Aldrich) and 138.34 mmol/L NH₄Cl (Merck), and gently mixed for 6 min at room temperature. After centrifugation (200 \times *g* for 10 min at 4 °C), cells were washed twice in control solution i.e. 300 μ L RPMI 1640, 1% bovine albumin fraction V and 0.2% NaN₃. A second incubation on ice was performed in the dark for 30 min with 50 μ L of the FITC-labeled secondary antibody, diluted 1/250 in control solution. Subsequently, 300 μ L PBS was added, samples were collected by centrifugation (200 \times *g* for 10 min at 4 °C), and cells were washed once in 300 μ L PBS.

2.4. Immunofluorescence staining of intracellular antigens

Surface stained cells were stabilized by leukocyte pellet suspension in 100 μ L of 4% paraformaldehyde in PBS and incubated for 10 min at 4 °C. Subsequently, 300 μ L PBS was added, samples were collected by centrifugation (200 \times *g* for 10 min at 4 °C), and cells were washed once in 300 μ L PBS. Cell membrane permeabilization was achieved with saponin (Sigma-

Aldrich). The fixed and washed leukocytes were exposed to 100 μ L of saponin (0.2–1.4%) dissolved in PBS containing 20% bovine pooled serum for 30 min at 4 °C, and then washed once in cold PBS by centrifugation (200 \times *g* for 10 min at 4 °C). Maximal binding of mAbs specific for L-selectin and Mac-1 was detected by flow cytometry after 1% saponin treatment (data not shown), a concentration that was used for all experiments. Following permeabilization, the same mAbs concentration employed to stain the cell surface was used, and cells were then incubated at 37 °C during 30 min diluted with 50 μ L PBS containing 20% bovine-pooled serum and 0.1% saponin. Subsequently, 300 μ L PBS with 0.1% saponin was added, cells were collected by centrifugation (200 \times *g* for 10 min at 4 °C) and washed once in the same buffer. A final incubation was performed in the dark for 30 min at 4 °C with 50 μ L of FITC-labeled secondary antibody in PBS containing 20% bovine-pooled serum and 0.1% saponin. Non-permeabilized samples were prepared without detergent. All samples were subjected to a final washing with PBS, collected by centrifugation (200 \times *g* for 10 min at 4 °C), resuspended in PBS, and immediately measured by flow cytometry.

2.5. Flow cytometric analysis of L-selectin and Mac-1

Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson). Analysis was performed on 10 000 cells using an argon laser beam (488 nm). The different cell subsets were characterized by forward and side light scattering characteristics. For the permeabilization experiments the different cell populations were further identified with CH138A, Du12-116b and VPM65 mAb, and neutrophil and eosinophil populations were separately gated (Fig. 1). We choose to present the relative mean fluorescence intensity (MFI) because this parameter is

