ABSTRACT

A comparative evaluation of in vitro phagocytic and metabolic activities of freshly isolated bovine blood neutrophils from 26 cows was performed. Degrees of phagocytosis and nitroblue tetrazolium reduction by neutrophils were measured using zymosan, Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma bovis, Salmonella sp., and Brucella abortus opsonized with fresh bovine serum. Percent phagocytosis and nitroblue tetrazolium reduction by the neutrophils were determined by microscopic evaluation of stained coverslip smears. Individual variation among cows was found in parameters measured, but age was not a contributing factor. Phagocytosis was high (73 to 81%) for E. coli, Salmonella sp., Strept. agalactiae, and zymosan; intermediate (64%) for Staph. aureus; and low (24 to 40%) for M. bovis and B. abortus. Nitroblue tetrazolium reduction was distinctly lower than zymosan (74%) for all microorganisms. Similar percentages (32 to 41%) of nitroblue tetrazolium reduction were observed for E. coli, Staph. aureus, Strept. agalactiae, and M. bovis; intermediate (28%) was obtained for Salmonella sp.; and lowest (11%) for B. abortus.

INTRODUCTION

The neutrophil leukocyte is the most active phagocyte in the body. A large number of circulating neutrophils enter the bovine mammary gland during inflammation and provide resistance against most mammary pathogens (11, 14, 15, 16). Cows vary in their ability to resist mammary infection as a result of microbial virulence (10), poor phagocytic ability of neutrophils in milk (12, 26, 28), individual variations between cows and between mammary quarters (9, 20), stage of lactation (11, 22), and physiologically stressful situations such as parturition (12).

In vitro studies have shown that bovine neutrophils from blood and milk can phagocytize and kill mammary pathogens such as Staphylococcus aureus (20, 21, 23, 30), Escherichia coli (11, 30), Klebsiella aerogenes (13, 17), and Streptococcus agalactiae (19, 27). However, simultaneous comparative evaluations with regard to phagocytosis and metabolic activity of bovine neutrophils for most of these organisms have not been performed. Such studies would provide information about relative competence of neutrophils to phagocytize and kill different mammary pathogens and in turn provide some insight about pathogenesis of mastitis caused by these microorganisms.

The main objective in the present study was to compare phagocytic activity and metabolic stimulation [nitroblue tetrazolium reduction (NBT)] of bovine blood neutrophils after in vitro interaction with zymosan and four common mammary pathogens: Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, and Mycoplasma bovis. Brucella abortus, a facultative intracellular parasite, survives intracellular killing by inhibiting the activity of the hydrogen peroxide-myeloperoxidase-halide microbicidal system and degranulation of primary granules in bovine neutrophils (4). Salmonella sp. inhibited oxygen consumption and oxidative metabolic burst of human neutrophils (6). Therefore, these two microorganisms were used also in this comparative evaluation.
of bovine neutrophil functions. Blood rather than milk neutrophils were used primarily because milk neutrophils are derived from blood, and milk constituents, such as milk fat or casein, may adversely affect the functional properties of neutrophils (24, 26, 28). Thus, comparative phagocytic and NBT reductive properties of bovine neutrophils for zymosan and six species of microorganisms are described in the present study.

MATERIALS AND METHODS

About 100 ml of blood were collected from each of 26 Holstein-Friesian cows by jugular venepuncture into 10 to 12 (10-ml capacity) EDTA vacutainers. Blood was collected also into two 10-ml vacutainers without anticoagulant to obtain fresh serum. Total and differential leukocyte counts were within the normal range of these parameters for the cow (12).

