Isolation and Phagocytic Properties of Neutrophils and Other Phagocytes from Nonmastitic Bovine Milk

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ABSTRACT

A technique for the separation of neutrophils from macrophages-epithelial cells in samples of nonmastitic bovine milk with low cell counts has been developed. The procedure is based on centrifugation in a discontinuous metrizamide gradient and is rapid, taking less than 40 min. The recovery of the neutrophils is about 30% and their viability about 90%. The isolated neutrophils showed an appreciable unstimulated luminol- and lucigenin-dependent chemiluminescence, which was due to NADPH oxidase rather than to xanthine oxidase. The neutrophils had a higher rate of ingestion of C3-opsonized particles than macrophages-epithelial cells, whereas no significant differences in phagocytosis of IgG-opsonized yeast or unopsonized yeast were detected between the two cell populations. The macrophages-epithelial cells produced no luminol-dependent chemiluminescence and induced considerably lower activity in the lucigenin-dependent system than neutrophils, indicating that these cells contain no myeloperoxidase. Analyses of the activity of the neutrophils in response to C3-opsonized yeast particles showed that the luminol-dependent chemiluminescence of cells isolated from residual milk increased significantly over the lactation period. Moreover, a tendency to a higher phagocytosis and chemiluminescence of neutrophils isolated from residual milk than from stripping milk was indicated. (Key words: milk, neutrophils, macrophages)

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INTRODUCTION

The phagocytic function of bovine milk neutrophils has been the subject of several studies. However, the general procedure in these investigations has been to increase the number of cells in the milk by different means, such as local deposition of oyster glycogen, endotoxin, foreign bodies, or other inflammatory promoters (7, 23, 31). It is well documented that neutrophilic granulocytes that are recruited by such procedures to other sites in the body show an altered functional activity compared with blood neutrophils (2, 36). Moreover, the few studies that have been done to date of the phagocytic function of neutrophils from nonmastitic bovine milk with low cell counts indicate that alterations in neutrophil function may occur in inflamed udders (3, 18, 19). The main reason for the scarcity of studies of neutrophils from nonmastitic milk has been the low numbers of neutrophils in such milk, because most of the existing phagocytic methods require large amounts of cells (1). Moreover, no technique to separate different phagocytes from bovine milk with low cell counts has been available.

We have earlier presented a method to separate neutrophils from bovine blood with high yield and purity (11) and have also developed a technique to study both phagocytosis and chemiluminescence of small cell populations (12). In this paper, these techniques, with some modifications, have been applied to the study of phagocytic cells from bovine stripping and residual milk collected from unmanipulated udders. By this procedure, populations of milk...
neutrophils devoid of macrophages have been isolated. Populations of macrophages-epithelial cells (MEC) uncontaminated by neutrophils were also obtained. Functional studies of the phagocytes isolated in this manner showed that the neutrophils caused an appreciable unstimulated chemiluminescence due to the activity of NADPH oxidase and that the MEC lacked myeloperoxidase activity. Moreover, the chemiluminescence of neutrophils from residual milk increased significantly over the lactation period.

MATERIALS AND METHODS

Animals

Apparently healthy cows, between 2 and 8 yr of age and of three different breeds (Friesian, Jersey, and Swedish Red and White), were used as blood and milk donors. Cell counts in whole udder milk from one morning milking and one evening milking were determined once a week for each individual by the Fossomatic technique (32). The 30 animals used for milk sampling all had \(1.5 \times 10^5\) somatic cells/ml in milk the last 4 wk before sampling and during the sampling week and had shown no signs of clinical mastitis during the lactation period.

Milk Sampling

For optimization of the separation method, stripping milk from one teat was collected immediately after morning milking, because this was the simplest procedure. In functional studies of neutrophils isolated from different lactation stages, cows from 8 to 289 d postpartum were used. Stripping milk was first withdrawn with a milking machine in these experiments. The cow was subsequently injected i.v. with oxytocin (10 IU), and residual milk was collected after 2 to 3 min from all four teats with a milking machine. For comparisons of macrophage and neutrophil function, stripping milk from one teat was removed by hand, and 70 to 100 ml of the residual milk were then collected by hand milking following an i.v. oxytocin injection as just described. To give a higher yield of cells in the later study, the cows were either in the beginning or in the end of the lactation period (\(\leq 3\) wk of \(\geq 38\) wk postpartum).