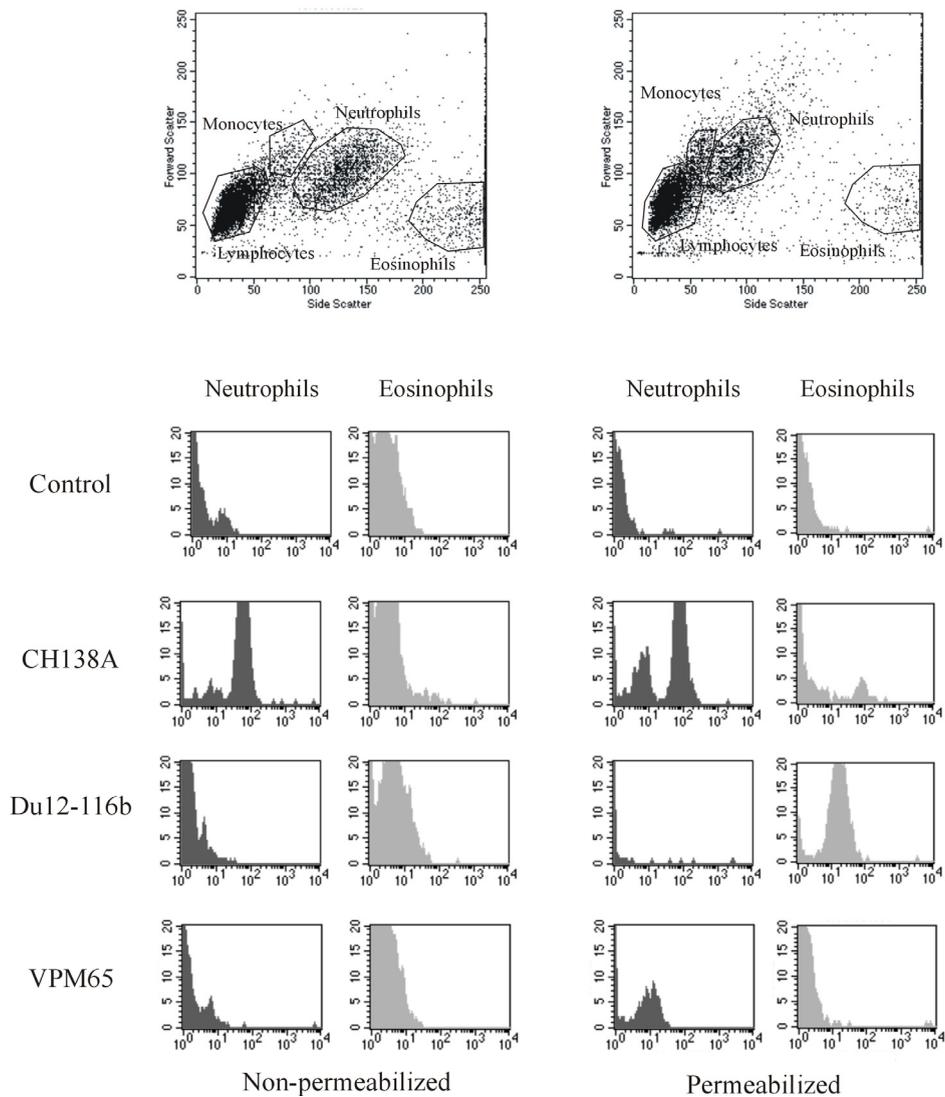


Figure 1. Dot plots of non-permeabilized (left) and permeabilized samples (right) after fixation with 4% paraformaldehyde. Neutrophil and eosinophil subsets were identified by forward and side light scattering characteristics in combination with specific mAbs (CH138A, Du12-116b and VPM65) and FITC-labeled secondary antibody. Cell-associated FITC mean logarithmic fluorescence intensity (x-axis) and cell number (y-axis) is illustrated for each panel. The results shown here are representative for 3 similar experiments.

much more discriminating for the experimental conditions studied than was the percentage of positive cells. The MFI provides an accurate measurement of the brightness of stained cells and is thus an indicator of the receptor number per cell. Non-specific background fluorescence was any fluorescence associated with granulocytes upon incubation with cross-matched isotypes and FITC-labeled secondary antibody.

2.6. Confocal microscopy of L-selectin and Mac-1 surface and total pool

Whole blood samples (90 μL) were stained, fixed and permeabilized as described for flow cytometric analysis. After the surface and/or intracellular labeling, cells were fixed in 1% paraformaldehyde in PBS and stored at 4 °C overnight. Cell nuclei were stained with 50 μL of propidium iodide (PI, 50 $\mu\text{g}/\text{mL}$, Sigma-Aldrich) for 20 min at 4 °C and washed three times with 300 μL of PBS (200 \times g for 10 min at 4 °C). Cells were then spread onto coverslips at 700 rpm for 5 min in a Cytospin 3 (Shandon, Astmoor, Runcorn, UK). Samples were air dried and immediately covered with a mixture of glycerol/PBS (9:1, v/v) supplemented with 25 mg/mL 1,4-diaza-bicyclo[2.2.2]octane (DABCO, Acros organics, NJ, USA). Slides were kept in the dark at 4 °C prior to analysis. Cellular distribution of L-selectin and Mac-1 was examined with confocal microscopy using a Leica DM IRB microscope equipped with a 63 \times magnification oil-immersion objective (Leica, Wetzlar, Germany) and a Leica TCS SP2 scan unit (Leica, Mannheim, Germany). Images were processed with Leica confocal software.

2.7. Sialic acid cleavage

Blood aliquots of 100 μL were lysed with 400 μL of cold Trizma/ NH_4Cl pH 7.4 solution in polystyrene round-bottom

tubes (Becton Dickinson). Cells were washed twice with RPMI 1640 medium containing 0.05 M HEPES (Sigma-Aldrich), 0.02% acetylated BSA (Promega, Madison, WI, USA) and 0.02% NaN_3 . Cells were resuspended in 90 μL of the same medium except for a change in acetylated BSA concentration to 0.1%, and the addition of 10 μL of sialidase buffer (50 mmol/L $\text{Na}^+\text{CH}_3\text{COO}^-$ pH 5.5, 9 mmol/L CaCl_2 and 154 mmol/L NaCl) containing 1, 10 or 25 mU/mL of *Clostridium perfringens* sialidase (Sigma-Aldrich), and incubated for 30 min at 37 °C. Subsequently, 300 μL of RPMI washing medium was added and cells were collected by centrifugation (200 \times g for 10 min at 4 °C). After washing twice, the cell pellet was stained at the cell surface as mentioned above.

