Preinfection Chemotactic Response of Blood Polymorphonuclear Leukocytes to Predict Severity of *Escherichia coli* Mastitis

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ABSTRACT

Experimental mastitis was induced by inoculating rear right quarters of 10 healthy cows with 10^3 cfu of *Escherichia coli*. The chemotactic responses of peripheral blood polymorphonuclear leukocytes at d –6, –5, –2, –1, and immediately prior to inoculation were measured. Chemiluminescence of polymorphonuclear leukocytes was measured immediately prior to inoculation. Severity of the experimental mastitis was assessed by bacterial growth in the inoculated quarters.

Results of this study indicated that severity of the experimental mastitis may be predicted by the chemotactic response in vitro of polymorphonuclear leukocytes isolated from the peripheral blood at d 2, d 1, and immediately prior to inoculation. The number of circulating polymorphonuclear leukocytes immediately prior to inoculation also showed a negative relationship with the severity of mastitis. No relationship existed between preinfection chemiluminescence of polymorphonuclear leukocytes and the severity of the experimental mastitis.

Preinfection chemotactic response of polymorphonuclear leukocytes and preinfection numbers of circulating polymorphonuclear leukocytes appeared to be valuable as predictors of severity of experimental *E. coli* mastitis in cows.
(**Key words**: polymorphonuclear leukocytes, chemotactic response, *Escherichia coli* mastitis)

Abbreviation key: **AUC** = area under the curve, **MP** = milk production, **PMNL** = polymorphonuclear leukocytes, **WBC** = white blood cells.

INTRODUCTION

Mastitis in well-managed dairy herds is often caused by opportunistic bacteria such as *Escherichia coli* (2, 20). Nonspecific host defense, in particular, the functional activity of polymorphonuclear leukocytes (**PMNL**), has been thought to play an important role in the defense against these pathogens (9, 10, 11, 17). Variation in functional activity of PMNL may help to explain the variation in susceptibility of cows to mastitis. A definition of markers for host defense mechanism capabilities could be valuable for studying differences in mastitis susceptibility among individual cows or herds (20).

In vitro bovine leukocyte function was characterized (6, 8, 12, 14, 15), but few studies exist that investigate preinfection peripheral blood PMNL function and the severity of subsequent (experimental) mastitis. Recent studies (3, 8) described a negative correlation between

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the capacity of blood neutrophils to generate reactive oxygen species and the severity of experimentally induced *E. coli* mastitis. A relationship was observed between severity of induced mastitis and the difference between the chemotactic differential of a white blood cell (WBC) suspension and a purified PMNL suspension (14). No relationship has been demonstrated between preinfection chemotactic response of PMNL and the severity of experimental infection.

The objective of the present study was to investigate the relationship between the chemotactic response of blood PMNL isolated from the peripheral blood prior to induction of experimental *E. coli* mastitis and the severity of induced *E. coli* mastitis. Additionally, the relationship between chemiluminescence of blood PMNL isolated from the peripheral blood immediately prior to inoculation and the severity of the disease was studied.

MATERIALS AND METHODS

Cows

Ten clinically healthy dairy cows of the Dutch Friesian breed or crossbred cows (Dutch Friesian × Holstein Friesian) in their third to sixth lactation and wk 3 to 6 of lactation were used. All cows had calved normally and showed no clinical signs of periparturient diseases. Foremilk samples from the inoculated (rear right) quarters and the control (rear left) quarters were bacteriologically negative for major pathogens and had SCC below 250,000 cell/ml. Cows were housed in a tie-stall barn and fed wilted grass silage and concentrates. Water was provided for ad libitum intake. Milkings were at 0100 and 1400 h.

Bacterial Suspensions

An encapsulated strain of *E. coli* 0:157 isolated from a cow with clinical mastitis (14) was used for intramammary inoculation. The strain was maintained on brain-heart infusion agar with glycerol and stored at −70°C. The day before inoculation, the bacteria were subcultured into brain-heart infusion broth CM 225 (Oxoid, Hampshire, England), grown at 37°C for 22 h, and washed three times (1000 × *g*, 4°C, 15 min) in pyrogen-free saline. Washed bacteria were resuspended in pyrogen-free saline to yield approximately 50 cfu/ml. Numbers of *E. coli* in inocula were determined in each experiment using a spiral plater (Lameris Laboratory, Breukelen, The Netherlands) on violet red bile glucose agar CM 484 (Oxoid).

