Interaction Between the Respiratory Burst Activity of Neutrophil Leukocytes and Experimentally Induced *Escherichia coli* Mastitis in Cows

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ABSTRACT

The respiratory burst activity of neutrophil leukocytes from bovine peripheral blood was studied before and during an experimentally induced *Escherichia coli* mastitis. The competence of neutrophils to generate reactive oxygen species following stimulation with opsonized particles prior to infection was negatively correlated with severity of subsequently induced *E. coli* mastitis. In the presence of the soluble activator, phorbol myristate acetate, no such correlation was obtained. However, combination of blood neutrophil numbers with phorbol myristate acetate induced respiratory burst competence, called reactive oxygen species-generating capacity, displayed a negative correlation with the intensity of a subsequent inflammation of the bovine mammary gland.

At the onset of mastitis, a concomitant reduction in blood neutrophil numbers, a strong shift in cell types, and a substantial decrease in production of reactive oxygen species occurred. Reestablishment and even enhancement of the respiratory burst activity coincided with the reappearance of mature neutrophils. Possible stimulatory effects on neutrophil superoxide generation are discussed. Data suggest that generation of reactive oxygen species by mature neutrophils may be of primary importance for microbial killing during the onset and recovery from mastitis.

(**Key words**: neutrophil leukocytes, respiratory burst, *Escherichia coli* mastitis)

INTRODUCTION

One of the most striking features to occur in acute bovine mastitis is the massive emigration of leukocytes from the circulating and the marginal pool toward the mammary gland. Although several antimicrobial systems exist in the bovine udder (17), it is generally accepted that the presence of neutrophil leukocytes in milk constitutes the most important natural defense mechanism.

Ingestion of bacteria by phagocytic cells triggers various bactericidal mechanisms including a burst of oxidative metabolism, which involves a marked increase in cyanide-insensitive oxygen consumption with generation of superoxide, hydrogen peroxide, and hydroxyl radicals (3). Moreover, these reactive oxygen species (ROS) constitute key components of the antimicrobial armamentarium of the phagocyte. Despite intensive investigation into the process of inflammation of the udder, data on the possible role of oxygen-dependent killing of invading pathogens by phagocytes during mastitis are scarce.

Under both clinical and experimental conditions, mastitic cows show a great variability in illness and a wide range of pathological responses (7). Preliminary studies in our laboratory on cows have demonstrated considerable variations in blood neutrophil numbers and neutrophil competence to produce ROS.

The present study was undertaken to investigate: 1) the relationship between respiratory burst competence of blood neutrophils from individual cows before infection and severity of inflammation following inoculation of *Escherichia coli* in the udder; and 2) the ability of cows experimentally infected with *E. coli* in the udder to generate oxygen radicals by neutrophils during several days.

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MATERIALS AND METHODS

Animals

Fourteen health second lactation cows of the East Flemish Red Pied breed between 3 and 5 wk after calving and ranging in weight from 520 to 670 kg were used. Animals were kept under controlled temperature (18 ± 1°C) in a stable with individual tie-stall bars. They had free access to hay and water and were fed 8 kg of concentrates each day. Cows were machine milked at 0830 and 2200 h. Their daily milk yield averaged 20 kg at the start of the experiment. Before experimentation, each udder was examined clinically, and quarter foremilk samples of all cows were taken for bacteriological examination and electronic SCC. All animals were free of udder infection and SCC and milk electrolyte concentration of individual quarters were acceptable for their stage of lactation. Blood was collected into acid citrate dextrose from a chronically implanted catheter (PE 90 Dubernard, Bordeaux, France) in the external jugular vein.

Bacteria

Escherichia coli P4:032 isolated from a clinical case of mastitis was used. This strain has been used by several researchers to induce mastitis in cows. The strain was held in stock on nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, Engl.) at 4°C. Cultures were frequently observed for viability and purity. Before experimentation, the bacteria were subcultured overnight in brain-heart infusion (Oxoid CM 225) during 3 successive d at 37°C. After three washings, the suspension was diluted in pyrogen-free saline to the desired concentration. Then the right quarters of the udder were inoculated aseptically by way of the teat canal with 10^4 cfu of the *E. coli* suspension. Bacterial counting was performed using the plate count method.