Total cell counts in whole milk were analyzed by the Fossomatic technique and by flow cytometry (10). Cell counts in milk were also determined by acridine orange staining by the addition of 20 \(\mu\)l of milk to 180 \(\mu\)l of 0.001% (wt/vol) acridine orange (3) in modified Gey's solution [MGS; (11)]. Immediately before the cells were counted in a fluorescence microscope, 5 \(\mu\)l of 0.25% (wt/vol) trypan blue in MGS were added to the sample to quench extracellular fluorescence.

Separation of Phagocytes

The isolation of the cells was done at room temperature and could be completed in about 40 min. Only polystyrene or polypropylene tubes and instruments were used throughout the procedure. Immediately after collection, 35 ml of milk were mixed with 15 ml of MGS in 50-ml tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). A volume of 5 ml of a solution of metrizamide (Nyegaard & Co., Oslo, Norway) with a density of 1.100 (11) was injected at the bottom of the tube, followed by 1 ml of a solution with a density of 1.145. The tubes were centrifuged at 1800 \(\times g\) for 20 min, and the bottom 10 ml of the contents were then collected in fractions of 1 to 3 ml (see Figure 2) by aspiration. The collected cells were suspended in 0.9% (wt/vol) NaCl and 0.5% (wt/vol) bovine serum albumin (BSA) to a final volume of 10 ml. After centrifugation for 5 min at 200 \(\times g\), the pellet was resuspended in 100 \(\mu\)l of MGS and 0.5% (wt/vol) BSA.

The isolated cell populations were counted in a Bürker chamber, using cover slips with a thickness of 0.3 mm. Total numbers and differential counts of milk neutrophil preparations were determined after fivefold dilutions of the cells in both Türk's solution and 0.001% (wt/vol) acridine orange (3) in MGS. When acridine orange was used, the cells were suspended to \(<5 \times 10^6/ml\) before dilution with the dye for maximal fluorescence. Smears stained with toluidine blue in 1 \(M\) sodium borate buffer were examined as a reference method for neutrophilic differential counts. In all three procedures, neutrophils were identified on the basis of cell size and the shape of nuclei and, in the acridine orange method, also from their
strongly fluorescent, yellow-green nucleus. Total numbers and differential counts of macrophage preparations were always determined by the acridine orange method. The criteria used for macrophages in this procedure were their larger size than the other cell types in the preparation and their yellow-reddish cytoplasm due to the high content of RNA.

The validity of the macrophage differential counts was confirmed by fixing macrophage preparations (obtained from the top 2.5 ml of the 10 ml collected after gradient centrifugation of cows in the beginning or the end of the lactation period) overnight in 2.5% (wt/vol) glutaraldehyde in .067 M cacodylate buffer, 500 mOsm, pH 7.2, and postfixing them in a 1% (vol/vol) aqueous solution of osmium tetroxide. Centrifuged pellets of these preparations were then routinely flat-embedded in Agar l00® plastic resin (Link Nordiska, Stockholm, Sweden) for transmission electron microscopy, and toluidine blue-stained semi-thin sections were analyzed quantitatively by light microscopy. The criteria used for macrophages in these analyses were a large, lobed, kidney-like nucleus containing both diffuse and condensed chromatin and a cytoplasm more than half the size of the nucleus, usually containing phagocytic vacuoles and long cytoplasmic processes. A qualitative and semi-quantitative confirmation of the composition of the cell preparations was also done by electron microscopy of thin sections cut along the spin axis. In this procedure, the presence of many lysosomes and the scarcity of other cytoplasmic organelles were used as additional criteria for macrophages.

The viability of the cells separated from milk was determined by light microscopy after a fivefold dilution of the cells in .5% (wt/vol) trypan blue in MGS.

Blood neutrophils were separated as described previously (11, 12). May Grünwald-Giemsa staining of smears showed that the preparations contained 95 ± 2.5% (SD) neutrophils with 4 ± 2% contamination of eosinophils.