Experimental Design

Experimental *E. coli* mastitis was induced by aseptic infusion of the right rear quarters with 20 ml of the diluted *E. coli* suspension (50 cfu/ml) at 0700 h. Infused quarters were not milked at the first milking after inoculation (1400 h). Rectal temperatures and heart rate were determined twice daily during a 5-d period before inoculation. From 4 h before inoculation to 12 h postinoculation, rectal temperature and heart rate were recorded every 30 min. From 12 to 36 h postinoculation, rectal temperature and heart rate were determined hourly. From 36 h postinoculation to 6 d postinoculation, rectal temperature and heart rate were determined three times daily. Rectal temperatures were measured with a mercury thermometer; heart rate was determined by counting the number of heart beats with a stethoscope for 30 s.

Quarter SCC and quarter milk production (MP) were measured twice daily from 5 d before to 6 d after inoculation and at d 14 postinoculation. Quarter MP was measured using a four-quarter milking unit; SCC of quarter foremilk samples were measured by the Fossomatic device (Foss Electric, Hillerød, Denmark). Quarter foremilk samples of all cows were obtained for bacteriological examination before experimental mastitis at d 5, d 1, and immediately prior to inoculation. Colony-forming units of *E. coli* were counted in aseptically collected milk samples from the infused right rear quarters at 8, 15, 22, 32, 46, 54, 72, 104, and 120 h postinoculation with a spiral plater.

Before inoculation (at d 6, 5, 2, and 1 and immediately prior to inoculation), jugular venipuncture blood samples for leukocyte differential counts were collected in vacutainer tubes (Venoject®; Terumu Corp., Tokyo, Japan) containing heparin as anticoagulant. Jugular vein blood samples for chemotaxis assay cell isolation were collected in polypropylene tubes (13) containing 1 ml of sodium tricitrate

(3.8%) solution at d 6, 5, 2, and 1 before inoculation and immediately prior to inoculation. Chemiluminescence was determined only in cell suspensions isolated immediately prior to inoculation. The PMNL in vitro chemotaxis and chemiluminescence were measured in WBC suspensions and in purified PMNL suspensions. Glucose, β-hydroxybutyrate, and cortisol concentrations were determined in blood samples collected immediately prior to inoculation.

Immediately after morning milking at d 6, 7, and 8 postinoculation, all cows were treated intravenously with 40 ml of a solution containing 40 mg of trimethoprim and 200 mg of sulfatroxazole/ml (Leotrox®; Leo, Emmen, The Netherlands), and the inoculated quarters were infused with 50 mg of polymixin B sulfate (Polymixin B®; Pfizer, Rotterdam, The Netherlands) dissolved in 250 ml of pyrogen-free saline.

WBC Isolation

White blood cells and purified PMNL were isolated from the peripheral blood as described previously (13). Blood samples were centrifuged for 20 min at 1000 × *g*. The plasma layer was discarded for isolation of WBC. The plasma and the buffy coat layers were discarded for isolation of purified PMNL. Erythrocytes were lysed in two steps by hypotonic lysis. After being washed twice in Eagle's minimal essential medium (Flow Laboratories, Irvine, England), the cells were resuspended in Eagle's minimal essential medium and adjusted to a concentration of 5×10^7 cells/ml by an automatic cell counter (Sysmex K-1000; Goffin, IJsselstein, The Netherlands).

Chemotaxis Assay

The in vitro chemotactic response of WBC or purified PMNL was determined using an under agarose technique (7, 16). Pooled bovine serum was used as chemoattractant. Serum was obtained from 10 normal healthy cows, pooled, and stored in 1-ml portions at –80°C. The same batch of pooled serum was used in all experiments.

Migration distance was measured using an ocular micrometer in a stereo microscope at 25× magnification. The chemotactic response was expressed as directed migration and chemotactic differential. The chemotactic differential was defined as the difference between directed and random migration (16). Results were averaged from five observations per sample.

Generation of Chemiluminescence

Chemiluminescence was measured for WBC and purified PMNL stimulated with Zymosan (2.5 mg/ml; Sigma Chemical Co., St Louis, MO), preopsonized in 100% pooled bovine serum, and phorbol myristate acetate (100 ng/ml, Sigma Chemical Co.). Chemiluminescence was measured in a luminometer (Type 1251; LKB, Wallac, Turku, Finland) in the presence of .5 m*M* lucigenin (bis-N-methylacridinium nitrate; Sigma Chemical Co.) and with a total volume of .5 ml. Chemiluminescence was determined every 60 s over 45 min.

Statistical Analysis

Rectal temperature and heart rate were expressed as the differences from the preinfection baseline. Baselines were defined as the average of rectal temperature and heart rate during the last 24 h before inoculation. Total daily MP and MP in infected and control quarters were expressed as percentages of the preinfection baseline determined for each variable and cow. The baselines for quarter MP were defined as the average MP during the last 4 d before inoculation.