Each experiment was performed in the tie stall of the animal. After a preinoculation control period of 1 wk, animals were infused intramammarily with the *E. coli* suspension after the morning milking in a volume of 20 ml of pyrogen-free saline. the glands were massaged to disperse the bacteria as much as possible. Then, 24 h later, the cows received a systemic and local antibiotic treatment: 1) after the morning milking polymyxin B (500,000 IU, Pomesul®, Bayer, Brussels) was injected into inflamed quarters; and 2) trimethoprim (1 g) plus sulfadoxin (5 g) was injected twice a day i.v. (Duoprim®, Coopers Agrovet, Aalst, Belgium). the antibiotic treatment was continued for 7 d.

Trial 1

Blood was collected for isolation of granulocytes in the healthy cows before *E. coli* was inoculated intramammarily. Respiratory burst competence of these blood neutrophils was measured and compared with severity of subsequently induced *E. coli* mastitis. The amounts of milk and α-lactalbumin production by secretory tissue over 3 d were taken as parameters for degree of inflammation, thus reflecting the functional loss of the mammary gland during illness.

Trial 2

Effect of acute *E. coli* mastitis on the respiratory burst activity of blood neutrophils was followed for several days after infection. A quantitative and differential analysis of blood neutrophils from 14 cows was performed daily.

Isolation of Blood Neutrophils

Before and after intramammary inoculation of *E. coli*, blood samples were taken daily for preparation of neutrophils. Polymorphonuclear leukocytes (PMN) were prepared as described (15). Briefly, granulocytes were isolated from blood by differential centrifugation followed by hypotonic lysis of the erythrocytes. Separation of eosinophils and neutrophils was achieved by passing the granulocyte suspension through a preformed continuous Percoll gradient (average density 1.103). This procedure caused the formation of two clearly visible cell bands. The individual cell populations were removed, washed, and resuspended to a concentration of 2.10^7 cells/ml in Hanks solution. Only samples containing more than 95% neutrophils and 80% eosinophils, respectively, were used for further experiments. Cell viability, as assayed by trypan blue exclusion, always exceeded 95%.

TABLE 1. Generation of superoxide anions[1] by isolated bovine blood polymorphonuclear (PMN) leukocytes, neutrophils, and eosinophils of normal periparturient cows under basal conditions and after activation with phorbol myristate acetate (PMA, 100 ng/ml) and opsonized zymosan (1 mg/ml).

Stimulus	Superoxide production[2]								
	Unseparated PMN leukocytes			Neutrophils			Eosinophils		
	$\bar{x}$	SEM	(n)[3]	$\bar{x}$	SEM	(n)	$\bar{x}$	SEM	(n)
Basal production	.2	.1	4	.1	.1	4	.5	.3	4
PMA	4.9	2.2	10	2.9	.9	14	19.1	6.6	14
Zymosan	1.8	.7	10	1.4	.3	14	4.3	1.1	14

[1]Samples assayed in triplicate.

[2]Expressed as nanomoles O_2^- /min per 10^6 cells.

[3]Number of experiments.

Respiratory Burst Competence of Blood Neutrophils

Respiratory burst activity was quantified after stimulation of the cells with phorbol myristate acetate (PMA, 100 ng/ml) and opsonized zymosan (1 mg/ml). Release of hydrogen peroxide from cells was monitored fluorometrically by means of peroxidase-catalyzed oxidation of scopoletin (19). Superoxide anion production was quantified continuously using PMA as activator and discontinuously in the presence of zymosan by following the superoxide dismutase inhibitable reduction of cytochrome c spectrophotometrically (2).

Clinical Signs and Milk Production

Clinical measurements and observations were carried out as described earlier (6) such as: rectal temperature and heart rate, pain and swelling of the mammary glands, and electrolytes in milk of the inflamed and uninflamed glands. Quarter milk production was measured daily before and after challenge with *E. coli* by means of a quarter milking device.

Analytical Methods

Electrolytes and lactose in milk of inflamed and uninflamed glands were determined. Milk chloride was measured using a chloridemeter (Corning EEL 920, Sunderland, England) and sodium and potassium were analyzed by flame photometry (IL 243, Instrumentation Laboratory Benelux, The Netherlands). Milk samples were analyzed for α-lactalbumin by the double-antibody radioimmunoassay procedure (1). Somatic cell counts were estimated with a Coulter Counter (Coulter Counter ZF, Coulter Electronics Ltd, Luton, England). Lactose and serum albumin were determined.

