Cell-surface lactoferrin as a marker for degranulation of specific granules in bovine neutrophils

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Objective—To develop a rapid and accurate flow cytometric method for measuring degranulation of specific granules in bovine neutrophils.

Sample Population—Blood samples obtained from four 6- to 18-month-old Holstein cattle.

Procedure—A monoclonal antibody (BL97) was generated against bovine lactoferrin and tested for applicability in ELISA, immunoprecipitation tests, immunofluorescence microscopy, and flow cytometric analyses. Using this antibody, cell-surface lactoferrin was measured concurrent with amount of secreted lactoferrin from bovine neutrophils activated with phorbol myristate acetate (PMA). Cell-surface lactoferrin also was measured on neutrophils in bovine whole blood stimulated with PMA, platelet-activating factor (PAF), N-formyl-methionyl-leucyl-phenylalanine (fMLF), and interleukin 8 (IL-8).

Results—Antibody BL97 recognized bovine lactoferrin in ELISA and western immunoblots and was useful for immunoprecipitation testing, immunofluorescence microscopy, and flow cytometric analyses of bovine leukocytes. Neutrophils activated with PMA had parallel increases in content of secreted lactoferrin (measured by ELISA) and cell-surface lactoferrin (measured by flow cytometry) with increasing PMA concentrations. In addition, fluorescein-conjugated BL97 antibody detected increases in cell-surface lactoferrin on neutrophils in bovine whole blood after activation with PMA, PAF, and IL-8. In contrast, increases in cell-surface lactoferrin were not detected on bovine neutrophils treated with fMLF.

Conclusion and Clinical Relevance—Measurement of cell-surface lactoferrin on bovine neutrophils by flow cytometry is a valid and rapid method for assessment of release of lactoferrin from specific granules in these cells and represents a means to rapidly measure neutrophil activation. This technique allows for investigation of mechanisms of neutrophil modification in isolated cells as well as in whole blood. (Am J Vet Res 2000;61:29–37)

Polymorphonuclear neutrophils (PMN) play an important role in the host defenses of cattle against pathogens. For example, infection of the mammary glands by bacterial pathogens is characterized by a rapid influx of a large number of PMN into the infected glands where they are responsible for phagocytosing and killing the microbes. This inflammatory response is essential in host defenses against mastitis-inducing pathogens, and a correlation between decreased PMN function and severity of mastitis has been documented in a number of studies.

For an effective host defense against pathogens, circulating PMN must be recruited to the area via a multiple-step process involving adherence of the cell to the vascular endothelium, diapedesis through the vessel wall, and migration into the infected tissue. Although mechanisms involved in PMN recruitment are complex, it is clear that this event is accompanied or preceded by changes in the cell-surface expression of adhesion molecules or receptors and that upregulation of many of these surface molecules in neutrophils results, in part, from the degranulation of intracellular granules and incorporation of proteins associated with granule membranes into the plasma membrane.

Once in the infected tissue in cattle, PMN use various microbicidal mechanisms to carry out their host-defense functions. After phagocytosing the microbe, activated PMN use oxygen-dependent mechanisms, such as the production of toxic oxygen metabolites (eg, superoxide anion, hypochlorous acid, and peroxynitrite), and oxygen-independent mechanisms, such as the release of proteases and cationic peptides, to destroy pathogens. Interestingly, both host-defense mechanisms use stores of PMN proteins that are located on or in cytoplasmic granules that are mobilized upon activation. This type of organization ensures that toxic agents generated by PMN used during microbicidal responses are regulated and only mobilized when required, thereby preventing inappropriate damage to host tissues.