2.8. Reagents for adhesion molecule mobilization

For adhesion molecule mobilization, endotoxin-free blood aliquots of 90 μL were incubated in pyrogen-free polystyrene round-bottom tubes (Becton Dickinson). PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, Sigma-Aldrich) was dissolved in analytical-grade chloroform and used at a final concentration of 10^{-7} mol/L following dilution with pyrogen-free saline (final chloroform concentration of $5 \times 10^{-4}\%$). Cytochalasin D (Sigma-Aldrich) was diluted in DMSO and used at a final concentration of 10^{-6} mol/L (final DMSO concentration of $8.5 \times 10^{-3}\%$). This concentration of cytochalasin D has been shown to disrupt actin microfilaments in human neutrophils while it had no effect on microtubular structures [16]. Colchicine (Sigma-Aldrich) was dissolved in water and used at a final concentration of 10^{-6} mol/L. According to Tsai et al. [26] this concentration will selectively affect the microtubular structures without having an effect on microfilaments. Cytochalasin D and colchicine were further diluted with pyrogen-free saline. All chemicals used were of the

highest purity available. Blood samples were incubated with PAF, cytochalasin D or colchicine at 37 °C during 1 h to evaluate adhesion molecule mobilization. When evaluating the effect of cytoskeleton perturbing agents on the PAF-induced adhesion molecule mobilization, samples were pretreated with cytochalasin D or colchicine for 10 min prior to stimulation with PAF for 1 h.

2.9. Statistical analysis

Statistical evaluation of the results was carried out using the Statistix[®] program package (Analytical Software, Thallahassee, FL, USA). The normal distribution assumption was checked by the Wilk-Shapiro test. Statistical analysis of the data was performed using analysis of variance. Comparison of means was executed with the Scheffe's test. Data are presented as mean \pm s.e. for *n* experiments with duplicate readings. The following statistical significances were considered: $P < 0.05$, $P < 0.01$ and $P < 0.001$.

3. RESULTS

3.1. Identification of bovine peripheral leukocytes and effect of saponin on cytogram distribution

Figure 1 shows the effect of saponin on light scattering properties of various cell populations. This mild detergent induced slight modifications in the morphological features of the different cell subsets as could be deduced from the intensity of forward and side scatter characteristics. Subsequent labeling of the cell populations of interest was carried out with a panel of 3 mAbs ($n = 3$). While most of the cells gated as neutrophils were CH138A positive (90%), this antibody only labeled circa 13% of the eosinophil population with or without permeabilization. The population identified as eosinophils by size and

granularity was Du-116b positive for 87% only upon saponin treatment, since this antigen is an intracellular protein. Permeabilization resulted in the appearance of VPM65 positive cells in less than 10% of the cells gated as neutrophils. Considering that approximately 5% of the neutrophils are CD14 positive [9], this would mean that the neutrophil population is blended with 5% of contaminating monocytes.

3.2. L-selectin and Mac-1 surface and total detectable pool in unstimulated granulocytes

The presence of an intracellular pool of L-selectin and Mac-1 was assessed in unstimulated blood granulocytes by flow cytometry ($n = 12$). The MFI of L-selectin and Mac-1 on neutrophils and eosinophils without or with permeabilization, reflected surface or the total detectable pool of both adhesion molecules, respectively (Fig. 2). The average MFI increased significantly for neutrophil L-selectin upon permeabilization: clone 11G10 from 28.5 ± 1.6 to 36.6 ± 2.1 ($P < 0.001$), and clone 2G8 from 29.3 ± 1.6 to 38.3 ± 2.5 ($P < 0.001$). A similar trend was observed for neutrophil Mac-1 expression, increasing from 27.1 ± 1.6 to 62.5 ± 2.8 ($P < 0.001$) upon permeabilization. The L-selectin and Mac-1 expression level was also increased upon permeabilization in eosinophils: L-selectin clone 11G10 from 23.1 ± 0.9 to 30.3 ± 1.5 ($P < 0.001$), clone 2G8 from 23.8 ± 1.0 to 30.6 ± 1.5 ($P < 0.001$), and Mac-1 from 24.4 ± 1.2 to 45.8 ± 1.7 ($P < 0.001$), respectively. A more substantial intracellular pool was detected for Mac-1 as compared to L-selectin for both granulocyte populations. L-selectin expression augmented approximately 30% for both clones and both granulocyte populations over that measured on non-permeabilized cells, while a larger increase ($P < 0.01$) was observed for Mac-1 on neutrophils (136%) as compared to the eosinophil population (93%).