Statistical Analysis

Means, standard deviations, standard errors, Student's *t*-test, regressions, and correlations were computed according to Snedecor and Cochran (23).

RESULTS

Data showing generation of superoxide anions by PMN leukocytes from healthy cows are shown in Table 1. Under basal conditions PMN leukocytes, neutrophils and eosinophils produced .2, .1, and .5 nmoles O_2^- /min per 10^6 cells, respectively. The normal ROS eosinophil: neutrophil ratio was about 5. After stimulation with PMA and zymosan, this ratio was 6.6 and 3.1.

Trial 1

Table 2 shows neutrophil numbers in blood as well as PMA and zymosan-induced ROS generation by neutrophils before infection and their relation to alterations in milk and α-lactalbumin production after infection. A significant negative correlation was observed between number of circulating blood neutrophils before infection and the production of milk and α-lactalbumin during the first 48 h following the inoculation of *E. coli*.

TABLE 2. Correlations between the phorbol myristate acetate (PMA) and zymosan induced respiratory burst of bovine neutrophils expressed as O_2^- generation/cell (nmoles O_2^-/cell) and O_2^- generation capacity (nmol O_2^- × neutrophil number) prior to the inoculation of *Escherichia coli* and the severity of mastitis.[1]

Characteristic of blood neutrophils	Decrease in production of	
	Milk	α-Lactalbumin
Neutrophil number	−.58*	−.48*
PMA-induced		
O_2^- generation/cell	−.16	−.22
H_2O_2 generation/cell	−.18	−.26
Zymosan-induced		
O_2^- generation/cell	−.80**	−.67*
PMA-induced		
O_2^- generation capacity	−.77*	−.72**
Zymosan-induced		
O_2^- generation capacity	−.90***	−.75**

[1]Severity of mastitis was considered as the percentage decrease in milk and α-lactalbumin production on d 1 and 2 after inoculation of *E. coli* into the udder.

[2]Correlation coefficients with probabilities *$P \leq .05$, **$P \leq .005$, ***$P \leq .001$ for 14 cows.

In presence of PMA, no correlation between ROS-producing competence of neutrophils and loss in milk production or α-lactalbumin synthesis during subsequent experimentally induced mastitis could be detected. In contrast, when zymosan particles were used, a negative correlation could be observed. The ROS-generating capacity of blood neutrophils (blood cell number × mean ROS producing competence) and severity of experimentally induced mastitis were tested in parallel (Table 2). There was a significant negative correlation between capacity of ROS production by neutrophils before infection and severity of a subsequently induced inflammation of the mammary gland expressed as percent decrease in milk and α-lactalbumin production. Correlations were significant in presence of both types of respiratory burst activators, as well for capacity of superoxide as hydrogen peroxide production ($r = -.79$ and $r = -.84$). Correlations were similar comparing capacity of ROS generation with decrease in α-lactalbumin production.

Trial 2

A marked swelling of infected quarters was observed about 3 h after inoculation. Cows became restless and showed symptoms of pain. Cows kicked at the inflamed udder half, and milk leakage occurred between 4 to 6 h after inoculation. About 10 h after inoculation, milk was nearly normal but contained some fine clots. At 15 h, milk became yellow and contained large purulent clots. Induction of mastitis elicited fever and tachycardia. Rectal temperature started to increase a few hours after inoculation, reaching a maximum at 10 h.

Based on loss in milk, α-lactalbumin production, alterations in milk composition, and systemic signs, a moderate and a severe response could be distinguished (Figure 1). Local and systemic changes were qualitatively the same in both groups and support the findings of Guidry et al. (11). However, differences were striking in degree and time of onset of the different phenomena (Figure 2). In the moderate response group, milk production of infected quarters decreased 60% the day after inoculation (Figure 1). The decrease of milk production in uninfected quarters amounted to 11%. In the severe response group a loss of milk production of 90 and 78% occurred in infected and uninfected quarters on d 1. Changes in milk electrolyte and lactose concentrations, which represented degree of damage of the interstitial milk barrier, tended to occur earlier in the moderate mastitis group. This was also the case for the influx of cells in milk and for the appearance of the systemic symptoms such as fever and tachycardia. On a quantitative basis, all these phenomena seemed to be more pronounced in the severe mastitis group.