To express bacterial growth in infected rear right quarters, the area under the curve (AUC) ($\log_{10}$ *E. coli* bacterial count time) was calculated for the first 5 d postinoculation. The AUC was calculated for every cow using the following:

$$\begin{aligned} AUC = {} & \{(t_i - t_{i-1}) \times f_{i-1}\} \\ & + \{.5 \times (t_i - t_{i-1}) \\ & \times (f_i - f_{i-1})\} \end{aligned}$$

where

t_i = time of observation,
t_{i-1} = previous time of observation,
f_i = $\log_{10}$ bacterial number at time i, and
f_{i-1} = $\log_{10}$ bacterial number at time i – 1.

Means, standard deviations, linear regressions, correlations, and paired *t* tests were computed on a microcomputer package (Statistix®; NH Analytical Software; Roseville, MN). The following linear model was used to analyze the relationship between preinfection chemotaxis and AUC:

$$Y = a + b_1 X_1 + e$$

where

Y = AUC,
a = intercept,
X_1 = preinfection chemotaxis,
b_1 = regression coefficient, and
e = error term.

RESULTS

Severity of Experimentally Induced *E. coli* Mastitis

Bacterial counts of the inocula were from 40 to 57 cfu/ml, corresponding to a total inoculum of 800 to 1140 cfu per quarter. At d 5 postinoculation, one cow developed acute necrotic *Staphylococcus aureus* mastitis in the right front quarter and was euthanatized. All data from this cow until death were used.

The SCC in milk from inoculated quarters increased to more than 1×10^6 cells/ml at 11 ± 2.5 h postinoculation. Rectal temperature and heart rate increased to maxima of 42.2 ± .1°C and 119 ± 13.1 beats per min, respectively, at 10.7 ± .6 h postinoculation. The maximum number of *E. coli* bacteria in secretion from infected quarters ranged from 1.9×10^5 to 2.1×10^9 at 15 to 22 h postinoculation.

The AUC ranged from 199 to 690 cfu/h. The correlation of AUC with rectal temperature and heart rate was high during the first 2 d of the experimental mastitis (Table 1). The AUC was negatively correlated with MP in the infected and the control quarters (Table 1). Because of these significant correlations, AUC was used as the parameter for judging severity of the experimental mastitis.

Relation of Preinfection Parameters and AUC

In vitro activities of WBC and purified PMNL immediately prior to inoculation are presented in Table 2. Chemotactic response expressed as directed migration was correlated with chemotactic differential (r = .97) in both purified PMNL and WBC; therefore, only correlations between the preinfection chemotactic differential and AUC are presented in Table 3. The negative relationship between AUC and the chemotactic differential of WBC isolated immediately prior to inoculation and at d 1 and 2 before inoculation was significant ($P < .005$) (Table 3, Figure 1). The relationship between AUC and chemotactic differential of purified PMNL was significant ($P = .05$) in suspensions isolated at d 1, 2, and 5 before inoculation (Table 3). The relationship between chemiluminescence of the purified PMNL or WBC and AUC was not significant. The number of circulating PMNL immediately prior to inoculation was correlated with AUC (r = .71, $P < .005$) (Table 3).

DISCUSSION

A significant relationship was demonstrated between the severity of experimental *E. coli* mastitis and the in vitro chemotactic response of PMNL that was determined immediately prior to inoculation and at d 1 or 2 before inoculation. This relationship appeared to be more pronounced when the chemotactic responses of WBC versus purified PMNL were

TABLE 1. Correlation between the area under the curve (AUC) (log_{10} bacterial counts versus time), milk production in control and infected quarters, rectal temperatures, and heart rates at d 1, 2, 3, 4, 5, and 14 postinoculation (n = 10).

Days postinoculation	Quarter milk production		Rectal temperature[2]	Heart rate[2]
	Control quarter[1]	Infected quarter[2]		
1	−.84***	−.50	.60	.83***
2	−.70*	−.53	.70*	.70*
3	−.81***	−.81***	.37	0
4	−.75*	−.84***	.02	.03
5	−.74*	−.75*	0	.35
14	.05	−.89***	. . .	. . .

[1]Percentage of baseline values.

[2]Difference from baseline values.

*$P < .05$.

***$P < .005$.

TABLE 2. Chemotactic response and chemiluminescence of white blood cells (WBC) and purified polymorphonuclear leukocytes (PMNL) immediately prior to inoculation (n = 10).

