Preinfection Functions of Blood Polymorphonuclear Leukocytes and the Outcome of Experimental *Escherichia coli* Mastitis in the Cow

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ABSTRACT

The relationship between preinfection functions of blood neutrophils and outcome of experimental *Escherichia coli* mastitis was studied in 11 cows. Random migration, chemotaxis, phagocytosis, and chemiluminescence by neutrophils were determined in white blood cell suspensions, and in purified neutrophil suspensions. The course of *E. coli* mastitis (10^4 *E. coli* 0:157 in rear quarters) was monitored using clinical parameters, counts of *E. coli* in mastitic secretion, and milk production. Regressions were calculated for areas under curves of these parameters and preinfection activities of neutrophils. Chemiluminescence by nonstimulated neutrophils in white blood cell suspensions was negatively correlated with counts of *E. coli* in secretion and with losses in milk production. The chemotactic differential in white blood cell suspensions minus the chemotactic differential in purified suspensions of neutrophils referred to as delta varied from −.66 to +.50, indicating, respectively, inhibition and stimulation of chemotactic activity of neutrophils in white blood cell suspensions. Delta correlated negatively with counts of *E. coli* in mastitis secretion, inhibition of the amplitude of rumen contractions, and losses in milk production. We hypothesize that a factor in white blood cell suspensions may be involved in the down-regulation of the migratory response of neutrophils during *E. coli* mastitis.

(**Key words**: *Escherichia coli* mastitis, blood polymorphonuclear leukocyte functions, chemotaxis)

INTRODUCTION

Escherichia coli mastitis is an important cause of morbidity and losses in milk production in dairy cows, and, therefore, has become of great economic importance to the dairy industry (4). *Escherichia coli* mastitis in cattle may be associated with systemic signs of disease, predominantly in the immediate postpartum period (7, 10).

Polymorphonuclear leukocytes (PMN), which migrate from the blood and enter the milk, play a key role in preventing or eliminating infections in the bovine udder (5, 19). The effectiveness of this defense system depends on the promptness and magnitude of the migratory response of the PMN and the phagocytic and bactericidal activities of these cells (5, 10, 11, 19).

Data are limited on in vitro activities of bovine blood PMN function before infection in relation to the course of subsequent experimental mastitis. In a recent study by Heyneman et al. (9), decrease in milk production during 48 h after experimental *E. coli* mastitis in newly calved, lactating cows was related to preinfection blood PMN functions. Preinfection values of nitroblue tetrazolium reduction of PMN (after stimulation with zymosan), and oxygen-dependent bactericidal index (number of circu-

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lating neutrophils multiplied with in vitro H_2O_2-producing capacity of PMN after stimulation with phorbol myristate acetate) were negatively correlated with decrease in milk production after infection. However, clinical signs during the acute phase of *E. coli* mastitis and long-term decrease in milk production were not mentioned.

The objective of this study was to investigate if in vitro activities of blood PMN before inoculation were related to severity of the disease and milk loss after experimental *E. coli* mastitis in the cow.

MATERIALS AND METHODS

Animals

Twelve clinically healthy, crossbred Holstein × Dutch Friesian cows in their first to eighty lactation were used. All cows had calved normally and had no history of retained placenta, mastitis, or periparturient disease. Cows were 20 to 30 d postpartum at time of inoculation, were housed in a tie-stall barn, and were fed wilted grass silage and concentrate. Water was provided for ad libitum access. Milkings were at 0730 and at 1530 h.

Induction of Experimental Mastitis

An encapsulated strain of *E. coli* 0:157 isolated from a case of clinical mastitis was used for intramammary infection. The strain was maintained on nutrient agar CM 3 (Oxoid Ltd., Basingstoke, UK) at 4°C. Organisms were subcultured twice into 5 ml of brain-heart infusion broth CM 225 (Oxoid), grown in 50 ml of brain-heart infusion broth at 37°C for 22 h, and washed three times. Washed bacteria were resuspended in saline to yield approximately 5×10^2 cfu/ml. Numbers of *E. coli* in inocula were determined in each experiment using a plate count method (3) on violet red bile glucose agar (VRBG) CM 485 (Oxoid) after 18 to 24 h incubation at 37°C. Inoculum size was 20 ml of this diluted culture. Actual counts of the inoculum were $8.8 \times 10^3 \pm 1.3 \times 10^3$ (mean ± SEM).