One day after inoculation of *E. coli*, blood PMN number was severely reduced (Figure 3) and a drastic shift in cell types (Table 3) was established. Metamyelocytes and myelocytes appeared frequently in the blood and even became the predominant cell types during the first days of inflammation. At the same time, there was a massive immigration of neutrophils into milk. There was a clear difference between the leukocyte kinetics of the moderate and the severe mastitis group. Depending on degree of inflammation, neutropenia disappeared after 3 to 8 d and a neutrophilia developed during which myelocytes were replaced by band cells and mature neutrophils. On one occasion (re-

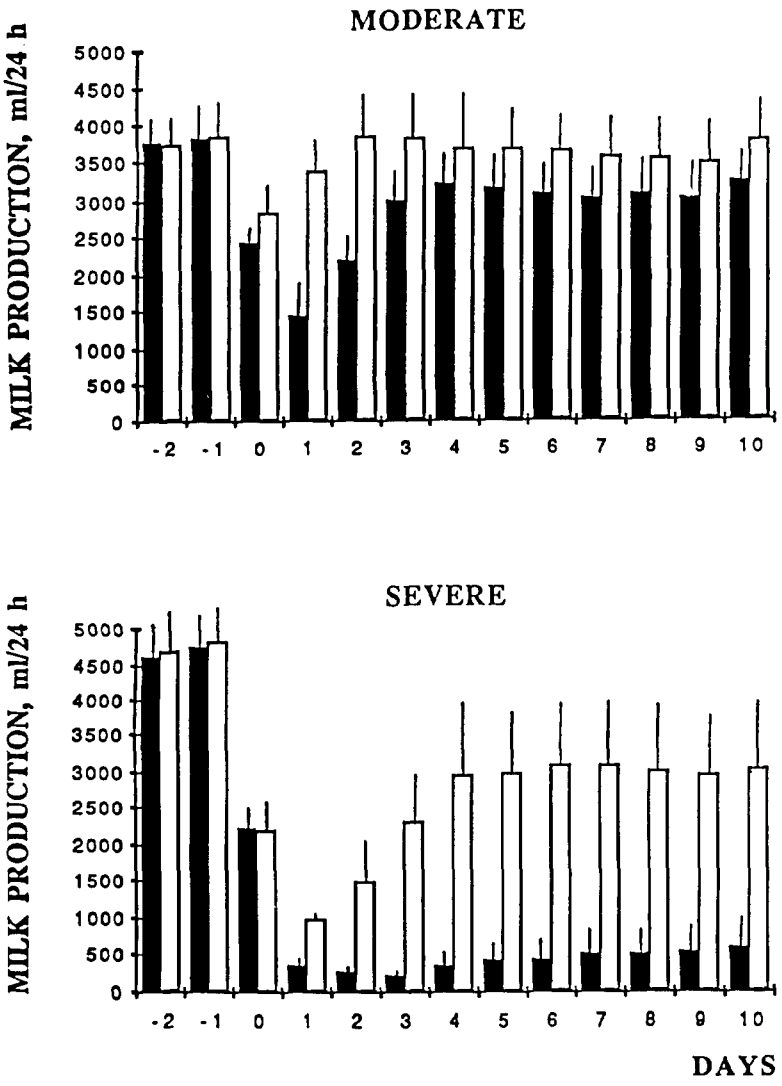

Figure 1. Quarter milk production in infected (■) and uninfected (□) glands of six cows after intramammary infusion of *Escherichia coli*. Cows were classified in moderate (n = 3) or severe (n = 3) mastitis groups. Each value is the mean ± SEM of six quarters. *Escherichia coli* (P4:O32, 10^4 cfu) was inoculated into each mammary gland of the right udder half on d 0; d −2 and −1 are preinoculation control days.

sults not shown), neutropenia lasted 2 wk. Following this was an extreme neutrophilia (>20,000 neutrophils/mm^3), characterized by the persistent presence of immature cell types in the blood. Rectal temperature remained elevated and the animal died after a few weeks.