Phagocytosis and Chemiluminescence

The method described previously for the parallel study of chemiluminescence and phagocytosis (12) was used with some modifications to characterize the function of the purified milk phagocytes. One of these was that the luminometer cuvettes were pretreated with 5% (wt/vol) polyvinyl pyrrolidone (PVP; A.H.T. Co. Philadelphia, PA) overnight at 4°C, followed by an upside down centrifugation at 200 x g for 5 min to prevent adsorption of the cells to the cuvette walls. Moreover, the isolated phagocytes were stored in MGS with .5% (wt/vol) BSA in tubes treated with PVP as described. The assay was performed by addition of 33 μl of the cells to a cuvette, which was then incubated for 15 s at 37°C in a luminometer (Lumac Biocounter R M 2010; Lumac systems AG, Basel, Switzerland), followed by addition 67 μl of a solution, prewarmed to 37°C, containing luminol or lucigenin (5-amino-2,3-dihydro-1,4-phthalazinedione and bis-N-methylacridinium nitrate, respectively; Sigma, St. Louis, MO) and either complement factor C3-opsonized yeast, IgG-opsonized yeast, or unopsonized yeast conjugated with fluorescein isothiocyanate. The resulting chemiluminescence was monitored graphically. After 10 min, 20 μl of a saturated solution of crystal violet in MGS, 50 mM EDTA, or, when lucigenin was the substrate, a 1:1 mixture of a saturated solution of crystal violet and saturated trypan blue in the same buffer was added to the cuvette to quench extracellular fluorescence and to stop phagocytosis. The number of ingested particles per neutrophil or macrophage was then determined by fluorescence microscopy by counting ≥200 cells. The phagocytosis results were corrected for the number of dead cells, obtained by trypan blue staining; both living and dead cells were shown to be counted in the fluorescence microscope. Total chemiluminescence was calculated by integration of the recorded graph.

In experiments designed to evaluate the involvement of xanthine oxidase in the chemiluminescent reaction, the milk cells were preincubated with .1 mM allopurinol for 15 min at 37°C or 10 μg/ml of cytochalasin B for 15 min at 20°C. Alternatively, xanthine or EDTA was added directly to the assay mixture to concentrations of 50 μM and 10 mM, respectively. Stock solutions of allopurinol (50 mM) and cytochalasin B (2 mg/ml) were in dimethylsulfoxide.
TABLE 1. Comparison of different staining techniques for neutrophil differential counts of purified milk neutrophil populations.

<table>
<thead>
<tr>
<th>Staining techniques</th>
<th>Number of comparisons</th>
<th>Mean 2</th>
<th>P&gt;3</th>
<th>Coefficient of correlation 2</th>
<th>P&lt;3</th>
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</thead>
<tbody>
<tr>
<td>Toluidine blue, acridine orange</td>
<td>14</td>
<td>45.47</td>
<td>.05</td>
<td>.96</td>
<td>.001</td>
</tr>
<tr>
<td>Toluidine blue, Turk's</td>
<td>14</td>
<td>45.45</td>
<td>.03</td>
<td>.91</td>
<td>.001</td>
</tr>
<tr>
<td>Acridine orange, Turk's</td>
<td>14</td>
<td>47.45</td>
<td>.05</td>
<td>.96</td>
<td>.001</td>
</tr>
</tbody>
</table>

1Neutrophil populations were separated from residual milk of 14 different cows, giving neutrophil differential counts ranging between 7.9 to 94.9% according to toluidine blue staining.

2The arithmetic means of the differential counts (percentage of the total number of cells) obtained by the two staining techniques compared. The P values refer to two-tailed paired t tests between the results of these two techniques.

3The coefficients of correlation obtained by linear regression between the two staining techniques compared. The P values describe the significance of the correlations.

Statistical Analysis

Statistical analyses were performed with the two-tailed Student’s t test for paired observations. Correlations between cell numbers and differential counts obtained by different staining techniques were calculated. Linear regression of measurements of neutrophil phagocytic function on lactation stage (days postpartum) and somatic cell count in milk was estimated.