Organization of neutrophil granules is complex, and several granule types have been characterized in bovine neutrophils. Some of these granules are analogous to granules found in human PMN (eg, specific and azurophil granules); however, 1 granule (known as the large granule) is only found in bovine cells. There is evidence of selective recruitment of granule types, depending on the stimulus, in human and bovine PMN. The specific (secondary) granule in particular is an important reservoir of adhesion molecules and receptors, including CD11b/CD18 and receptors for fibronectin, laminin, and tumor necrosis factor. Many activating agents can cause degranulation of specific granules in human PMN as well as bovine PMN.
Although mobilization of specific granules in bovine PMN plays an important role in recruitment of PMN to the udder as well as for the microbicial activity of these cells during mastitis, little is known about regulation of the degranulation process in bovine PMN and the points in the host-defense process at which specific granules are mobilized. One reason for this lack of understanding is that there are few assays that provide accurate data on the in vivo degranulation process in PMN. The most common method of measuring degranulation of specific granules is to measure release of lactoferrin (an iron-binding protein in specific granules) by PMN in an in vitro ELISA. 22,30 Although precise and often quantitative, this method requires isolation of the cells and a lengthy assay procedure. Because the amount of handling involved in isolating cells from whole blood can influence a number of neutrophil functions, including oxidative burst activity, phagocytosis, chemotaxis, and expression of cell-surface antigens,31-33 it has become essential that new assay methods be developed that can be used to evaluate PMN function in whole blood. 31-33

Recently, Afeltra et al.21 reported that lactoferrin could associate with the human PMN cell-surface membrane and that the relative amount of surface-associated lactoferrin, as determined by flow cytometric analysis, increases with exposure to agents that cause degranulation of specific granules. In the study reported here, we generated an anti-bovine lactoferrin monoclonal antibody and used it in a similar flow cytometric assay to document that lactoferrin also associates with the plasma membrane in bovine PMN. Using this method, we directly compared changes in neutrophil cell-surface lactoferrin with the amount of lactoferrin secreted by the same neutrophils (as measured by ELISA). Our objective was to develop a method that will provide a reliable means of rapidly estimating degranulation of specific granules in bovine neutrophils in isolated preparations and whole blood.

Materials and Methods

Animals and blood collection—Blood was collected from four 6- to 18-month-old Holstein cattle. Tubes contained 3 mM EDTA as the anticoagulant. When isolated neutrophils were required, erythrocytes were removed from EDTA-treated blood by use of hypotonic lysis, and neutrophils were separated from mononuclear cells on a twostep gradient, as described elsewhere. 31 All procedures were approved by an institutional Animal Care and Use Committee.

Production and labeling of monoclonal antibody BL97—Anti-bovine lactoferrin monoclonal antibodies were generated by inoculating BALB/c mice (30 µg of bovine lactoferrin emulsified in adjuvant; IP 2 times with a 3-week interval between inoculations). Three days prior to fusion, the mice were given a booster (50 µg of bovine lactoferrin, IV). Spleen cells from the inoculated mice were fused with SP2/0 tumor cells,36 and hybridoma supernatants were screened 9 days later.

For hybridoma screening, microtiter plates were coated with 50 µl of 10 to 20 µg of bovine lactoferrin/ml dissolved in 0.1M bicarbonate buffer, pH 9.6. Plates then were incubated overnight at 4 C. After washing with Dulbecco’s phosphate-buffered saline (DPBS)37 solution containing 0.5% fetal bovine serum and 0.5% Tween 20 (wash buffer), 50 µl of the hybridoma supernatants was added to the wells, and the plates were incubated for 2 hours at 25 C. Plates then were washed, incubated for 2 hours at 25 C with alkaline phosphatase-conjugated goat anti-mouse secondary antibody, washed again, developed by use of an alkaline phosphatase substrate,37 and read with a microtiter plate reader.38 One of the wells with positive results was expanded and subcloned to obtain monoclonal antibody BL97.

To label monoclonal antibody BL97, the antibody was initially purified by ion-exchange chromatography on a column equilibrated with 20 mM tris, pH 8.0, and eluted with a 0.0 to 0.5M NaCl gradient in the same buffer. The purified antibody was conjugated with fluorescein isothiocyanate (FITC), 39 using standard protocols.37 The FITC-conjugated antibody was then exchanged into DPBS buffer by use of gel chromatography on a 10DG desalting column,37 and aliquots of approximately 2 mg/ml were stored at –80 C until use.

ELISA procedures—Two types of ELISA procedures were used: a single-antibody antigen capture assay and a two-antibody antigen capture assay. Both procedures are variations of standard methods described by Harlow and Lane. 38 For the single-antibody procedure, bovine lactoferrin standards and experimental samples were allowed to bind for 3 hours at 24 C in 96-well plates.40 Plates were washed 3 times with DPBS, blocked overnight at 4 C in DPBS containing 3% bovine serum albumin, and washed again with DPBS; 10 µg of antibody BL97/ml then was added to each well. After incubating for 2 hours at 24 C, plates were washed 4 times with DPBS, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:3,000 dilution) for 2 hours at 25 C, and washed 4 additional times with DPBS. Plates were developed by adding 75 µl of substrate solution (10 mM diethylamine inside 25 C; plates were then ready for a fluorescence microtiter plate reader, using excitation and emission wavelengths of 355 and 460 nm, respectively.