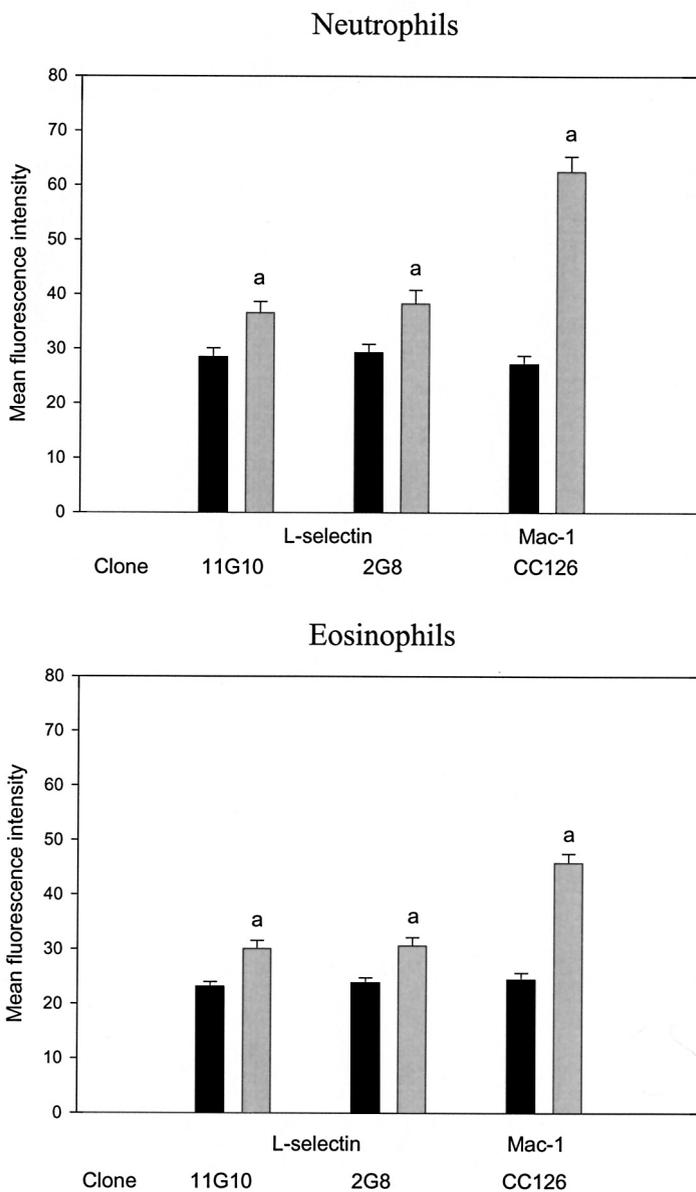


Figure 2. L-selectin (clones 11G10 and 2G8) and Mac-1 (clone CC126) mean fluorescence intensity of unstimulated bovine neutrophils and eosinophils. Non-permeabilized samples (black bars) represent the surface expression and cells permeabilized with 1% saponin (shaded bars) represent the total pool. Data are means \pm s.e. of 12 cows. The values are corrected for non-specific binding. Statistically significant differences $P < 0.001$ (a) between the permeabilized vs. non-permeabilized samples are indicated.

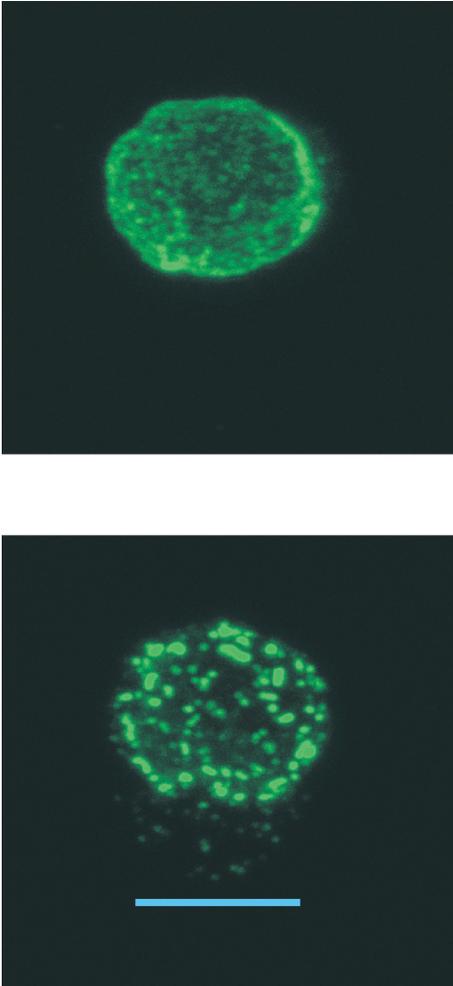


Figure 3. 3-D reconstruction of serial images obtained by confocal microscopy of surface L-selectin (clone 11G10 top) and Mac-1 (bottom) from a representative unstimulated peripheral blood bovine neutrophil. The polymorphic shape of the nuclei by PI staining identifies the cells as neutrophils (not shown). Bar indicates 10 μm .