RESULTS

Comparison of Different Staining and Cell-Counting Techniques

The reliability of the techniques to stain cells and to determine cell counts and differential counts in whole milk, as well as in purified leukocyte suspensions, was of crucial importance for the evaluation of both the isolation procedure and the functional studies of milk phagocytes. We therefore compared different staining techniques to find the most reliable methods.

For selection of the best method to determine the recovery of milk neutrophils after separation, cell numbers in whole milk were analyzed by the Fossomatic technique, flow cytometry, and fluorescence microscopy after acridine orange staining. All three methods were highly correlated (r = .96 to .97; data not shown). The Fossomatic procedure gave the same results as direct counting in a fluorescence microscope, whereas the flow cytometry technique (10) resulted in significantly higher (−50%) counts than the other two methods. For this reason and because the Fossomatic method is well documented (32), simple, and standardized weekly in collaborative studies, we chose this method for analyses of total cell counts in whole milk in subsequent studies. For calculations of recovery of neutrophils, the Fossomatic procedure was combined with differential counts obtained by flow cytometry.

The percentages of neutrophils in purified cell populations determined by counting in either Turk’s solution or acridine orange were both highly correlated with the number of neutrophils obtained by toluidine blue staining of smears (Table 1), and no significant differences could be detected among the three methods in paired t tests. We chose acridine orange as the differential staining method in most of our experiments because of its slightly higher reliability and because counting of cells in suspension consumed considerably fewer cells.

Macrophage differential counts obtained by acridine orange staining of purified cell populations were confirmed by examination of toluidine blue-stained sections of embedded cell preparations and by electron microscopy. Preparations enriched in macrophages (see Materials and Methods) from six different cows were judged to contain 81.4 ± 10.7, 9.9 ± 7.9, and 8.6 ± 7.9% (SD) of macrophages, lymphocytes, and neutrophils, respectively, by differential counting with acridine orange. A quantitative examination of the same preparations by toluidine blue staining of spin-axis sections of the embedded material similarly gave 74.5 ±

TABLE 2. Recovery, purity, and viability of neutrophil populations isolated from bovine stripping and residual milk from eight cows.

<table>
<thead>
<tr>
<th></th>
<th>Stripping milk</th>
<th>Residual milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>$10^6$ x cells/ml in milk (Fossumatic)</td>
<td>.379</td>
<td>.027-2.149</td>
</tr>
<tr>
<td>Days postpartum</td>
<td>101</td>
<td>8-289</td>
</tr>
<tr>
<td>Neutrophil enrichment</td>
<td>42</td>
<td>11-100</td>
</tr>
<tr>
<td>Percentage recovery</td>
<td>26</td>
<td>16-44</td>
</tr>
<tr>
<td>$10^6$ x Neutrophils recovered</td>
<td>1.92</td>
<td>.122-9.7</td>
</tr>
<tr>
<td>Percentage neutrophils</td>
<td>66</td>
<td>36-82</td>
</tr>
<tr>
<td>Percentage MEC</td>
<td>1.6</td>
<td>0-3</td>
</tr>
<tr>
<td>% Trypan blue exclusion</td>
<td>90</td>
<td>75-98</td>
</tr>
</tbody>
</table>

1The neutrophil to macrophage ratio determined by staining in acridine orange after the isolation procedure, divided by the same ratio determined by flow cytometry before separation.

2Neutrophils recovered after separation in percentage of neutrophils in the milk sample. The latter value was calculated from total counts by the Fossumatic method and differential counts obtained by flow cytometry. Neutrophil counts after separation were determined by acridine orange staining.

3Number of neutrophils obtained after separation of 35 ml of milk.

4Percentage neutrophils and percentage macrophages-epithelial cells (MEC) of whole leukocyte suspensions were determined by counting in a fluorescence microscope after staining with acridine orange.

5Percentage of whole leukocyte population excluding trypan blue.