For the two-antibody procedure, wells were coated initially with antibody BL97 (50 µl of a solution of 20 µg/ml per well) for 2 hours. Plates were washed 3 times with DPBS, blocked, and washed again as described earlier. Pure bovine lactoferrin or experimental samples (diluted 1:2 in blocking buffer) were incubated in the wells for 3 hours at 25 C; plates were washed 4 times with DPBS, rabbit anti-human lactoferrin (1:750) was added to the wells, and plates were incubated for 2 hours at 25 C. After addition of alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2,500) was added to the wells, and plates were incubated for 2 hours at 25 C. Plates then were washed, developed, and read as described earlier.

Flow cytometry—Flow cytometric analysis was performed as described earlier. 38 Neutrophils were gated from mononuclear cells on the basis of their forward- and side-scatter properties, and 10,000 gated events were obtained for each sample.

Flow cytometry was performed on samples of whole blood, using the method of McCarthy and Macey, 41 as modified by our laboratory group for bovine neutrophils. Briefly, 50-µl aliquots of blood (containing approx 1 X 107 neutrophils) were incubated at 37 C for 5 minutes. Treatments (phorbol-12-myristate-13-acetate [PMA], 42 platelet-activating factor [PAF]; 1-O-hexadecyl-2-acyl-sn-glycerol-3-phosphocholine),43 interleukin 8 [IL-8], 44 N-formyl-methionyl-leucyl-phenylalanine [fMLP],45 or buffer) were added (5-jl volume), and the incubation was continued at 37 C for 15 minutes. During the final 2 minutes, LDS 751 was added.
at a final concentration of 20 µg/ml. Samples then were incubated at 4 °C for 30 minutes with FITC-conjugated BL97 (4 µl/tube). Samples then were diluted with 500 µl of DPBS containing 0.2% bovine serum albumin and analyzed by use of flow cytometry. Red blood cells were discriminated by a lack of FL3 fluorescence (ie, LDS-751 nuclear staining), and neutrophils then were gated as described. Results for all flow cytometric assays were expressed as mean fluorescence intensity of gated neutrophils.

Degranulation assays—Bovine neutrophils (5 × 10⁶ cells/ml of Hank’s balanced salt solution [HBSS]) containing 1 mM CaCl₂ were placed in polystyrene tubes (400 µl/tube) and incubated at 37 °C for 5 minutes. The PMA or buffer control solution was added (40 µl of a 10X solution), and cells were incubated at 37 °C for 15 minutes with shaking. Cells then were centrifuged at 250 × g for 5 minutes at 4 °C. Supernatants were removed and used for analysis of secreted lactoferrin, using the ELISA described earlier. The cell pellet was resuspended in 200 µl of cold DPBS, and 3 µl of FITC-conjugated BL97 was added to each tube. Cells were incubated on ice for 30 minutes, approximately 3 ml of cold DPBS was added to each tube, and cells were centrifuged as described earlier. The cell pellet then was resuspended in 400 µl of cold DPBS and analyzed, using flow cytometry as described earlier. Analysis of the binding of exogenous lactoferrin was performed in the same manner, except that bovine lactoferrin (from 10 mg/ml) was added to unstimulated cells.

Immunofluorescence microscopy—Isolated neutrophils were washed with DPBS, applied to glass slides, and fixed in DPBS containing 3% formaldehyde for 15 minutes. Staining of the cells was performed, using the saponin-permeabilization method of Sander et al. Briefly, slides were washed with DPBS, blocked with DPBS containing 2% bovine serum albumin, washed again, and stained with FITC-conjugated BL97 diluted 1:200 in DPBS containing 2% bovine serum albumin and 0.1% saponin. Slides then were washed twice with DPBS containing 0.1% saponin, followed by 2 washes with DPBS. Digital images of the stained cells were acquired with a digital camera on an inverted microscope (40X objective). Nonspecific binding was determined by use of an irrelevant FITC-conjugated mouse IgG antibody. This isotype control monoclonal antibody against p47-phox was generated and characterized in our laboratory.