Isolation of Neutrophils from Bovine Blood

The neutrophil isolation procedure was that of Carlson and Kaneko (5). Briefly, blood was centrifuged at 2000 × g for 15 min. Plasma, the buffy coat, and the top 1/4 of the red cell layer were discarded. The remaining packed red cell mass was pipetted into 30 ml of sterile distilled H2O in 50-ml centrifuge tubes and mixed for about 40 s to lyse red cells. Then 10 ml of sterile phosphate buffered (.0132 M, pH 6.8) 2.7% NaC1 solution were added to the cell suspension to restore isotonicity. Tubes were mixed gently and centrifuged at 200 × g for 10 min. The supernatant was discarded and the pellet was resuspended in 35 ml of sterile .8% NaC1 in .0132 M phosphate buffer (PBS) pH 6.8 and recentrifuged. Washing was repeated once more and the pellet, which consisted primarily of neutrophils, was resuspended in 5 ml of phosphate buffered (PBS) pH 6.8 and recentrifuged. Washing was repeated once more and the pellet, which consisted primarily of neutrophils, was resuspended in 5 ml of sterile buffered saline solution (.8% NaCl in .0132 M phosphate buffer (PBS) pH 6.8 and recentrifuged. Washing was repeated once more and the pellet, which consisted primarily of neutrophils, was resuspended in 5 ml of sterile buffered saline solution (.8% NaCl in .0132 M phosphate buffer, pH 6.8, BSS). Percent transmission was measured at 540 nm in a Bausch & Lomb 20 spectrophotometer. Serial dilutions from 10-1 to 10-5 were prepared by transferring initially 1 ml of washed culture suspension to 9 ml of BSS and then each dilution was plated on duplicate bovine blood agar plates to obtain colony counts. This procedure was repeated to obtain a specific percent transmission of the initial washed culture suspension to yield 1 × 10^6 microorganisms/µl. Percent transmissions for E. coli, Staph. aureus, and Salmonella sp. were 5, 7.5, and 9.6%, respectively. For
PHAGOCYTOSIS BY BOVINE NEUTROPHILS

Strep. agalactiae, one isolated bacterial colony was suspended initially in 1 ml of BHI and incubated at 37°C for 18 h. Washed bacterial suspension of this culture in 200 µl of BSS yielded bacterial counts of $1 \times 10^6$/µl. These bacterial concentrations were used in all experiments to have neutrophil:bacteria of approximately 1:50.

For Mycoplasma bovis, a large colony from a 5 to 7-d culture grown on selective agar at 37°C in 10% CO₂ (18) was cut and placed in selective broth and incubated similarly for 5 d. The culture suspension was centrifuged at 30,000 x g for 45 min at 4°C and resuspended in 100 µl of BSS. This preparation yielded an average of $2.6 \times 10^8$ cfu.

For Brucella abortus, fresh cultures were obtained by growing organisms on tryptose agar slants at 37°C for 24 h. Bacterial growth was washed off with sterile BSS, centrifuged at 7000 x g for 15 min, resuspended in BSS, and diluted to obtain $1 \times 10^6$ organisms/µl by adjusting the percent transmission at 610 nm. All experiments with B. abortus were carried out with optimum precautions.

Opsonization procedure

One hundred microliters each of zymosan and the six bacterial suspensions were aliquoted into 7.5 cm x 1-cm glass tubes. Then 100 µl of fresh bovine serum were added to each tube. Tubes were incubated in a shaking water bath at 37°C for 30 min for opsonization.

Preparation of Nitroblue Tetrazolium Solution

Twenty milligrams of NBT (#0298 Polysciences, Inc.) were mixed in 10 ml of PBS, sonicated for 1 min and filtered through #40 filter paper. This solution containing .2% NBT (29) was kept in the dark at 4°C and used within 1 wk of preparation.

Phagocytosis and Nitroblue Tetrazolium Reduction

Two hundred microliters of NBT solution were added to each tube containing 200 µl of zymosan (positive control) or various microorganisms opsonized with fresh bovine serum. Then 400 µl of neutrophil suspension were added to each tube. A negative control (to assess the possibility of spontaneous NBT reduction by neutrophils) consisted of 200 µl of NBT solution, 100 µl each of PBS and serum, and 400 µl of neutrophil suspension. All tubes were incubated at 37°C in a shaking water bath for 15 min. Then tubes were centrifuged at 60 x g for 5 min at room temperature to concentrate neutrophils. The supernatant was discarded and coverslip smears were made from the pellet for microscopic evaluation.