Experiment Procedures

Blood for evaluation of PMN function was collected from the jugular vein at 1 h before inoculation. At the start of the experiment (0930 h, 2 h after the morning milking), 20 ml of pyrogen-free saline solution containing approximately 10^4 cfu *E. coli* 0157 were infused in both rear quarters of all cows. Cows were not milked at the afternoon milking following infection. All cows received antibacterial treatment at 7 d postinfection immediately after the morning milking (16).

Systemic Clinical Signs, Milk Production, and Determination of Viable Counts of *Escherichia coli* in Mastitis Secreta

Rectal temperatures were recorded with a thermocouple; heart rate was determined by counting heart beats with a stethoscope for 1 min. Rectal temperature and heart rate were determined every 30 min during the first 36 h postinfection. Rumen motility was recorded continuously up to 36 h post-infection as described previously (16). Frequency and amplitude of rumen contractions were calculated from recordings over 30-min periods. Quarter milk production was measured twice daily with a four-quarter milking unit from 7 d before to 21 d postinfection.

At 7, 4, 2, and 1 d, and at 1 h before infection, the udder was examined clinically and quarter foremilk samples of all cows were obtained for bacteriological examination and electronic somatic cell counting (Model F, Coulter Electronics, UK) (12, 17). Milk from infected rear quarters was used to determine viable counts of *E. coli*. Samples were collected aseptically (17) immediately before inoculation; at 4, 6, 9, 12, 15, 18, and 21 h postinfection; and thereafter at every milking up to 7 d after infection. Duplicate bacterial counts of *E. coli* in milk were determined by a plate count method (3) utilizing VRBG and a 24-h incubation at 37°C.

Preparation of White Blood Cell and Purified Polymorphonuclear Leukocytes Suspensions

Blood was collected from the jugular vein in heparinized tubes (Venoject®, Terumo Corp., Tokyo, Jpn). To prepare white blood cell (WBC) suspensions, blood was centrifuged at 400 × *g* for 10 min, and the plasma was

withdrawn. Red blood cells were lysed by a hypotonic flash lysis procedure (8). The blood cells were again centrifuged (10 min at 400 × *g*), and the leukocyte pellets were washed three times in Krebs-bicarbonate buffer (pH 7.4).

To isolate PMN, the WBC suspensions were carefully layered onto Percoll (1.077 g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden). After centrifugation for 20 min at 400 × *g*, the mononuclear cell layer at the interface was removed. The PMN pellet was washed three times. The concentrations of WBC and PMN suspensions were adjusted to 5×10^6 cells/ml of Krebs-bicarbonate buffer. Viability of PMN was determined with trypan blue exclusion.

Binding and Ingestion Assay

Escherichia coli strain PC 2166 was used to measure binding and ingestion. Bacteria were grown overnight at 37°C in 5 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, MI) to which .02 μCi of [^{3}H]thymidine (specific activity 5 Ci/mmol; Amersham, UK.) had been added (21). Bacterial suspensions were prepared as described previously (8) and were opsonized in diluted pooled bovine serum by incubating on a rotator for 30 min at 37°C. Samples of .2 ml of WBC and PMN suspensions (5×10^6 cells/ml) were mixed with .2-ml aliquots of the suspension of opsonized (1% pooled bovine serum) *E. coli* (10^8 bacteria/ml). These mixtures were incubated in a shaking water bath at 37°C for 30 min to study binding and ingestion activities of PMN (9, 22). The final ratio of bacteria to bacteria was 200:1. The PMN together with cell-associated bacteria were separated from unassociated bacteria by differential centrifugation. The amount of cell-associated radioactivity was expressed as a percentage of the total radioactivity added. Previous studies have shown that 90% of the cell associated bacteria were ingested by PMN (8).

Chemotaxis and Random Migration of Polymorphonuclear Leukocytes

Chemotactic activity of PMN in WBC and PMN suspensions were determined using an underagarose technique (8, 18). Suspensions of WBC and PMN (5 μl; 5×10^7 cells/ml) were placed in one well (diameter 2.4 mm) cut into agarose; another well (2.7 mm apart) contained 5 μl of zymosan-activated pooled bovine serum. Plates were incubated at 37°C for 18 h in a humidified atmosphere of 5% CO_2:95% air. Migration distances (mm) were measured from projected images. The chemotactic differential was calculated as directed migration of the leading edge toward serum minus random migration in the opposite direction (14, 18). The chemotactic index was defined as directed migration toward serum divided by random migration (18). Values for each parameter were calculated from a minimum of eight observations per cow.