3.3. Distribution of L-selectin and Mac-1 in unstimulated granulocytes

The distribution of L-selectin and Mac-1 was detected with FITC-conjugated secondary antibodies using confocal micros-

copy. The morphology of the nuclei was visualized by PI staining. Background staining for FITC was minimal in control neutrophils, however, eosinophils showed higher background fluorescence probably related to their well-known strong autofluorescence. Non-permeabilized neutrophils displayed a specific staining at the cell surface for L-selectin and Mac-1. L-selectin was present in clusters that were evenly distributed over the entire neutrophil surface, while Mac-1 cell surface distribution was different, showing a more patched-like pattern (Fig. 3). Confocal microscopy of permeabilized neutrophils indicated the presence of an additional amount of both L-selectin and Mac-1 molecules (Fig. 4). L-selectin was located close to the cell membrane, in contrast to Mac-1 which was largely distributed throughout the cytoplasm. Similar localization patterns for L-selectin and Mac-1 were observed for the eosinophil population without and with permeabilization.

3.4. Effect of sialidase treatment on L-selectin and Mac-1 expression on granulocytes

To verify that the observed increase in fluorescence for L-selectin and Mac-1 in bovine granulocytes was related to an intracellular pool, and not to the unmasking of possible hidden epitopes at the cell surface due to the permeabilization step, cells were treated with sialidase. L-selectin MFI values increased slightly when treated with 1 mU sialidase ($n = 6$; Fig. 5). A maximal increase of 20% was observed upon incubation with 10 mU of sialidase ($P < 0.001$). On the other hand, granulocyte Mac-1 expression was minimally affected by sialidase treatment.

3.5. Effect of PAF on L-selectin and Mac-1 expression

Blood samples were incubated with PAF to evaluate its effect on L-selectin and

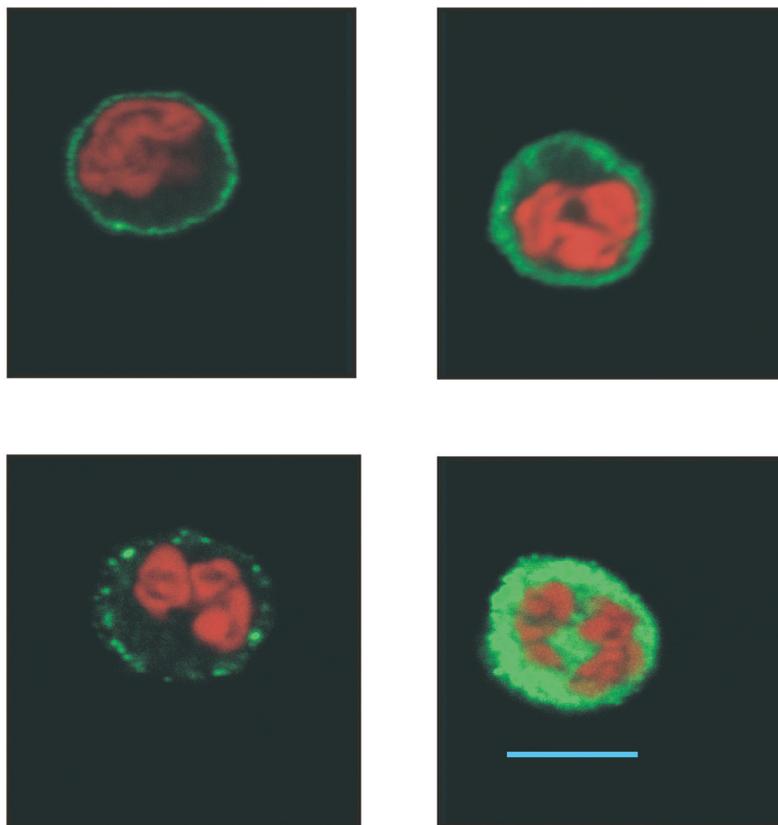


Figure 4. Central sections obtained by confocal microscopy of L-selectin (clone 11G10 top) and Mac-1 (bottom) in a representative unstimulated bovine neutrophil without (left) and with (right) permeabilization. PI stain is used to visualize the multilobulated morphology characteristic for the neutrophil nucleus. Bar indicates 10 μm .