Staining Procedures

Thin, air-dried coverslip smears of leukocytes interacted with zymosan, E. coli, Staph. aureus, Strep. agalactiae, M. bovis and Salmonella sp. were fixed in buffered methanol for 60 s. Excess fixative was drained and the coverslips were dipped in Harleco Diff Quik solution 1 (Contains 1 g xanthene dye/L, #64851, Dade Diagnostics Inc.) for 60 s. Smears were dipped in distilled H₂O once and counter stained with 2% methyl green (C. I. 42590, Sigma) for 45 to 60 s (29). Coverslips were rinsed quickly in distilled H₂O and air dried immediately. For M. bovis, a second coverslip smear was fixed in buffered methanol and stained with Giemsa stain (G4507, Sigma) for 45 min (18).

Coverslip smears for B. abortus were fixed in buffered methanol and stained by modified Ziehl-Neelsen method (1). Briefly, fixed coverslip smears were dipped in 1:10 solution of carbol fuchsin (C. I. 42500, J. T. Baker Chemical Co.) for 10 min and washed in tap H₂O. Then coverslips were dipped in .5% acetic acid for 30 s, rinsed in tape H₂O, and counter stained with 2% methyl green.

All stained coverslip smears were mounted with "Protexx" synthetic mounting medium (M7635, American Scientific Products) on glass slides and examined under an oil immersion objective.

Microscopic Examination

A good differential staining was achieved using a combination of Diff Quik and methyl green. Zymosan particles stained pink, various microorganisms acquired different degrees of blue, reduced NBT (formazan) appeared as a blue-black precipitate, and neutrophil nuclei stained light green. Mycoplasma bovis in Giemsa-stained smears could be recognized easily as purplish pleomorphic organisms at

Journal of Dairy Science Vol. 71, No. 6, 1988
intracellular and extracellular locations. *Brucella abortus* stained red with carbol fuchsin.

The percentage of phagocytically active neutrophils (cells with ingested zymosan or microorganisms) and the percentage of neutrophil neutrophils showing NBT reduction (cells with blue-black formazan) were determined by counting 500 neutrophils under oil immersion objective (x 1250). These values were determined from Diff Quik-methyl green stained smears for zymosan, *E. coli*, *Staph. aureus*, *Strep. agalactiae* and *Salmonella sp.*, and carbol fuchsin-methyl green stained smears of *B. abortus*. Percent phagocytosis for *M. bovis* was determined using Giemsa-stained smears, while NBT reduction was determined using smears stained with Diff Quik and counter stained with methyl green. Statistical analyses were performed by parametric methods using the "Number Cruncher" computer program (Number Cruncher, Interactive statistical analysis system, 1983, version 3.0. copyright by Jerry L. Hinitze, 865 East 400 North, Kaysville, UT 84037).

**RESULTS**

Data for phagocytosis and NBT reduction observed in various experiments are presented in Table 1. Percent phagocytosis represents number of neutrophils engaged in active phagocytosis. Because NBT reduction in the form of blue-black formazan occurred primarily in phagocytically active cells, the number of neutrophils showing NBT reduction was expressed as percentage of all neutrophils exhibiting phagocytosis. On average, 3% (0 to 10) neutrophils in the negative controls showed tiny specks of formazan in the absence of bacterial or zymosan phagocytosis.