Generation of Chemiluminescence

Chemiluminescence by PMN in WBC and PMN suspensions (1×10^6 PMN) was measured in a luminometer (Type 1251, LKB Wallac, Turku, Finland) in the presence .5 m*M* lucigenin (bis-N-methylacridinium nitrate; Sigma Chemical, St Louis, MO) in a total volume of .5 ml of Zymosan (2.5 mg/ml), preopsonized in 100% pooled bovine serum, and phorbol myristate acetate (100 ng/ml) were used as stimuli. Chemiluminescence was determined every 60 s over a 45-min period.

Statistical Analysis

Differences between means were tested with a two-sample *t* test. Statistical significance was determined at $P<.05$. Area under curve (AUC) was calculated for clinical parameters, total daily milk production, milk production from rear quarters, and bacterial counts of *E. coli* in left and right rear quarters. Data on clinical signs and milk production were expressed as percentage of a preinfection baseline (rectal temperature was expressed in degrees Celsius); bacterial counts were expressed as $\log_{10}$ of cfu). These data were plotted against time after infection. The AUC of each parameter was calculated for every cow as:

$$\Sigma_1 = \{(t_i - t_{i-1}) \times f_{i-1}\} + \{.5 \times (t_i - t_{i-1}) \times (f_i - f_{i-1})\}$$

where:

t_i = the time of observation,
t_{i-1} = the previous time of observation,
f_i = the value of parameter at time i,

f_{i-1} = the value of clinical parameter at time i–1, and
f_0 = the mean of the values of the parameter before infection.

The predictive value of PMN functions for parameters was tested in the general linear model:

parameter = a + b × PMN function + e

where:

a = the general mean,
b = the regression coefficient, and
e = the error term, presumed normal distribution.

RESULTS

Preinfection Activities of Polymorphonuclear Leukocytes Suspensions

The WBC suspensions contained 64.1 ± 5.26% PMN (mean ± SEM). Purified PMN suspensions contained 95.4 ± .57% PMN. Viability of PMN exceeded 95%.

Chemotaxis, binding and ingestion, and chemiluminescence by PMN in WBC and PMN suspensions are in Table 1. Summary data on migration of PMN from the blood of different animals show a wide range in random migration, chemotactic activity (directed migration toward activated serum), chemotactic differential (directed migration minus random migration in the opposite direction), and chemotactic index (directed migration divided by random migration). This variation was observed for PMN in WBC and PMN suspensions. Delta was defined as the chemotactic differential of PMN in WBC suspensions minus chemotactic differential of PMN suspensions. Values for delta were – .17 ± .09 (mean ± SEM), and ranged from –.66 to .50. Values of delta greater than 0 indicate stimulation, whereas values less than 0 indicate inhibition of chemotactic differential of PMN in WBC suspensions as compared with PMN suspensions.

Clinical Signs and Milk Production Before Infection

Clinical signs before inoculation revealed no abnormalities. Mastitis pathogens were not

TABLE 1. Summary data for preinfection activities of polymorphonuclear leukocytes (PMN) isolated from the blood of 11 cows[1] in purified PMN and white blood cell (WBC) suspensions.

	PMN Suspension			WBC Suspension		
PMN Function	$\bar{X}$	SEM	Range	$\bar{X}$	SEM	Range
Binding and ingestion of opsonized *E. coli*[2] (200:1 Bact:PMN ratio), %	52.7	2.1	40–60	51.3	2.6	37–68
Peak chemiluminescence,[3] mV/10^6 PMN						
No stimulus	31.8	7.4	13–69	33.27	7.5	5–80
PMA	700.2	121.4	323–1344	664.5	74.3	353–1034
Zymosan	751.7	161.5	205–1650	681.0	105.1	268–1484
Migration[4]						
Chemotaxis, mm	1.80[a]	.06	.99–2.65	1.59[b]	.07	.6–2.65
Spontaneous migration, mm	.38	.01	.21–.61	.35	.01	0–.56
Chemotactic index	5.54	.4	3.05–7.44	4.49	.5	2.76–8.22
Chemotactic differential, mm	1.42	.05	.78–2.28	1.24	.06	.58–2.24

[a,b]Means differ ($P<.05$).

[1]Blood was sampled 1 h before induction of experimental mastitis.