Figure 3 shows the average PMA-induced superoxide basal release from blood neutrophils during moderate and severe mastitis. A sudden decrease in specific activity of the respiratory burst was noticed 1 d after infection of the mammary gland. Activity was rapidly restored in the case of moderate inflammation but continued for several days in cows suffering from severe mastitis. This period of diminished burst activity roughly correlated with the appearance

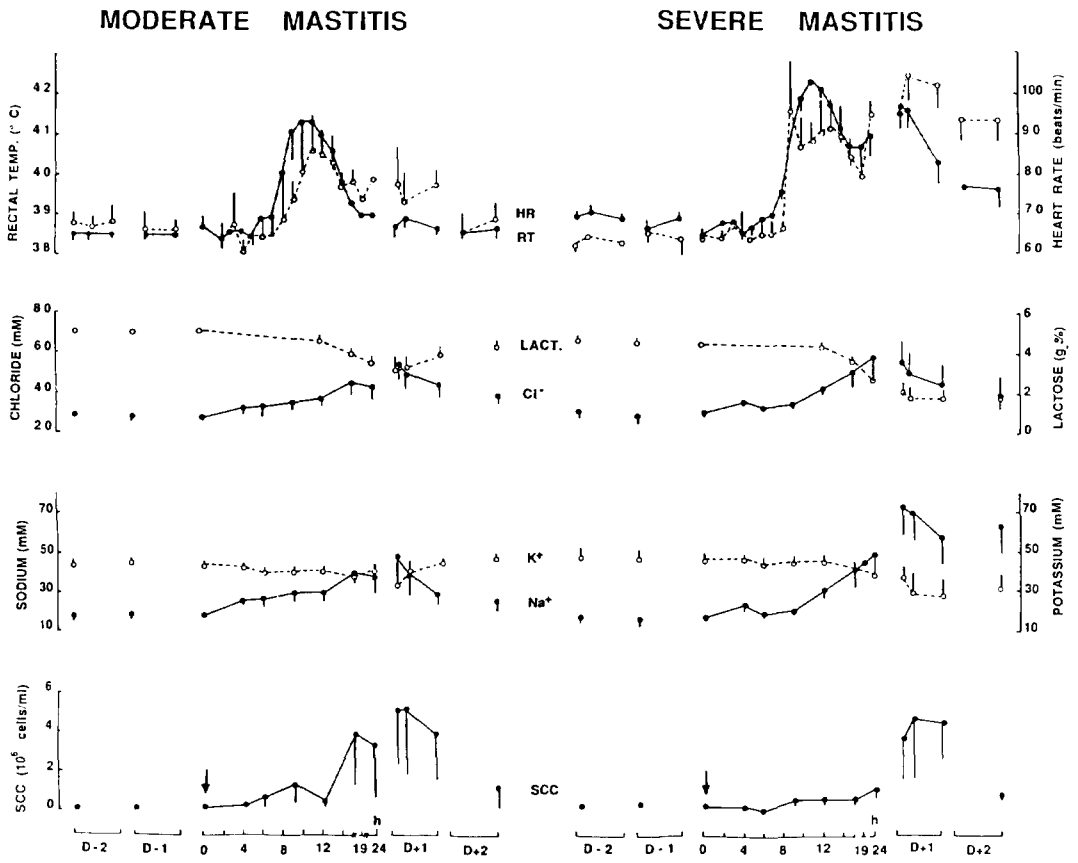

Figure 2. Overall results of rectal temperature and heart rate, SCC, chloride, sodium, potassium, and lactose concentrations in milk in the moderate and the severe mastitis group. Each value is the mean ± SEM of three cows. Infected (●) and uninfected (○) glands.

of immature neutrophil forms (myelocytes and metamyelocytes). Reestablishment of the respiratory burst activity was followed by a significant enhancement of superoxide production and the reappearance of mature neutrophils in the blood. Stimulation of isolated neutrophils with zymosan particles was followed by a similar superoxide response during the development of *E. coli* mastitis. However, zymosan did not generate a superoxide enhancement during the reappearance of mature neutrophils in circulation (Figure 3).