Mac-1 mobilization. Surface L-selectin MFI values decreased with 18.3% ($P < 0.05$) and 16.9% ($P < 0.05$) for neutrophil and eosinophil populations, respectively ($n = 6$; Fig. 6). The total L-selectin MFI diminished with 19.8% ($P < 0.001$) and 16.9% ($P < 0.001$) for neutrophils and eosinophils, respectively. Surface Mac-1 expression was not significantly altered in neutrophils, while a significant increase of 17.6% was observed in eosinophils ($P < 0.05$, Fig. 6). Total Mac-1 expression was not significantly affected by PAF in neutrophils nor in eosinophils.

3.6. Cytoskeleton involvement in L-selectin and Mac-1 mobilization

Cytochalasin D and colchicine were used to evaluate the involvement of the actin and microtubule cytoskeleton structures in the mobilization of L-selectin and Mac-1. Surface and total L-selectin expression were not significantly altered by both cytoskeleton-disrupting agents in neutrophils and eosinophils. The efficiency of Mac-1 mobilization varied depending on the cell type and the specific treatment.

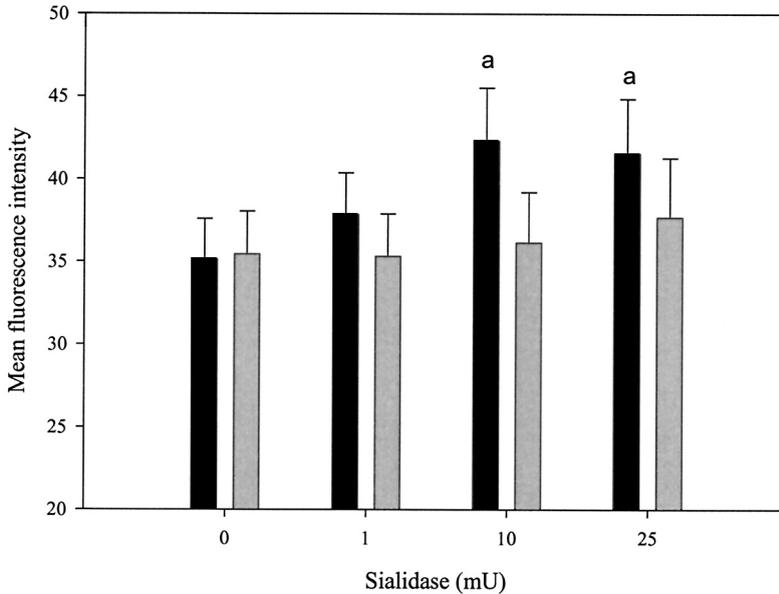


Figure 5. Effect of increasing sialidase concentration on L-selectin (clone 11G10, black bars) and Mac-1 (shaded bars) surface expression on granulocytes (mainly neutrophils blended with a small amount of eosinophils). Data are means \pm s.e. of 6 cows. Statistically significant differences $P < 0.001$ (a) between the sialidase treated samples and the control sample are indicated.

Cytochalasin D significantly up-regulated the Mac-1 surface expression averaging 10% ($P < 0.05$) and 25.1% ($P < 0.001$) on neutrophils and eosinophils, respectively (Fig. 6). On the other hand, colchicine only exerted an up-regulatory effect of 18% on eosinophil Mac-1 ($P < 0.05$). In permeabilized samples, no significant increase of Mac-1 MFI was observed upon cytochalasin D or colchicine treatment.

3.7. Cytoskeleton involvement in the PAF-induced L-selectin and Mac-1 mobilization

The ability of cytochalasin D and colchicine to influence adhesion molecule expression in PAF-stimulated cells was investigated. The cytoskeleton perturbing agents did not have a significant effect on the granulocyte L-selectin decrease following PAF activation (Fig. 6). When

added alone, PAF had no effect on neutrophil Mac-1 expression, and no difference was observed in combination with the different tested drugs. In contrast, cytochalasin D and colchicine elicited an additive effect on Mac-1 surface expression when administered in combination with PAF of non-permeabilized eosinophils ($P < 0.05$).