[2]PMN or WBC suspensions (10^6 PMN) were incubated with radiolabeled, opsonized (1% serum) *Escherichia coli* (2×10^7 bacteria) for 30 min at 37°C. Binding and uptake of *E. coli* was calculated from the amount of PMN-associated radioactivity.

[3]Chemiluminescence by PMN was measured in a luminometer in the presence .5 m*M* lucigenin in a total volume of .5 ml PMN (100 ng/ml) and zymosan (2.5 mg/ml) preopsonized in 100% pooled bovine serum were used as stimulus.

[4]Assay of chemotactic activity was performed under agarose at 37°C for 18 h.

TABLE 2. Means and range[1] of clinical signs, milk production, and numbers of *Escherichia coli* in mastitis secretion in 11 cows after infusion of 10^4 cfu of *E. coli* 0:157 into rear quarters.

Parameter	$\bar{X}$[1]	SEM	Range	
			Minimum	Maximum
Heart rate,[2] %[3]	11.81	2.51	1.97	23.64
Rectal temperature, °C	1.01	.18	.28	2.36
Rumen frequency, %[3]	−18.55	3.31	−32.25	−3.72
Rumen amplitude, %[3]	−29.78	6.92	−56.48	−.48
Total daily milk production[4] %[3]	−25.64	8.91	−92.37	5.09
Milk production in rear quarters, %[3]	−41.00	10.52	−97.73	1.05
Counts of *E. coli* in secreta from[5]				
Left rear quarter	3.36	.54	.95	6.66
Right rear quarter	2.93	.56	.40	5.70

[1]Means and range refer to average changes of parameters during the entire observation period.

[2]Clinical symptoms were recorded during 36 h postinfection.

[3]Data are expressed as percentage of preinfection values.

[4]Quarter milk production (L) was recorded during 21 d postinfection.

[5]Counts of *E. coli* in secretion were determined up to 125 h postinfection.

demonstrated and foremilk SCC were <300,000/ml. Average rectal temperature at the start of the experiment was 38.8 ± .02°C, and average heart rate was 82 ± 1.0 beats/min (n = 44). The characteristic powerful rumen contraction sequences occurred about once a minute. Milk production ranged from 16.8 L/d to 31.7 L/d; the average daily milk production was 23.5 ± .44 L/d (mean ± SEM; n = 77). Milk production in rear quarters was 55.8 ± 1.8% of total daily milk production.

Clinical Signs, Milk Production and Counts of *Escherichia coli* in Mastitis Secreta After Infection

One cow was excluded from the results because clinical mastitis was detected in the right front quarter at 25 h after infection. A strain of *E. coli* (not serotyped) was isolated from the secretion.

Overall averages and ranges of heart rate, rectal temperature, rumen frequency, and rumen amplitude were calculated over the entire observation period (Table 2). Variation in the duration and severity of clinical signs associated with experimental infection was considerable. Marked swelling of all infected quarters was observed from 3 to 4 h postinfection, and quarters were warm and sore. From 10 h postinfection, appearance of secretions varied widely; in animals with moderate clinical signs, secretion was milk-like with some small clots; in severely ill animals, secretions were yellowish, serous, and contained large clots.

In general, induction of *E. coli* mastitis elicited fever, tachycardia, and inhibited rumen frequency and rumen amplitude. Rectal temperature began to increase at 6 h postinfection and reached mean maximum values of 41.6°C (range 41.0 to 42.0°C) at about 9 h postinfection (results not shown). Maximum increases in heart rate were 29.3% (range 7.0 to 40.0%) of baseline (preinoculation) values (results not shown). Frequency and amplitude of rumen contractions started to decrease from 7 h postinfection with mean maximum decreases of 37.4% (range 9.5 to 75.0%) for rumen frequency and 52.8% (range 9.5 to 75.0) for rumen amplitude reached at 11 h postinfection (results not shown). In three cows, clinical parameters returned to baseline values at 15 h postinfection and remained within preinfection range during the remainder of the observation period; 4 other cows showed depression, anorexia, fever, tachycardia, and decreased rumen motility up to 36 h postinfection.

Overall average numbers of *E. coli* in mastitis secretion of all samplings are in Table 2. Counts of *E. coli* in secretion from rear quarters initially increased in all cows. Maximum counts for individual cows ranged from 3.3 ×

10^1 to 1.2×10^9 cfu/ml. Secretions of 3 cows animals were bacteriologically negative by 29 h post-infection, whereas secretions of 4 others contained large numbers of *E. coli* at all samplings.