DISCUSSION

Techniques utilized to separate PMN leukocytes from bovine blood are often based upon fractionation of the packed red cell layer after centrifugation or on a density gradient centrifugation derived from the original procedure described by Böyum (5). However, since blood from adult cattle occasionally contains a large percentage of eosinophils (15), these methods often yield granulocyte preparations containing elevated numbers of eosinophils. Results from Table 1 clearly demonstrate the influence of a minor population of eosinophils on the assay of the respiratory burst from neutrophils. This emphasizes the necessity for highly purified neutrophils in obtaining adequate data on their respiratory burst competence.

The normal ROS eosinophil:neutrophil ratio was about 5. In contrast, stimulation with PMA

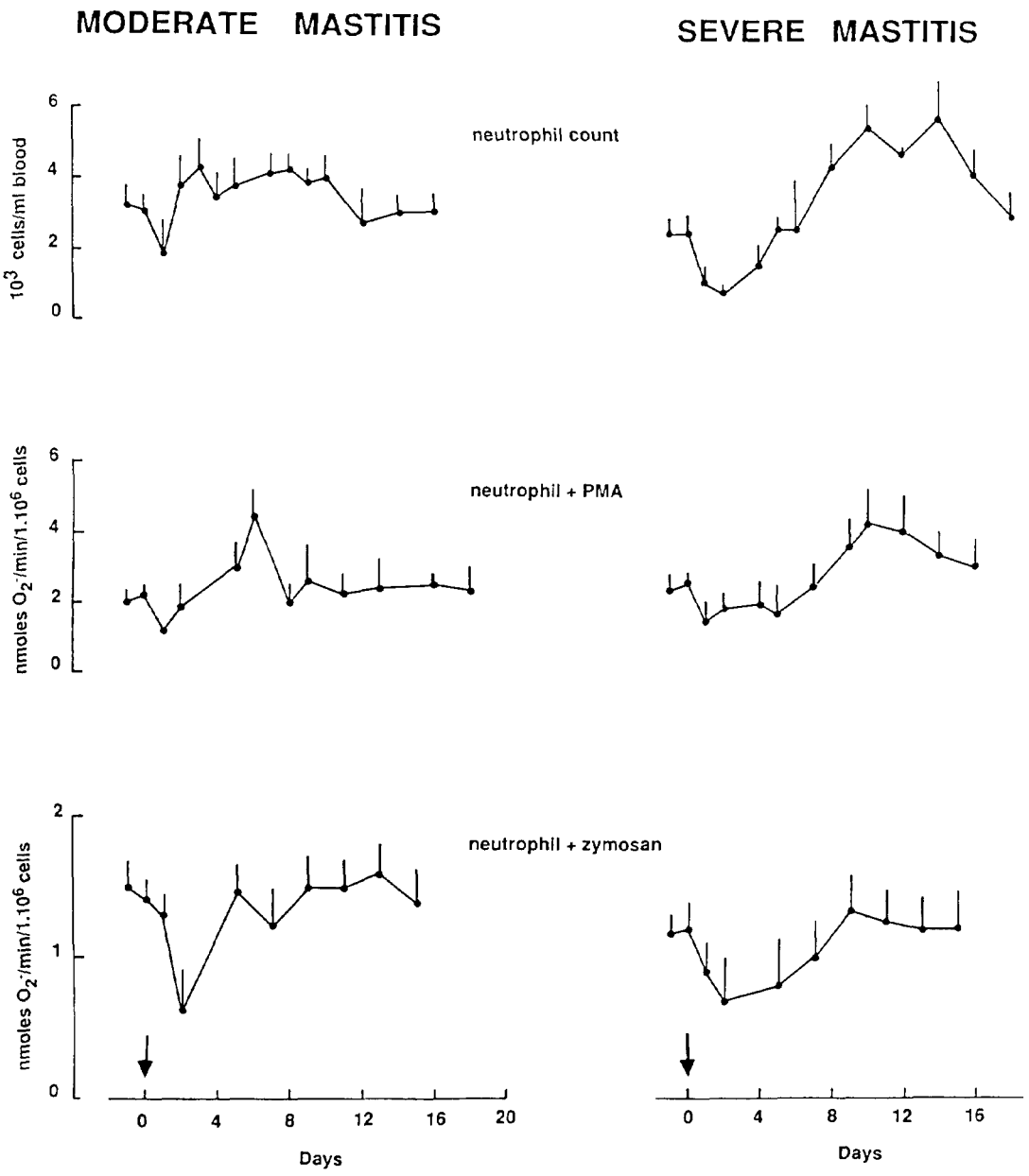

Figure 3. Blood neutrophil number and superoxide anion generation by blood neutrophils isolated from three cows with moderate and three cows with severe *Escherichia coli* mastitis. Cells are activated for superoxide release with phorbol myristate acetate (PMA) and zymosan.