4. DISCUSSION

Following activation, L-selectin is shed from the granulocyte cell surface by rapid proteolytic cleavage, while cellular Mac-1 expression typically increases [14, 17]. However, a number of studies indicate that L-selectin levels on granulocytes can increase as well upon activation [3, 4, 6, 18, 20, 24, 25]. An intracellular reservoir of L-selectin in granulocytes has not yet been

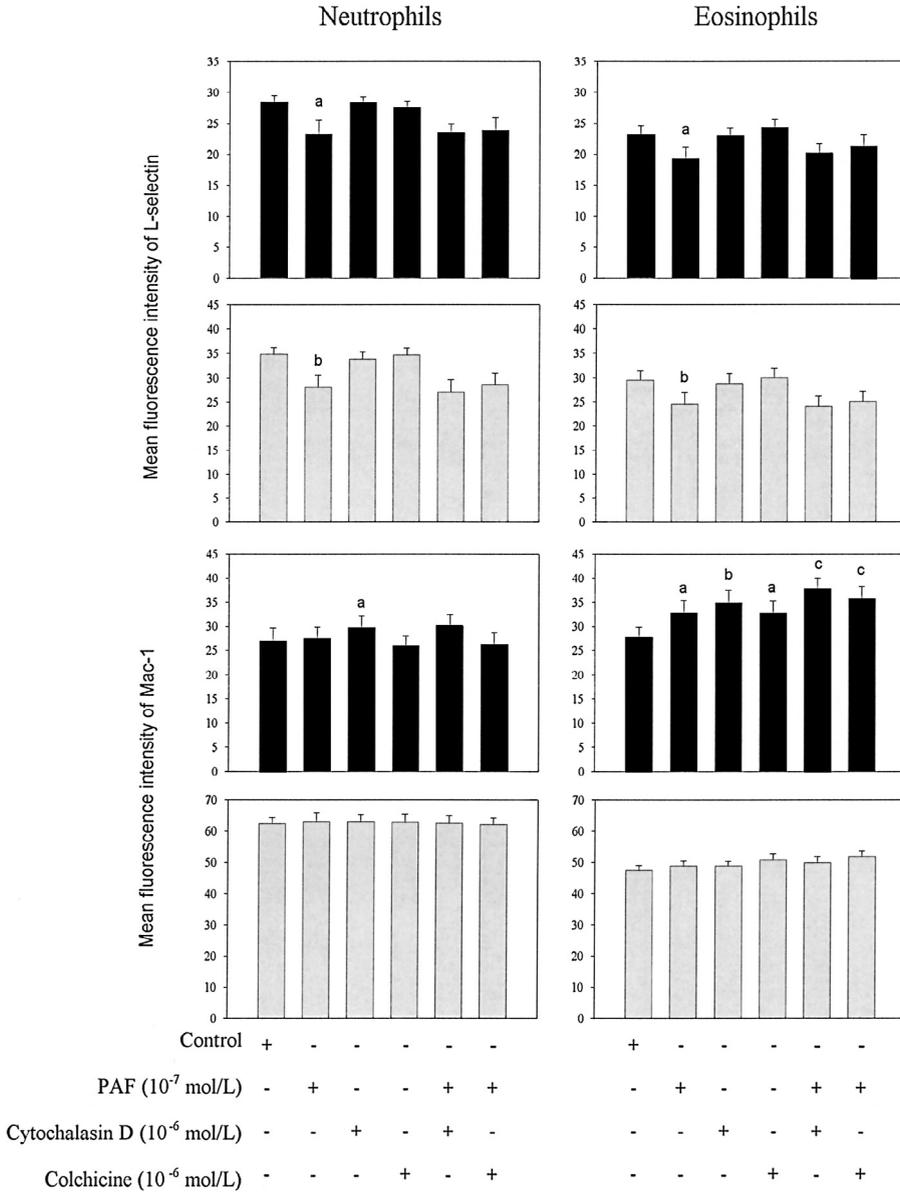


Figure 6. L-selectin and Mac-1 expression on neutrophils and eosinophils following single treatment (PAF, cytochalasin D, or colchicine) or combined treatment (PAF with cytochalasin D or PAF with colchicine). Non-permeabilized samples (black columns) represent the extracellular pool of L-selectin or Mac-1, and cells permeabilized with 1% saponin (shaded columns) represent the total pool. Data are means \pm s.e. of 6 cows. The values are corrected for non-specific binding. Statistically significant differences $P < 0.05$ (a) and $P < 0.001$ (b) between single treatment vs. control samples are marked. Combined treatment vs. single treatment results in a significant additive effect $P < 0.05$ (c) where indicated.

documented. Such an intracellular pool could provide an explanation for the observed rapid augmentation of L-selectin expression on granulocytes. In contrast to reports on L-selectin, a pool of Mac-1 in cytoplasmic granules has already been demonstrated in human granulocytes [13, 21]. In addition, several studies in the bovine have suggested that the increased surface expression of β_2 -integrins on neutrophils originates from cytoplasmic pools [10, 23], by analogy with their human counterpart.