Immunoprecipitation test—Granules from bovine neutrophils were prepared by differential centrifugation of bovine neutrophil homogenates, using methods previously described for porcine neutrophils. The isolated granules were lysed by 3 cycles of freezing-thawing, by adding DPBS buffer containing 0.1% Triton X-100, or by adding DPBS buffer containing 0.1% NP-40. Granule lysates or pure bovine lactoferrin were incubated overnight at 4 °C with 50 µg of monoclonal antibody BL97 or an antibody isotype control antibody (anti-p47-phox). Samples then were incubated for 3 hours at 25 °C with 100 µl of a 50-50 slurry of protein-G beads in DPBS or DPBS plus the same detergent used for lysing the granules. After 4 washes with the same buffers, the beads were boiled for 5 minutes in 120 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed, using SDS-PAGE and western immunoblots analysis.

SDS-PAGE and western immunoblots analysis—Samples were separated by SDS-PAGE on 10% polyacrylamide gels and stained with Coomassie blue stain or transferred to nitrocellulose membrane for western immunoblot analysis, as described elsewhere. Briefly, immunoblots were blocked with DPBS containing 10% normal goat serum and 0.2% Tween 20. After blocking, immunoblots were probed with antibody BL97, which was followed by alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody; immunoblots were developed by use of an alkaline phosphatase development kit. Blocking buffers that contain milk cannot be used for analysis of lactoferrin immunoblots. For reference, preblotted molecular weight standards were included on all gels.

Results

Characterization of monoclonal antibody BL97—Bovine lactoferrin was used as the antigen to immunize BALB/c mice for the purpose of generating monoclonal antibodies, one of which (BL97) proved to be useful for the detection of bovine lactoferrin in several applications. In an ELISA format, BL97 bound in a linear fashion to bovine lactoferrin that was previously adsorbed to polystyrene wells over the range of 2.5 to 160 ng of lactoferrin/well (Fig 1A). However, when supernatants from stimulated neutrophils were analyzed with this procedure, background fluorescence was unacceptably high, perhaps as a result of neutrophil-derivied alkaline phosphatase binding to the

Figure 1—Development of a lactoferrin ELISA with BL97 monoclonal antibody. A single-antibody ELISA method that used blocking, addition of BL97 antibody, and addition of alkaline phosphatase-conjugated secondary antibody was developed to indicate the amount of lactoferrin bound to microtiteration plate wells (panel A). A two-antibody sandwich ELISA method used antibody BL97 bound to microtiteration plate wells, followed by blocking, addition of lactoferrin standards (or samples), addition of rabbit anti-lactoferrin antibody, and addition of alkaline phosphatase-conjugated secondary antibody (panel B). Each point represents mean ± SEM of 3 measurements. Data are representative of 4 (panel A) and 3 (panel B) independent experiments.
granule extract (5 µg of total protein), respectively, were analyzed by use of western immunoblotting with approximately the location of lactoferrin on the immunoblots. Representative of 2 independent experiments. Arrowheads indicate standards (STD) are in the first lane of both panels. Data are representative of 3 independent experiments.

The BL97 subclone was chosen because of its ability to recognize cell-surface lactoferrin via immunostaining and flow cytometry. Isolated bovine neutrophils stained with BL97 followed by FITC-conjugated secondary antibody had fluorescence slightly higher than cells stained with an isotype control antibody (Fig 3). When these cells were activated with PMA, however, there was a substantial increase (three- to fourfold) in fluorescence of the activated cells. The fluorescence histogram was slightly broader for PMA-stimulated cells, and a subpopulation of the cells with bright fluorescence was observed. To verify that the increased fluorescence of PMA-stimulated cells was not attributable to increased nonspecific binding of antibodies or leakage of intracellular contents, an isotype control antibody against a bovine neutrophil cytosolic protein (p47-phox) was used. Treatment with PMA (10 ng/ml) resulted in an increase in mean fluorescence for p47-phox that was only 2% of the increase in lactoferrin mean fluorescence intensity, thereby confirming the specificity of staining (data not shown).