	$\bar{X}$	SD	Range
Directed migration, mm			
WBC	5.1	2.1	.9–7.5
PMNL	6.8	1.3	4.2–8.6
Migration differential, mm			
WBC	4.2	1.9	.8–6.9
PMNL	5.1	1.1	3.3–6.8
Chemiluminescence of WBC[1]			
Zymosan stimulation	76.7	49.5	15.7–176
PMA[2] Stimulation	92.0	58.4	27.0–205
Chemiluminescence of PMNL[1]			
Zymosan stimulation	154.8	108.9	39.5–341
PMA Stimulation	238.7	107.0	87.2–404

[1]Peak chemoluminescence (millivolts per 10^6 cells).

[2]Phorbol myristate acetate.

measured (Table 3). Factors or cells in the WBC suspensions may have influenced PMNL chemotaxis in vitro. The chemotactic response determined at d 1 and 2 before inoculation showed greater correlation with the severity of the experimental mastitis than chemotactic response determined immediately prior to inoculation. Stress, induced by the more intensive sampling schedule before inoculation, may have been responsible for this phenomenon.

TABLE 3. Correlation between preinfection parameters determined immediately prior to inoculation (d 0) and at d 1, 2, 5, and 6 before inoculation and the area under curve.[1]

Preinfection parameter[2]	Days before inoculation	r
Number of PMNL in peripheral blood	0	−.71*
	1	−.61
	2	−.62
	5	−.22
	6	. . .
Chemotactic differential of WBC	0	−.83***
	1	−.90***
	2	−.88***
	5[3]	−.55
	6[3]	−.16
Chemotactic differential of PMNL	0	−.60
	1	−.72*
	2	−.74**
	5[3]	−.92***
	6[3]	.13

[1]$\log_{10}$ bacterial counts versus time (n = 10).

[2]PMNL = Polymorphonuclear leukocytes, WBC = white blood cells.

[3]n = 7.

*$P < .05$.

**$P < .01$.

***$P < .005$.

A previous study (14) showed that no direct relationship existed between chemotactic response and the course of experimental mastitis. In that study (14), chemotaxis of PMNL may have been influenced by the use of heparin as anticoagulant (4, 5) or Ficoll-Hypaque in the PMNL isolation procedure (19). Therefore, in the present study, citrate was used as the anticoagulant, and a density gradient was not used for isolation. Chemiluminescence has been used to study respiratory burst activation, which is an important aspect of bactericidal activity of PMNL (1). A relationship between preinfection respiratory burst of blood PMNL and severity of experimental mastitis has been described (8). Eosinophils had a great influence on respiratory burst capacity of PMNL suspensions (8). In our study, no relationship existed between the severity of the experimental mastitis and generation of chemiluminescence by either cell suspensions, possibly because, in our study, eosinophils were not separated from other blood cells. Removal of eosinophils from PMNL suspensions would have required density gradient centrifugation, which could influence chemotactic response of the PMNL (13, 19).

The number of circulating PMNL immediately prior to inoculation was negatively

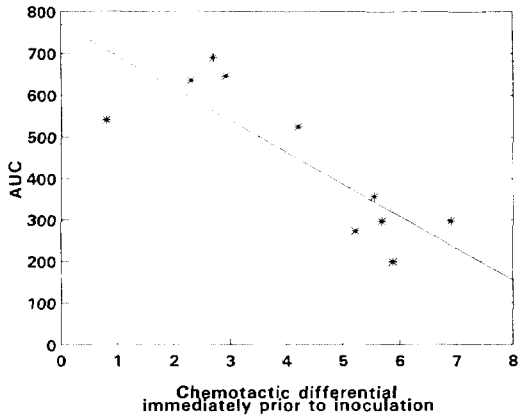

Figure 1. Relationship between the chemotactic differential of white blood cells isolated immediately prior to inoculation and the area under the curve (AUC) (bacterial count time); * = cow. Y = -76.6X + 770.5 + error; r = .83.

related to the AUC ($P < .005$) (Table 3). In other studies, a relationship has been demonstrated between the number of circulating PMNL before infection and the severity of an experimental *E. coli* mastitis (8) or increased incidence of other postpartum disease in cows (18).

The results of the present study suggest that, in healthy cows, the chemotactic response of WBC isolated from peripheral blood immediately prior to inoculation and at d 1 and 2 before inoculation and the number of circulating PMNL immediately prior to inoculation were negatively related to the severity of experimental *E. coli* mastitis. No relationship was demonstrated between the severity of the experimental mastitis and generation of chemiluminescence by either cell suspensions.

CONCLUSIONS

Preinfection chemotactic response of PMNL and preinfection numbers of circulating PMNL appeared to be valuable as predictors of severity of experimental *E. coli* mastitis in cows. Chemotactic response of blood PMNL may be considered to be a marker for host defense capabilities of cows, e.g., in studies concerning differences in susceptibility for mastitis among individual cows or herds of cows. Further investigations are necessary to establish the value of these markers for use in field conditions.

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