Overall average decrease in milk production of individual animals was calculated from milk production data during the 21 d following infection (Table 2). All cows dropped in milk production during the first days after induction

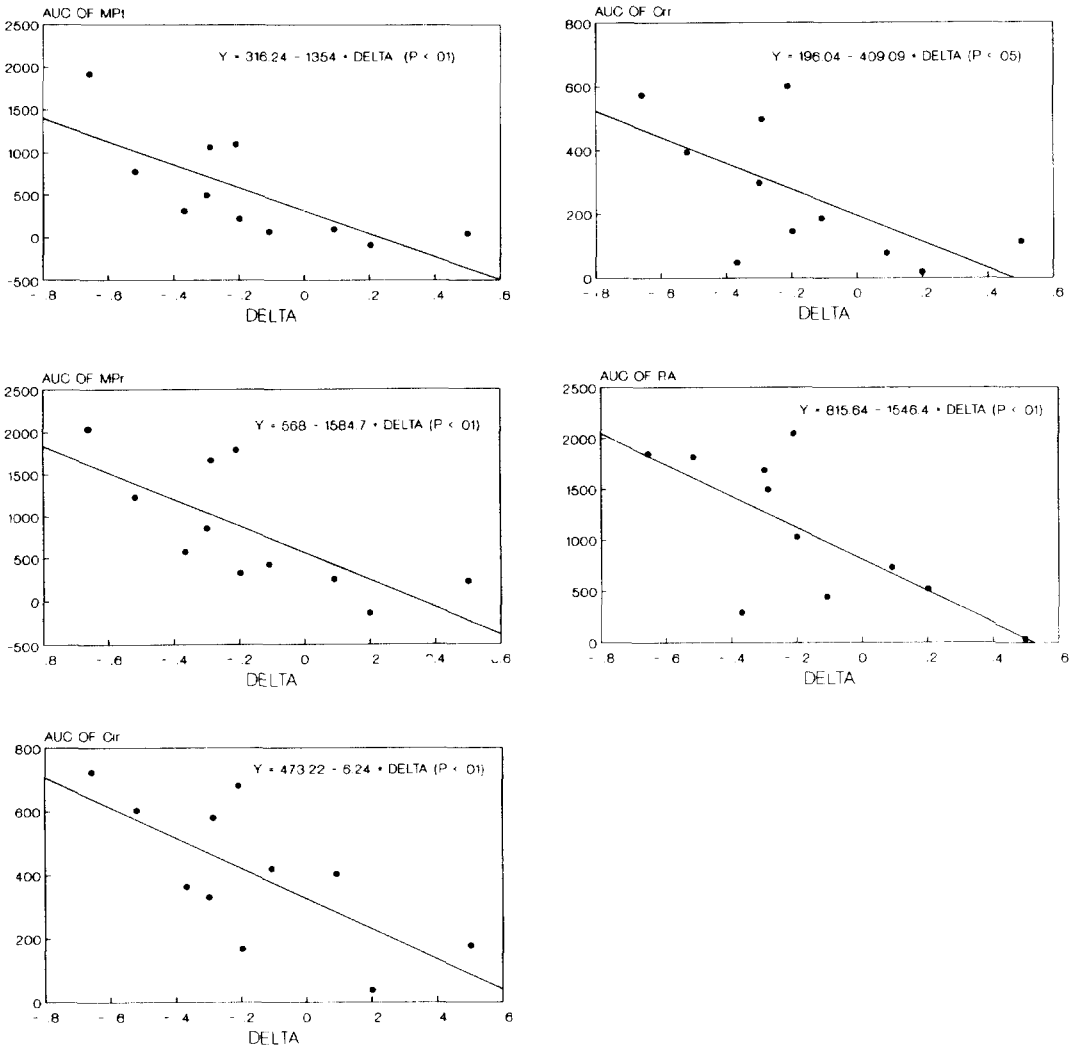

Figure 1. Regressions of preinfection delta values on area under curve (AUC) of milk production, counts of *Escherichia coli* in secretion from infected quarters, and rumen amplitude during experimental *E. coli* mastitis induced by infusion of 10^4 cfu of *E. coli* 0:157 in rear quarters in 11 cows. Delta (mm) denotes chemotactic differential of polymorphonuclear leukocytes (PMN) in white blood cells (WBC) suspension minus chemotactic differential of PMN from the same cow in purified PMN suspension. Values for each cow are the mean of 8 observations. AUC of MPt and AUC of MPr = Area under curve of daily milk production and AUC of daily milk production from rear quarters during 21 d postinfection, respectively. AUC of Clr and AUC of Crr = AUC of counts of *E. coli* in secreta from left and right rear quarters during 125 h post-infection, respectively. AUC of RA = AUC of amplitude of rumen contractions during 36 h postinfection.