To simplify immunostaining, BL97 was directly conjugated with FITC. Staining with FITC-conjugated BL97 resulted in a similar pattern when using flow cytometry, although the amount of background fluorescence was slightly higher than with unconjugated BL97. As a control standard, a FITC-conjugated isotype control antibody against a bovine neutrophil cytosolic protein (p47-phox) was used. Treatment with PMA (10 ng/ml) resulted in an increase in mean fluorescence for p47-phox that was only 2% of the increase in lactoferrin mean fluorescence intensity, thereby confirming the specificity of staining (data not shown).

Figure 2—Western immunoblot and immunoprecipitation analysis of bovine lactoferrin with BL97. In lanes 1 and 2 of panel A, samples of bovine lactoferrin (2.5 µg) and bovine neutrophil granule extract (5 µg of total protein), respectively, were analyzed by use of western immunoblotting with approximately 50 µg of antibody BL97/ml. In Panel B, samples of bovine neutrophil granule lysates prepared by freezing-thawing (lane 1), lysis with Triton X-100 (lane 2), or lysis with NP40 (lane 3), as well as samples of pure bovine lactoferrin at 1 mg/ml (lane 4), 0.2 mg/ml (lane 5), and 0.1 mg/ml (lane 6), were immunoprecipitated with antibody BL97. A sample of 1 mg of bovine lactoferrin/ml was immunoprecipitated with an isotype control antibody (lane 7). Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue stain. Prestained molecular weight standards (STD) are in the first lane of both panels. Data are representative of 2 independent experiments. Arrowheads indicate the location of lactoferrin on the immunoblots.

wells. To quantitate lactoferrin from a complex mixture, it was necessary to develop a sandwich ELISA, using BL97 and a commercially available anti-human lactoferrin antibody. This assay was quantitative over the range of 2.5 to 40 ng of bovine lactoferrin/ml (Fig 1B). Range of detection of this assay is suitable for analyzing supernatants from degranulated bovine neutrophils and also would be appropriate for testing blood and 10 mg/ml of undiluted milk.

Monoclonal antibody BL97 was useful for detecting bovine lactoferrin in samples analyzed by SDS-PAGE and western immunoblotting (Fig 2A); however, staining on immunoblots required higher antibody concentrations (approx 50 µg/ml), which increased the amount of nonspecific background staining. In contrast, BL97 was able to efficiently immunoprecipitate bovine lactoferrin from a complex mixture of proteins. The BL97 immunoprecipitated lactoferrin was evident in samples of mixed bovine granules that were lysed by several methods, including repeated cycles of freezing-thawing and detergent lysis (Fig 2B). These methods of lysis were evaluated to determine the one that was most effective for releasing lactoferrin from the granules; however, analysis of our results suggested that all methods were equally effective for lysing the granules and that BL97 could immunoprecipitate the protein in the presence or absence of detergents. Immunoprecipitation with antibody BL97 effectively cleared bovine lactoferrin from solutions containing a range of lactoferrin concentrations (1, 0.2, and 0.1 mg/ml).
Degranulation assays—To compare changes in cell-surface lactoferrin with those of the secreted form of lactoferrin, we quantitated lactoferrin secreted by isolated bovine neutrophils treated with a range of PMA concentrations and also analyzed the same cells with flow cytometry. Increases in secreted lactoferrin are closely paralleled by increases in detectable cell-surface lactoferrin (Fig 5). This parallel increase was evident even though there was the usual animal-to-animal and day-to-day variation in the amount of PMA required for a measurable response. A substantial increase in lactoferrin (secreted and cell-surface) was evident at 1 ng of PMA/ml, whereas other samples required up to 10 ng of PMA/ml before a substantial response in secreted and cell-surface lactoferrin was detected. However, in none of the samples was there a divergence of the results of the flow cytometric assay, compared with results for the ELISA.