### Comparative Phagocytosis and Nitroblue Tetrazolium Reduction

Percent phagocytosis and NBT reduction for various microorganisms were compared against similar values obtained for zymosan, which was used as a positive control in all experiments. Phagocytosis was highest for *E. coli* and *Salmonella sp.* followed by *Strep. agalactiae*, zymosan, *Staph. aureus*, *B. abortus*,

<table>
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<th>Zymosan/organism</th>
<th>Phagocytosis (%)</th>
<th>NBT reduction (%)</th>
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<tr>
<td>Zymosan</td>
<td>72.77 ± 19.3</td>
<td>74.33 ± 15.9</td>
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<tr>
<td>Escherichia coli</td>
<td>81.27 ± 11.4</td>
<td>39.90 ± 21.1</td>
</tr>
<tr>
<td>Salmonella sp.</td>
<td>80.61 ± 11.2</td>
<td>27.86 ± 13.6</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>76.96 ± 21.4</td>
<td>31.70 ± 10.7</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>63.62 ± 18.0</td>
<td>33.85 ± 19.5</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>40.44 ± 10.7</td>
<td>11.36 ± 07.6</td>
</tr>
<tr>
<td>Mycoplasma bovis</td>
<td>23.63 ± 12.7</td>
<td>40.99 ± 21.8</td>
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<tbody>
<tr>
<td>Zymosan</td>
<td></td>
<td></td>
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<tr>
<td>Escherichia coli</td>
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<tr>
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<tr>
<td>Streptococcus agalactiae</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>.0001*</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma bovis</td>
<td>.0001*</td>
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</table>

1 Mean ± SD and the range in parenthesis. The range represents extremes of variations among cows.

2 *P* = Probability when compared with zymosan controls.

*Significantly different from zymosan controls (*P*<.05).

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Journal of Dairy Science Vol. 71, No. 6, 1988
and M. bovis (Table 1). Nitroblue tetrazolium reduction was highest with zymosan followed by M. bovis, E. coli, Staph. aureus, Strep. agalactiae, Salmonella sp., and B. abortus. Phagocytosis was significantly lower for B. abortus and M. bovis, and NBT reduction was significantly lower for all six microorganisms than for zymosan.

Analysis of variance between means of percent phagocytosis for various microorganisms revealed that phagocytosis for E. coli, Strep. agalactiae, and Salmonella sp. was not significantly different. Phagocytosis for Staph. aureus, M. bovis, and B. abortus differed significantly. Similarly, analysis of variance between means of percent NBT reduction for various microorganisms showed that NBT reduction with B. abortus was significantly lower than that for other microorganisms. The percent NBT reduction for Salmonella sp. was significantly lower than that for E. coli and M. bovis. No significant difference was seen for percent NBT reduction among E. coli, Staph. aureus, Strep. agalactiae, and M. bovis.

Influence of Lactation Age

Data from various experiments were analyzed to determine the influence of lactation age of cows on variations in phagocytic and NBT reductive properties of neutrophils. Cows were arranged into four lactation groups—heifers, first, second, and third plus fourth lactations and results were compared in relation to those for heifers. No differences (P>.05) were observed in percent phagocytosis for various microorganisms. The degree of NBT reduction also did not show any significant differences except for a single occurrence of a higher NBT reduction with Staph. aureus in the third plus fourth lactation group.

DISCUSSION

Phagocytosis and bactericidal activities of neutrophils are important components of cellular immunity to microbial infection. Phagocytosis is promoted by opsonins, particularly specific antibodies and certain complement components (12). Phagocytic and microbicidal activities of neutrophils may differ for various microorganisms. Phagocytic events stimulate metabolic processes in the neutrophils leading to activation of oxygen-dependent and oxygen-independent microbicidal mechanisms. Microbial resistance to phagocytosis and intracellular killing by neutrophils are dependent on a variety of bacterial properties such as capsule, cell wall composition, toxins, and metabolic inhibitors (10).

The degree of NBT reduction depends primarily upon the generation of electron donors such as superoxide anions in the phagolysosome (25). Failure to reduce NBT indicates a block in the oxidative metabolic processes and inability of the phagocyte to inflict oxidative damage to the ingested microorganisms. Hence, NBT reduction may be considered an indirect measure of the oxygen-dependent microbicidal activities of neutrophils (8, 12).