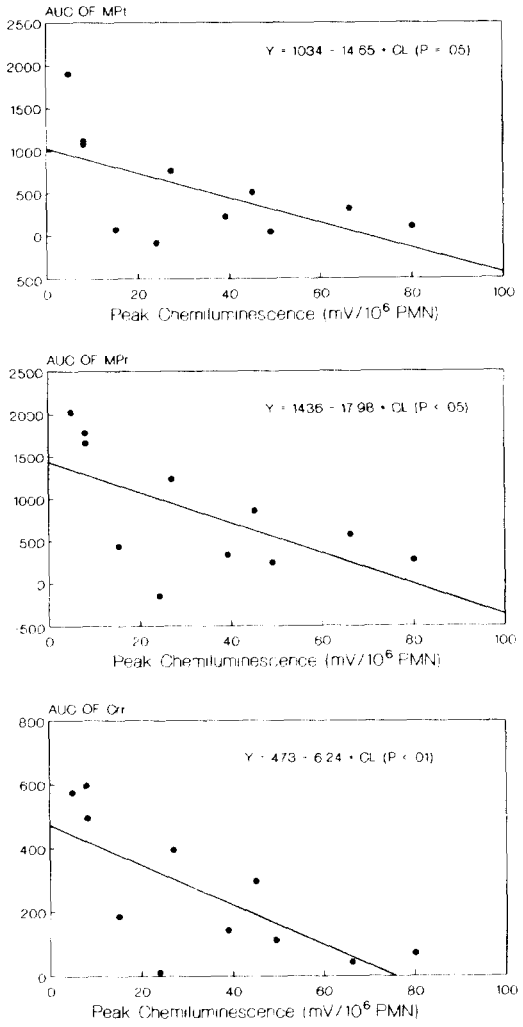

Figure 2. Regressions of preinfection generation of chemiluminescence by polymorphonucleocytes in white blood cell suspensions on area under curve (AUC) of milk production and counts of *Escherichia coli* in mastitis secretion during experimental mastitis induced by infusion of 10^4 cfu of *E. coli* 0:157 in rear quarters in 11 cows. AUC of MPt and AUC of MPr = Area under curve of daily milk production, and AUC of daily milk production from rear quarters during 21 d postinfection, respectively. AUC of Clr and Crr = AUC of counts of *E. coli* in secreta from left and right rear quarters, respectively, during 125 h postinfection.

of mastitis. Average change in total daily milk production during 21 d varied from –92.4 to +5.09% of baseline milk production; for milk production in rear quarters values ranged from –97.7 to +1.05%. Average changes in heart rate, rumen amplitude, and total daily milk production, and counts of *E. coli* in secreta from rear quarters showed a tendency to be smaller in heifers (n = 2) compared with multiparous cows, but differences were not significant.

Preinoculation Blood Polymorphonuclear Leukocytes Functions

Baseline milk production (corrected for parity) and parity were not related to PMN functions mentioned in Table 1.

For every cow, AUC were calculated for clinical parameters, for counts of *E. coli* during 36 and 125 h postinfection, and for milk production during 21 d postinfection. Regressions were calculated for AUC of the parameters mentioned and blood PMN functions mentioned in Table 1. Only PMN functions that were correlated ($P<.05$) to AUC are in Figures 1 and 2.

A positive value of delta was correlated with smaller decrease in rumen motility ($P<.01$), lower counts of *E. coli* in secretions from infected quarters ($P<.05$), and smaller losses in total daily milk production and milk production in rear quarters ($P<.01$) (Figure 1). Cows with lower values of delta showed higher numbers of *E. coli* in secretions and a more pronounced decrease in rumen amplitude, total daily milk production, and milk production from rear quarters ($P<.05$).