To test the hypothesis of intracellular adhesion molecule reservoirs in bovine granulocytes, we studied the subcellular distribution of L-selectin and Mac-1 by means of fluorescent labeling. In these experiments L-selectin and Mac-1 reservoirs were clearly demonstrated flow cytometrically on both neutrophils and eosinophils. Further analysis by confocal microscopy of adhesion molecule expression showed that permeabilization of granulocytes led to an increase in FITC labeling close to the cell surface for L-selectin, while Mac-1 specific fluorescence was distributed throughout the cytoplasm. Our results are in agreement with Borregaard et al. [1], who retrieved human neutrophil L-selectin exclusively at the cell membrane level after cell fractionation. It may well be the case that differences arise in the availability of epitopes of L-selectin on the cell membrane surface upon saponin treatment. To rule out this possibility, leukocytes were treated with sialidase in order to cleave terminal sialic acid residues, which may potentially mask cell surface epitopes [22]. The observation that sialidase treatment caused an increase in surface exposure of L-selectin may indicate that the detected L-selectin pool is primarily located at the cell surface, probably hidden by glycolipids containing sialic acid. On the other hand, the Mac-1 reservoir appears to be totally located inside the cell as no increase in fluorescence was detected upon sialic acid cleavage.

Under the current experimental conditions PAF down-regulated L-selectin surface and total expression in both neutrophils and eosinophils to the same extent. Because the decrease in L-selectin surface expression paralleled the decrease in the total detectable pool, L-selectin is probably cleaved from the granulocyte surface upon activation by PAF. These data therefore provide evidence for the constitutive shedding of L-selectin from the cell surface in PAF-stimulated granulocytes. Our observations are supported by other reports in which a decreased L-selectin cell surface expression correlated with an increase of extracellular soluble L-selectin [15, 17]. In contrast, PAF stimulation could only increase Mac-1 surface expression on eosinophils, whereas neutrophil Mac-1 surface expression remained unaltered. These data indicate that a qualitative difference exists in responsiveness of both granulocyte populations to stimulation with PAF. Moreover, our study supports the idea that different signaling pathways regulate L-selectin and Mac-1 expression on bovine neutrophils.

Our further experiments were carried out to evaluate the role of important cytoskeleton components in the mobilization of L-selectin and Mac-1 adhesion molecules in both unstimulated and PAF-stimulated granulocytes. A significant increase for both neutrophils and eosinophils was only observed for Mac-1 surface expression after incubation with cytochalasin D. Although the Mac-1 surface expression was similar on both granulocyte populations, eosinophils mobilized a larger portion of their total Mac-1 pool. Colchicine also caused elevated cell surface Mac-1 expression, although only on the eosinophil population. These results provide further evidence that in bovine granulocytes actin microfilaments are the most important cytoskeletal components involved in controlling surface expression of Mac-1. Microfilaments probably act by regulating the transport to the cell surface of Mac-1 containing granules, as shown

before in other models [12, 27]. Interestingly, microtubules seem to be involved in controlling Mac-1 surface expression specifically in the eosinophil population. In contrast to Mac-1, cytochalasin D and colchicine minimally affected L-selectin expression on granulocytes. The different effects among the cytoskeleton perturbing agents further suggests that the adhesion molecule reservoirs under study do not colocalize in the same subcellular structures. It is likely that the increased surface expression of Mac-1 on granulocytes upon PAF activation or cytoskeletal disruption was not related to *de novo* protein synthesis, because no significant increase in the total Mac-1 pool was detected upon permeabilization. When granulocytes were exposed to cytochalasin D or colchicine prior to stimulation with PAF, surface Mac-1 expression was significantly enhanced in the eosinophil population. Our data suggest an additive action between the cytoskeleton disrupting agents and PAF on Mac-1 exocytosis in bovine eosinophils.

In summary, using the described flow cytometric analysis protocol to quantify the intracellular pool of bovine adhesion molecules, it was possible to show the existence of separate L-selectin and Mac-1 reservoirs in bovine granulocytes. Confocal microscopic analysis and sialidase treatment described in this study provide a first means to examine the localization of both adhesion molecules. While L-selectin seems to be mostly located at the cell surface potentially hidden by sialic acid residues, Mac-1 appears to be contained predominantly in intracellular structures. Our further results support the hypothesis that mobilization of neutrophil and eosinophil L-selectin and Mac-1 adhesion molecules are selectively regulated, and that the molecule reservoirs are located in different subcellular structures. Measurement of the intracellular pool of these adhesion molecules should be considered in future studies on the immunomodulatory effects of anti-inflammatory agents.

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