14.7, 6.3 ± 6.1, and 6.9 ± 5.5% (SD) of macrophages, lymphocytes, and neutrophils, respectively. In addition, 11.5 ± 16.6% (SD) of a population of large round cells (>20 µm in size), classified as desquamated alveolar epithelial cells, was also observed. This cell type had a large, round-oval nucleus and a large number of vesicles of varying sizes in the cytoplasm. The semi-quantitative study by electron microscopy confirmed that >75% of the isolated cells were macrophages and also suggested that alveolar cells were present in the cell preparations. These cells showed all the basic characteristics of milk alveolar cells, i.e., a round-oval nucleus, large vesicles that seemed to contain fat, and smaller vesicles with electron-dense particles similar to the secretory vesicle (casein) of alveolar epithelial cells. Mitochondria, large amounts of rough endoplasmatic reticulum, and conspicuous Golgi complexes were also present in the cytoplasm, intermingled with microfilament bundles. Most cells appeared isolated, and no junctional complexes were seen. Because macrophages and the epithelial cells were not distinguished by the acridine orange method used normally for macrophage differential counting, the cell population containing these two cell types will be denoted as MEC below.

Isolation Procedure

Initial experiments showed that neutrophils in milk were considerably lighter than blood neutrophils (11), necessitating the development of different conditions of the metrizamide gradient used for their isolation (see Materials and Methods and legend to Figure 1). Analyses of the fractions from the centrifuge tube after separation under optimal conditions (Figure 1) showed that a satisfactory yield and purity of neutrophils could be obtained from the bottom 4 ml of the tube, whereas populations consisting predominantly of macrophages could be obtained from the top 2.5 ml of the 10-ml column collected. The low density of the neutrophils resulted in an appreciable contamination of lymphocytes in the neutrophil preparations (Figure 1). However, the use of higher metrizamide densities or greater volumes of metrizamide dramatically decreased the neutrophil yield, whereas a decrease of the density or an increase of the centrifugation time resulted in an unacceptably high macrophage contamination.

Eight samples of stripping and residual milk from different cows in different stages of lactation were separated (Table 2). The purity and yield of the isolated neutrophils varied somewhat with the cellular composition of the start-
Figure 1. Differential counts of leukocytes in different fractions of the metrizamide gradient. A volume of 35 ml of stripping milk, diluted with 15 ml of modified Gey’s solution, was separated on 5 ml of a metrizamide solution with a density of 1.100 on top of 1 ml of a solution with a density of 1.145. The bottom 10 ml of the tube were collected, beginning at the top, in fractions of 2.5, 1.5, 1, 1, 1, and 3 ml. Differential counts were done in Türk’s solution. Filled bars: percentage neutrophils; hatched bars: percentage macrophages-epithelial cells; open bars: percentage lymphocytes. Values are given as means and standard deviations of four separations from different cows.

Phagocytic Studies

In the assay procedure developed for blood neutrophils (12), in which phagocytosis and chemiluminescence by cells in suspension are analyzed in parallel, only about 60% of the milk cells were recovered for microscopic analysis of the number of phagocytized particles after a 10-min experiment in spite of the addition of .5% (wt/vol) BSA to the suspensions. This low recovery, which could have biased the results of the phagocytic studies, was increased to 95% by pretreatment of plastic tubes and cuvettes with PVP. To evaluate the effect of PVP treatment combined with the storage of neutrophils in .5% (wt/vol) BSA, we compared the phagocytic function of blood neutrophils stored under these conditions with the same cells stored and assayed in untreated tubes with albumin added in the assay only. Phagocytosis was essentially unaffected, as was luminol-dependent chemiluminescence, whereas lucigenin-dependent chemiluminescence was significantly decreased by -40% when PVP-treated tubes were used.

Suspensions of milk neutrophils showed an appreciable, unstimulated chemiluminescence, amounting to 39 and 28% of that toward unopsonized yeast with luminol and lucigenin, respectively (Figure 2). For optimal reproducibility, the luminogenic substrate and yeast parti-
we found no stimulating effect on either luminol- or lucigenin-dependent chemiluminescence by xanthine (Figure 2). Moreover, the lucigenin-enhanced chemiluminescence was totally abolished by EDTA, which has been reported to have no inhibitory effect on xanthine oxidase but to inhibit the neutrophil NADPH oxidase (5, 29). An analogous inhibition of the luminol-dependent chemiluminescence by EDTA was also observed, which, however, could be explained by the chelation of Fe^{2+} in the heme group of myeloperoxidase. Further analyses showed that pretreatment of the cells with cytochalasin B, an inhibitor of actin polymerization (33), greatly decreased the chemiluminescence, whereas allopurinol, a known inhibitor of xanthine oxidase (26), had no effect (Figure 2).