and zymosan changed these ratios to 6.6 and 3.1. In accordance with other animal species, including man (18), bovine eosinophils produce much higher amounts of ROS than neutrophils under basal conditions and after stimulation. The ROS ratio was only slightly elevated after stimulation with PMA. However, stimulation with opsonized zymosan gave considerably lo-

TABLE 3. Blood neutrophil differentiation[1] during the evolution of an experimentally induced *Escherichia coli* mastitis in cows soon after parturition.

	Moderate mastitis			Severe mastitis		
Days after infection	Myelo[2]	Band	Segmented	Myelo[2]	Band	Segmented
0	0	0	100	0	1	99
1	46	36	18	75	13	12
3	42	26	32	65	21	14
5	7	37	56	53	36	1
9	3	5	92	13	33	5
14	0	2	98	4	18	78

[1]Average percentage of values from four cows.

[2]Myelo refers to total myelocyte and metamyelocyte population.

wered ratios. Contrary to PMA, which enters the phagocyte and activates the respiratory burst by initiating a series of intracellular events (20), zymosan becomes attached to the plasma membrane before being engulfed and triggers membrane associated respiratory burst enzyme by an unknown transducing mechanism. The fact that eosinophils produced more ROS after stimulation with PMA than after zymosan can be explained by the less efficient phagocytic activity of these cells.

The existence of considerable variation in leukocyte numbers for individual cows has been shown (21). Cow variation was also noted in the responsiveness of cells to PMA and opsonized zymosan regarding the amount of superoxide anions released. Finally, variation between PMA and zymosan-induced generation of superoxide was also noticed for individual animals, which was not surprising, since each agent activates the respiratory burst at different levels. Although the zymosan-dependent activity is related to the phagocytic ability of the neutrophil, PMA induced burst is presumably the result of an intracellular stimulus-associated phosphorylation of one or more proteins thought to be essential in the assembly of the active respiratory burst oxidase (20).

Results presented in Table 2 suggest that a significant correlation exists between the ability of circulating neutrophils to produce ROS (ROS-producing competence) in presence of opsonized particles and severity of inflammation following inoculation of pathogenic bacteria into the udder. No such correlation is observed with PMA as a stimulus. However, when the ROS-generating capacity of blood neutrophils (ROS-producing competence multiplied with the individual neutrophil number) was tested in parallel with the decrease in milk and α-lactalbumin production during mastitis, a significant negative correlation was obtained with both stimuli. From these results, it was concluded that: 1) a high phagocytic potency and 2) elevated blood neutrophil numbers in combination with a high ROS-producing competence limited bacterial replication in the mammary gland. 3) The considerable variation in ROS-generating capacity of blood PMN might be due to the underlying mechanisms, which are responsible for individual variation in susceptibility to *E. coli* mastitis, a phenomenon observed during early, but not late, lactation in cows. 4) Because correlations between ROS-producing competence and milk production are well-established for secretory capacity of the whole udder, as well as for that of infected and uninfected glands, ROS activity seems to be very important in inhibiting resorption of toxins from the inflamed glands. The decrease in milk production in uninfected glands must be considered as a consequence of resorption of toxins, such as endotoxins, in the udder (6).

Recruitment of PMN into the udder is not dependent solely on number in circulation and in tissue pools. Speed of PMN mobilization can dramatically influence severity of infection (13). The second trial of the present study confirmed this finding. In the moderate mastitis group, this phenomenon may be responsible for immediate changes in milk composition and an early inflammatory reaction leading to restriction of the severity of mastitis. In the moderate mastitis group, milk production from uninfected quarters dropped temporarily with a tendency to restore completely. In the severe group, this production loss was much more

evident and was not restored to its original yield.