Generation of chemiluminescence by resting PMN in WBC suspensions was negatively correlated with bacterial counts in secretion from right rear quarters with losses in total daily milk production, and with losses in milk production from rear quarters ($P<.05$) (Figure 2).

DISCUSSION

Mean values of chemotactic activity of PMN in WBC suspensions were lower than in purified PMN suspensions (Table 1). Similar effects on PMN migration in WBC suspensions were observed in other studies (15, 20, 22). We introduced delta to evaluate the effect of the

WBC suspension on the chemotactic differential of PMN from individual cows. Delta ranged from −.66 to +.50, indicating, respectively, an inhibition and stimulation of chemotactic differential in WBC suspensions.

Severity of clinical signs and losses in milk production after experimental *E. coli* mastitis have been reported to vary widely among individual cows (10, 11). This variation may be influenced by stage of lactation at the time of infection (10) and differences in the functional capabilities of humoral and cellular defense mechanisms (5, 10). In our study, all animals were within 20 to 30 d postpartum to rule out the effect of stage of lactation on the function of PMN (13) and the effect of stage of lactation on the course of *E. coli* mastitis (10).

The results indicate that high susceptibility to experimental *E. coli* mastitis was correlated with some PMN functions before infection (Figures 1 and 2). Chemotaxis, chemotactic differential, and chemotactic index of PMN in WBC or PMN suspensions were not correlated to severity of clinical signs and loss in milk production. Delta, however, was correlated negatively ($P<.05$) with numbers of *E. coli* in mastitis secreta, decrease in rumen amplitude, losses in total daily milk production, and milk production in rear quarters. Part of the drop in milk production during the first days was likely caused by the missed afternoon milking following infection.

The importance of rapid migration of PMN into the lumen of the mammary gland for the outcome of *E. coli* mastitis has been well established (7, 10, 11). Failure or delay of PMN mobilization (established by SCC in milk or by postmortem histological observations) was associated with persistently high counts of *E. coli* in secretions from infected quarters, resulting in persistent systemic signs of disease and great losses in milk production (7, 10, 11). The latter data are in agreement with our results.

In a recent study (22), migratory properties of blood PMN before infusion lipopolysaccharide (LPS) were compared with increases in SCC in milk upon infusion of a very low dose (.1 ng) of LPS of *E. coli* into quarters of lactating cows. In cows that responded with a significant increase in milk SCC, values for chemotactic differential of PMN in purified PMN suspensions were high and were not decreased in WBC suspensions (resulting in values of approximately zero for delta). Purified PMN from nonresponders had lower chemotactic differential values, which decreased even more in WBC suspensions (negative delta). We hypothesize that a factor in WBC suspensions may be involved in the down-regulation of the migratory response of PMN during *E. coli* mastitis. In this regard, it is surprising that differences in most of the other capacities of WBC and PMN suspensions were not markedly different (Table 1). Migratory properties of PMN in WBC suspensions and in enriched PMN suspensions were also observed to differ in other studies (15, 22). This suggests a distinct action of chemotactic factors on the cell types present in WBC and PMN suspensions.

Generation of chemiluminescence by phagocytes is the light emission that accompanies the production of highly unstable oxygen metabolites as a result of respiratory burst activation (2). Chemiluminescence has been used to study to study oxygen-dependent microbicidal systems of phagocytes, because the oxidative metabolism by PMN is an important aspect of bactericidal activity (1). Generation of chemiluminescence of blood PMN in cows is correlated with their in vitro efficiency of intracellular killing of *Staphylococcus aureus* (23). This is in agreement with the negative relationship of chemiluminescence with numbers of *E. coli* in mastitis secretion and with the losses in milk production in rear quarters observed in our study (Figure 2).

The aim of our experiments was to relate preinfection blood PMN function with the course of subsequent experimental *E. coli* mastitis in order to predict susceptibility of to *E. coli* mastitis. The results in this paper indicate that some in vitro PMN functions, especially delta, may prove suitable for this purpose. Previous studies (13) have shown that PMN functions decreased dramatically during the periparturient period. These data emphasize the limitations of single-time samplings (6) to monitor immune cell function of individual animals. Therefore, longitudinal epidemiological studies on the relationship between blood PMN functions and incidence and severity of *E. coli* mastitis are indicated to validate our results. Moreover, study of the mechanism of inhibition or stimulation of chemotactic differential of PMN in WBC suspensions may result in more fundamental insight into the pathogenesis of *E. coli* mastitis.

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