A Comparison of Phagocytic Function of Neutrophils and MEC from Milk

Neutrophil function was compared with the function of MEC isolated from the same milk samples from seven different cows (Figure 3). The differential counts of neutrophil and macrophage populations were 58 ± 20% (SD) and 7 ± 4% neutrophils and 70 ± 18% MEC, respectively. The MEC apparently had no or very low luminol-dependent chemiluminescence, because the low activity measured could be completely accounted for by the neutrophils contaminating the cell suspensions. In contrast, the MEC produced a measurable activity in the lucigenin-dependent system that could not be explained by the small neutrophil contamination. However, the activity was significantly lower, only about 30% (P < .05) of that evoked by the milk neutrophils. Neutrophils phagocytized about twice as many C3-opsonized yeast particles per cell as the MEC (P < .05), whereas no significant differences in phagocytosis of IgG- and unopsonized yeast was observed between the two cell types. Both phagocytic and chemiluminescent activity was unaffected by the mixing of neutrophils and MEC (data not shown).

Applications of the Technique to Milk Neutrophils

Phagocytic function with C3-opsonized yeast and luminol as chemiluminescent substrate was studied in neutrophils isolated from
stripping and residual milk from eight different cows in different stages of lactation (Table 3). A high variation in neutrophil activity among individuals was apparent in these studies. Correlations of activity with days postpartum and somatic cell count in the milk samples were investigated as possible explanations of this variability. The chemiluminescence of neutrophils isolated from residual milk was positively correlated with the number of days postpartum (an increase of ~6%/d of the extrapolated value at parturition, P < .001; Figure 4). Higher activity in both phagocytosis and chemiluminescence of neutrophils from residual milk compared with the activity of the cells from stripping milk (~74% and ~90%, respectively), although not significant, was also observed (Table 3).

DISCUSSION AND CONCLUSION

To date, no sufficiently simple method has been developed to characterize the phagocytic function of neutrophils and macrophages from milk with normally low cell counts. Thus, only a few studies have been done of phagocytosis by such cells isolated from nonmastitic milk (3, 18, 19). To our knowledge, this is the first study in which the two phagocytic cell types from unmanipulated lactating udders have been isolated and their respiratory burst activity analyzed.

The two main phagocytic cells present in milk were separated on the basis of their different densities by gradient centrifugation. However, during migration to milk, the neutrophils lose weight, and they were thus collected at an appreciably lower density than blood neutrophils (11), making the separation from lymphocytes difficult. It seems unlikely that the presence of lymphocytes in the neutrophil preparations would have affected the results of the phagocytic studies, because the addition of blood lymphocytes does not influence the phagocytic activities of blood neutrophils (12). The reason for the low density of milk neutrophils could be the ingestion of fat (16, 21). An alternative explanation is that the neutrophils may have released a considerable amount of granule components when migrating into milk (25), making them lighter.

In a comparison of methods for the examination of total cell counts in milk, analysis by flow cytometry gave significantly higher results than the other two methods tested. This was an unexpected finding, because this method has been suggested to stain vital cells only (11). Possibly the higher values were due to the population of degenerated neutrophils in normal milk recently reported by others in studies with flow cytometry (28). Although the Fossomatic procedure was chosen as the best method for analysis of total cell counts in calculations of the recovery of neutrophils, this
method had to be combined with differential counting by flow cytometry, which was the only procedure available to us for such counting of cells in whole milk. The estimated neutrophil recovery of about 30%, therefore, should be interpreted with some caution, because it would have been affected by the accuracy of these differential counts. In methods presented earlier for the isolation of unseparated bovine milk leukocytes, the recovery has been 33 to 58% (23, 31). Thus, a considerable proportion of the neutrophils is not recovered in all isolation procedures developed so far. One possible explanation for the loss of cells could be that some cells are associated with milk fat (21, 30).