The antimicrobial systems of the PMN can be divided into oxygen-dependent and oxygen-independent systems. The experiments reported here investigated the potential role of oxygen-dependent mechanisms in natural defense against mastitis. This postulation has been questioned, however, because oxygen concentrations in mastitic milk appear to be several times lower than in venous blood (14) and fall below the apparent Michaelis constant of the respiratory burst oxidase for oxygen (≈10 μM) (3). This may reduce oxygen radical production severely, and, consequently, impair neutrophil oxygen-dependent bactericidal activity. The fall in milk oxygen may be associated with oxygen utilization by the large amounts of neutrophils migrating to the mammary gland (14). To our knowledge, no data on oxygen concentrations in mammary gland tissue are available. However, a biphasic increase in mammary blood flow during experimentally induced *E. coli* endotoxin mastitis was observed in cows and goats, and it was postulated (6) that this phenomenon was the result of a sudden decrease in local blood flow that split the initially induced vasodilation into two peaks. The decrease in blood flow, which varied considerably between individuals, coincided with the strong leukopenia and an increase in SCC in milk. During both increase and decrease in mammary blood flow, uptake of oxygen by the mammary gland might be severely altered. However, the superoxide-forming system can still play an important function during the initial phase of inflammation. As infiltration of cells proceeds, nonoxidative, killing mechanisms may grow in relative importance.

From the second trial, it is clear that stimulated blood neutrophils from cows with acute *E. coli* mastitis showed a sudden and marked decrease in ROS production during the first days of infection, coinciding with a severe neutropenia and shift in cell type. Both responses are commonly seen in early stage of acute mastitis (22). Significantly diminished nitro blue tetrazolium (NBT)-reducing values of bovine neutrophils have been described recently at the onset of acute clinical mastitis (16). The biochemical basis of NBT reduction is due to superoxide anions and is an expression of the respiratory burst activity. Diminished burst activity can most probably be attributed to appearance of immature cell types (Table 3), because oxygen-dependent microbial killing is a late manifestation of functional differentiation. Only segmented and, to a lesser degree, band neutrophils showed a substantial respiratory burst (9). However, immature neutrophils are as phagocytic as mature neutrophils (10).

Depending on the severity of mastitis, respiratory burst values returned to normal after 3 to 10 d and even exceeded normal for some days. Elevated values were mainly observed after stimulation with PMA and coincided with reappearance of mature neutrophils, which was most pronounced in the moderate mastitis group. Enhanced release of ROS may be ascribed to a stimulation of cells released from the bone marrow by bacterial endotoxins (lipopolysaccharides) or other inflammatory mediators. However, the most pronounced decrease in milk production was observed in animals presenting severe systemic clinical signs. This phenomenon might be related to absorption of endotoxins. Therefore, it seems that the "priming phenomenon", which is most pronounced in the moderate mastitis group, must be due to factors other than endotoxins. Different endogenous inflammatory mediators and hormones might be involved. A "priming" effect has been described in phagocytes that have been in contact with very low concentrations of several inflammatory mediators (12).

During experimentally induced *E. coli* mastitis in cows, different hormones, e.g. somatotropin, are released (8). Furthermore, we recently demonstrated that bovine recombinant somatotropin exerted a positive influence on restoration of the blood milk barrier and milk production during experimentally induced *E. coli* mastitis (7) and stimulated the in vivo superoxide generation of PMN cells from healthy cows. Therefore, we suggested that elevated somatotropin concentrations in blood may favor proliferation and generation of a neutrophil population, which is "primed" for an augmented oxidative response. Although these mediators are not stimuli themselves, treated cells are predisposed to an elevated release of toxic oxygen metabolites upon subsequent stimulation. The biochemical events underlying the "priming" phenomenon are still unknown, and a more fundamental knowledge of the

working mechanism of the respiratory burst enzyme is needed.

The findings reported here suggest that the generation of ROS by neutrophil leukocytes is of considerable importance, at least in the early phase of defense against *E. coli* infection of the mammary gland during the periparturient period. The results also support the concept that an acute inflammatory reaction is able to modulate the oxidative metabolism of cells not only at the site of inflammation but at points distant from it (4). The herein reported existence of mutual interferences between the inflammatory process and the generation of ROS is far from understood and certainly requires further investigation.

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