When exogenous bovine lactoferrin was added to unstimulated, isolated bovine leukocytes and cells were washed and probed with FITC-conjugated BL97, substantial increases in mean cellular fluorescence were seen in neutrophils as well as in monocytes and lymphocytes (Fig 6A), documenting that some exogenously added lactoferrin could bind to resting PMN. However, comparison of the increase in fluorescence resulting from the addition of exogenous lactoferrin to resting cells with that seen in PMA-stimulated neutrophils revealed that a substantially higher amount of lactoferrin was bound to the PMA-stimulated cells (Fig 6B). Thus, when the percentage increase in fluorescence was plotted against the solution lactoferrin concentration (determined by ELISA in the cell supernatant of PMA-stimulated cells), it was apparent that the amount of cell-surface lactoferrin was much higher than would be expected simply from the binding of released lactoferrin.
Analysis of whole-blood samples—When samples of whole blood were treated with a range of PMA concentrations and examined for FITC-conjugated BL97 staining with a flow cytometric assay, substantial increases in mean fluorescence of neutrophils could be seen at PMA concentrations >100 ng/ml (Fig 7A). This technique for whole blood was also useful for detecting degranulation of specific granules provoked by other, more physiologic activators. Treatment with PAF and IL-8 resulted in substantial increases in cell-surface lactoferrin that were comparable to those seen after stimulation with 1 µg of PMA/ml (Fig 7B). The formulated peptide fMLF did not cause an apparent degranulation response in bovine neutrophils, which is consistent with reports that fMLF does not activate other processes (eg, chemotaxis or the oxidative burst) in bovine neutrophils.

Discussion

Degranulation of neutrophils is often a graded response to specific stimuli. In humans and cattle, secretory and specific granules can be released at a lower concentration of stimulus than is required for release of azurophil granules. To facilitate further examination of this phenomenon, we developed a novel assay to measure degranulation of specific granules in bovine neutrophils that can be used for whole-blood samples as well as isolated neutrophils.

Lactoferrin is the marker protein commonly associated with the release of specific granules. Lactoferrin is a 70- to 80-kd glycoprotein that is found in milk, tears, other secretions, and neutrophils. Although lactoferrin is commonly dismissed as simply a potentially bacteriostatic iron-binding protein, the list of its immunoregulatory and host-defense functions is growing constantly.

The most common method of determining degranulation of specific granules is to measure, usually with an ELISA, the quantity of lactoferrin released...
by stimulated neutrophils in vitro. An alternative approach is to permeabilize treated neutrophils and to estimate the relative intracellular concentration of lactoferrin with a labeled antibody. A third alternative that we exploited is to analyze cell-surface lactoferrin by use of flow cytometry. Lactoferrin can be detected on the surface of human neutrophils, and the relative amount of cell-surface lactoferrin increases with PMA stimulation. Similar to human neutrophils, bovine neutrophils contain lactoferrin, albeit reportedly at lower amounts than in human neutrophils, and lactoferrin has been used as a marker of specific granules in bovine neutrophils. Recently, we documented that bovine neutrophils also have cell-surface lactoferrin and that the relative amount of cell-surface-associated lactoferrin increases with PAF stimulation.

In the study reported here, we documented that the appearance of lactoferrin on the surface of PMA-stimulated bovine neutrophils closely paralleled the amount of lactoferrin secreted by those same cells. Furthermore, stimulation with increasing doses of PMA resulted in proportional increases in secreted and cell-surface lactoferrin. However, high doses of PMA (1 μg/mL) caused bovine neutrophils to take on a greater range of size and granularity, and, therefore, they became excluded from the neutrophil gate in the flow cytometric measurements. Because the study of Salagar et al revealed that bovine neutrophils have a peak oxidative burst at much lower doses of PMA (approx 50 ng/mL), we concluded that the methods reported here can be used on cells stimulated with doses of PMA that typically are used in studies on bovine neutrophils. Thus, measurement of cell-surface lactoferrin on bovine neutrophils by use of BL97 represents an accurate method for rapid measurement of the degranulation of specific granules of bovine neutrophils.

It is interesting that lactoferrin staining of stimulated bovine neutrophils was characterized by a subpopulation of cells that had bright fluorescence, compared with the main population of stained cells. The reason for the high degree of staining in this subpopulation of cells is currently unknown but may relate to the known heterogeneity of neutrophil populations in blood. Another possibility is that this heterogeneity may reflect a population of cells that had been previously exposed in vivo to a priming stimulus, because exposure of neutrophils to a priming agent can mobilize certain granules and substantially alter the expression of cell-surface adhesion molecules.