We found that the majority of cells in the MEC preparations were macrophages. In addition, an appreciable number of the cells were classified as desquamated alveolar epithelial cells, the typical morphology of such cells being confirmed by transmission electron microscopy of sections cut through the spin-axis of the fixed pellets. Alveolar epithelial cells have been reported to be present in milk from other species (17), although there is a disagreement concerning the number of these cells in bovine milk (16, 24). A previous study by electron microscopy, in which no such cells were observed, was done with centrifuged samples (16), which were cut transversally. This procedure may have introduced a severe bias in the results, because the cells investigated may not have been representative of the entire population because of layering of the cells during centrifugation. In our study, the sections were obtained solely along the centrifugation axis of the pellet and thus represent the entire cell population. It is also possible that differences in the procedures for milk collection may have influenced the number of alveolar cells present in the milk (16, 17, 24). In our work, the residual milk was collected for electron microscopy after administration of oxytocin. The contraction of the udder tissue caused by this drug may have increased the shedding of alveolar cells into the milk.

The appreciable unstimulated luminol-dependent chemiluminescent activity reported earlier for neutrophils isolated from milk (7) was confirmed and further studied in this work. A certain unstimulated lucigenin-dependent activity was also observed. The unstimulated activity could not be increased by addition of xanthine, nor was it abolished by allopurinol. However, the lucigenin-dependent chemiluminescence was abolished by the addition of EDTA, known not to inhibit xanthine oxidase. Moreover, both luminol- and lucigenin-dependent chemiluminescence was inhibited by cytochalasin B, an inhibitor of actin polymerization (32) in a slightly higher concentration than that used by others (7). Based on these results, we propose that the basal oxygen radical formation of unstimulated cells, as revealed by luminol- and lucigenin-dependent chemiluminescence, is not due to xanthine oxidase but to the NADPH oxidase present in the neutrophil membrane (5).

The absence of a luminol-enhanced chemiluminescence indicates that MEC isolated by our method contain no myeloperoxidase (8), in accordance with the findings reported by others (16). However, this absence is in contradiction with earlier results of studies of macrophages isolated from the udder in the dry period (13, 16). One explanation for these discrepancies could be that the macrophage populations studied in the latter investigations were not free of neutrophils (14, 20). The milk MEC were highly active phagocytes, a finding that may be of considerable pathophysiological importance in mastitis, especially in infections caused by Staphylococcus aureus and Escherichia coli. The invading bacteria thus may be effectively ingested by MEC but may survive inside this cell type because of the absence of a myeloperoxidase-mediated killing mechanism (4). Therefore, phagocytosis of S. aureus by macrophages may actually protect the microorganism from being killed by neutrophilic granulocytes.

Neutrophils isolated from milk of different cows by our procedure and studied in a combined assay for phagocytosis and chemiluminescence exhibited highly variable activities, an observation in accordance with findings reported earlier (22, 34). In the case of chemiluminescence by cells isolated from residual milk, the variation was largely explained by an increased activity as a function of the number of days postpartum, a correlation that was highly significant, although the study was done with only eight animals. The low chemiluminescent activity of the milk neutrophils isolated from cows at an early stage of lacta-
tion indicates that oxygen-dependent killing mechanisms are severely impaired in early lactation and could be related to the well-documented higher incidence of mastitis in the beginning of the lactation (6, 27). An increased intracellular survival of *S. aureus* in bovine neutrophils thus has been associated with a reduced chemiluminescence (35). In cases of a defect of neutrophil oxygen-dependent killing activity in humans, it is also well documented that *S. aureus* and *E. coli*, the two most commonly isolated bacteria in acute bovine udder infections (9), are common pathogens (15). The lower phagocytic activities of neutrophils from stripping milk than of the same cell type from residual milk, although not significant, are consistent with neutrophils in residual milk representing a population that have left the udder tissue more recently than the same population in stripping milk, their activity having declined to a larger extent by the influence of milk components (21).

In summary, the method presented provides the possibility to study the phagocytic function of different cell populations in milk with low cell counts. The finding that neutrophil chemiluminescent activity is low in early stages of lactation and increases during the lactation period is highly interesting and deserves a further, detailed study.

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