In vivo and in vitro studies have shown that neutrophils are important in protecting cows from mammary pathogens (2, 3, 14, 15, 16, 23). Important mammary pathogens isolated from mastitic milk include E. coli, Staph. aureus, Strep. agalactiae, and M. bovis. In vitro phagocytic studies with bovine blood neutrophils have been reported for E. coli, Staph. aureus (30), and Strep. agalactiae (19). These studies evaluated neutrophil killing ability by measuring the percent survival of bacteria after interaction with neutrophils, but the extent of phagocytosis was not determined.

In the present study, a comparative evaluation of the phagocytic and oxidative bactericidal properties of bovine blood neutrophils for six microorganisms was made. Observations reported herein revealed that bovine blood neutrophils vary in their phagocytic ability for E. coli, Staph. aureus, Strep. agalactiae, and M. bovis, but their degree of NBT reduction for these microorganisms is not significantly different. Although phagocytic activities for E. coli, Staph. aureus, and Strep. agalactiae were similar to zymosan, NBT reduction was nearly half of that for zymosan. With regard to M. bovis, phagocytosis was lowest (about one-third of that for zymosan) but NBT reduction was similar to that for the above three microorganisms. These results indicate that in bovine neutrophils the respiratory burst does not occur to its fullest extent following phagocytosis of viable E. coli, Staph. aureus, Strep. agalactiae, and M. bovis. Consequently, oxygen-dependent microbicidal activities of bovine neutrophils may not be fully
operative for these mammary pathogens. Mechanisms responsible for reduced metabolic stimulation of bovine neutrophils following phagocytosis remain to be elucidated. It is possible that factors such as bacterial toxins may inhibit some aspects of metabolic stimulation of phagocytically active neutrophils (10). In the case of M. bovis infections, lower host resistance may also depend on the poor phagocytic activity of bovine neutrophils for this microorganism.

Nitroblue tetrazolium reduction did not vary between catalase-positive (Staph. aureus) and catalase-negative bacteria (Strep. agalactiae), which indicates that bacterial catalase may not inhibit the formation of oxygen moieties inside the phagolysosomes. Previous studies with phagocytic cells have shown that gram-positive bacteria are destroyed more readily than gram negative bacteria (6, 7). Observations in the present study showed no significant differences in the degree of NBT reduction between gram-positive (Staph. aureus and Strep. agalactiae) and gram-negative (E. coli and Salmonella sp.) bacteria. Therefore, it is possible that oxygen-independent mechanisms (such as cationic proteins) rather than oxygen-dependent mechanisms of neutrophils may be more important in selective killing of these bacteria (12).

It has been reported that Salmonella sp. inhibit oxygen consumption of human neutrophil (6), and virulent strains of B. abortus inhibit the hydrogen peroxide-myeloperoxidase-halide microbicidal system of bovine neutrophils (4). Thus, these bacteria are not efficiently killed following phagocytosis by neutrophils. In the present study, phagocytosis of Salmonella sp. was similar to E. coli but NBT reduction was lower than that for E. coli and M. bovis (Table 1). In contrast to a study that used a different strain of B. abortus (4), phagocytosis of B. abortus strain 19 was markedly lower and NBT reduction was the lowest of all microorganisms tested. Brucella abortus not only underwent limited phagocytosis but inhibited intracellular generation of oxygen moieties as evidenced by minimal NBT reduction. These observations reemphasize that reduced phagocytosis and metabolic stimulation of neutrophils may compromise host resistance to microbial infection.

Present observations showed also that although individual variations occur in the phagocytic ability and NBT reduction by bovine blood neutrophils, lactation age of the cows for the most part does not appear to contribute to this variation. Similar observations were made in previous studies (22, 29).

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