The technique reported here is also applicable to bovine whole-blood samples when the samples are analyzed by use of flow cytometric methods. In this way, degranulation of specific granules can be examined in whole blood, thus avoiding potential artifacts that result from isolation of specific cell types (eg, neutrophils). In fact, one possibility suggested by analysis of our results is that the isolation of PMN makes them more sensitive to the degranulating effects of PMA, because whole blood consistently required a 10- to 100-fold higher concentration of PMA, compared with isolated PMN, to have a substantial increase in cell-surface lactoferrin. This finding is consistent with that in a study on human PMN in which isolation of cells from blood samples results in alterations in the expression of cell-surface antigens, such as CD11b, that play a role in PMN activation. This problem has not been systematically studied in bovine neutrophils; however, analysis of preliminary results suggests that isolation of bovine neutrophils can result in down-regulation of cell-surface L-selectin expression, suggesting possible isolation-induced priming effects (data not shown).

The nature of the association between lactoferrin and the neutrophil membrane is not clear. Afeltra et al examined cell-surface lactoferrin in human neutrophils and found that the same epitope of lactoferrin always was exposed on the neutrophil surface, whereas a second epitope was never accessible to the specific antibody when lactoferrin was membrane-associated. Analysis of the secondary structure of lactoferrin does not suggest that it would have a membrane-spanning conformation, and the molecule consists of 2 similar globular, glycosylated domains connected with an extended alpha-helix. Therefore, rather than being directly inserted into the membrane, a more likely explanation is that lactoferrin is binding (perhaps in a stereo-specific manner) to sites on the surface of the cell during the process of granule exocytosis or after being released into the extracellular media. There are reports that many cell types, including leukocytes, possess lactoferrin receptors; however, there is considerable discrepancy between reported affinities and the molecular weights of these putative receptors. In addition, there is debate as to whether the actual function of these molecules is to serve as lactoferrin receptors. Regardless of the nature of this binding, analysis of our results indicated that exogenous lactoferrin can bind to bovine neutrophils (as well as monocytes and lymphocytes) and can be detected as cell-surface lactoferrin with the FITC-conjugated BL97 antibody, even after the cells are washed. This increase in cell fluorescence was notable at lactoferrin concentrations ≥ 20 nM, which are similar to the concentrations of secreted lactoferrin that resulted in measurable amounts of lactoferrin bound to the surface of PMA-stimulated PMN. However, it is clear that the binding capacity of unstimulated neutrophils for lactoferrin in solution is not sufficient to completely account for the increase in cell-surface lactoferrin measured in PMA-stimulated neutrophils. One possible explanation is that binding of lactoferrin by cells and plasticware represents a sink for released lactoferrin. Thus, measurement of lactoferrin remaining in solution from stimulated cells may not accurately reflect the amount of lactoferrin that was actually released by the cells. Birgens et al estimated that human monocytes have approximately 1.6 × 10⁶ lactoferrin binding sites/cell, with a dissociation constant of approximately 4.5 × 10⁻⁹. Based on those figures, 2 × 10⁵ monocytes in 0.4 mL could bind approximately 25% of the lactoferrin, providing the concentration of the original solution was 20 nM. Although neutrophil lactoferrin receptors are believed to have lower affinity than those of monocytes, the combined binding capacity of the cells themselves could represent a substantial sink for released lactoferrin. Another possible explanation is that the number or
affinity of receptors or other surface molecules that bind lactoferrin are increased in stimulated cells.

Assuming that cell-surface lactoferrin is, in part, a result of secreted lactoferrin binding to sites on the PMN membrane, 3 cautions should be considered for this assay. First, there appears to be a slight and often variable amount of cell-surface lactoferrin on unstimulated cells. Therefore, an unstimulated-cell control sample from the same cell preparation or blood sample is essential so that the relative amount of lactoferrin released above background can be determined. Second, because lymphocytes and, especially, monocytes also bind lactoferrin (which is then detected by antibody BL97), these cells can compete with neutrophils for lactoferrin that is released by degranulating PMN. Therefore, comparison of degranulation responses may only be valid within the same sample type (ie, isolated PMN vs total leukocytes vs whole blood). Third, because milk is rich in lactoferrin secreted from mammary cells, binding of that lactoferrin would possibly mask changes in cell-surface lactoferrin on cells obtained from milk. Comparison with control neutrophils in milk may be sufficient to overcome this problem. Otherwise, experiments that use this assay would need to be confined to examine changes in the PMN prior to their migration into the udder.

References


