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Plasma Corticosteroid, Circulating Leukocyte and Milk Somatic Cell Responses to *Escherichia coli* Endotoxin-Induced Mastitis[1] (37850)

M. J. PAAPE, W. D. SCHULTZE, CLAUDE DESJARDINS[2] AND R. H. MILLER

Animal Physiology and Genetics Institute, Agricultural Research Center ARS, USDA, Beltsville, Maryland 20705; and Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74074

Acute coliform mastitis can be produced in dairy cows by intramammary infusion of *Escherichia coli* (*E. coli*) endotoxin (1). Such an approach is desirable from an experimental standpoint because it evokes coliform mastitis without causing permanent damage to mammary secretory tissue or lowering of subsequent milk production (2).

Leukocyte profiles in blood and milk as well as overt clinical signs following *E. coli* endotoxin-induced mastitis have been elucidated (1, 2). In general, these include: (i) marked depressions in circulating leukocytes consisting primarily of neutropenia, beginning 2 hr postinfusion, (ii) hyperthermia, (iii) marked swelling and hypersensitivity of the infused quarter, and (iv) occurrence of abnormal milk (clots, flakes, off-color).

Pathological changes accompanying an acute exposure to bacterial endotoxins may be ameliorated, in part, by administering corticosteroids (3). In view of this, one might hypothesize that the naturally occurring defense response of the cow during the early stages of mastitis would be one of heightened adrenocorticosteroid secretory activity. This hypothesis was examined in the present study by relating changes in plasma corticosteroid concentration during *E. coli* endotoxin-induced mastitis to changes in leukocyte concentrations in blood and milk.

Materials and Methods. Six Holstein-Friesian cows in the second (4 cows) and the seventh (2 cows) postpartum month of lactation were used. Cows were free from pathogens known to cause mastitis as determined by cultures which were made twice weekly throughout the experiment.

Cannulas (PV-400, Clay Adams, Inc., New York, NY) were inserted into the jugular vein of each cow 24 hr before the start of the study (4). One quarter of each of 4 cows was infused 3 hr after milking with 0.5 mg endotoxin (Lipopolysaccharide B, *E. coli* 026:B6, Catalog No. 3920-25, Difco Laboratories, Detroit, MI) dissolved in 3 ml of 0.85% NaCl prepared in sterile pyrogen free water (Cutter Laboratories, Inc., Berkeley, CA). The solution was infused into the cistern of the mammary gland by inserting a blunted 19 gauge needle through the streak canal. Infused quarters were massaged to disperse the endotoxin throughout the lower portion of the mammary gland. Sterile syringe and needles were used for each infusion. The remaining two cows served as controls and were infused with the vehicle alone.

Blood samples (30 ml) were obtained at 30 min intervals 1 hr before intramammary infusion through 6 hr postinfusion, at hourly intervals through 12 hr postinfusion, and every 24 hr through 216 hr postinfusion. Blood samples were not collected dur-

[1] Journal article 2728 of the Oklahoma State University Agricultural Experiment Station, Stillwater, OK 74074.

[2] Present address: Department of Zoology, The University of Texas, Austin, TX 78712.

ing the 60 min period following milking, because the milking stimulus provokes marked alterations in plasma corticosteroid (4) and circulating leukocyte concentrations (5).

Blood was transferred to 50-ml centrifuge tubes containing 36 mg of disodium ethylenediaminetetraacetate, mixed and placed in an ice bath. A 5 ml portion was taken for total erythrocyte and total and differential leukocyte counts. Total cell counts were made electronically with a Model B Coulter counter using an upper threshold setting of 9. Blood films were prepared for differential leukocyte counts and stained with Wright's stain (5). Blood plasma was obtained after centrifuging the remaining sample at 5000*g* for 30 min at 5°. Aliquots of this sample were subjected to solvent extraction and competitive protein binding analysis to quantify total plasma corticosteroids and all values were corrected for 100% recovery (6). In addition, samples of blood sera were collected from one of the endotoxin-infused cows at −1, 0, 1, 4, 5, 6, 8 and 10 hr postinfusion for glucose analysis (Glucostat, Worthington Biochemical Corp., Freehold, NJ 07728).

Foremilk samples (20 ml) were collected immediately after taking the blood samples from an infused and uninfused quarter (within cow control) of each of the 4 endotoxin-infused cows, and from one quarter of the 2 control cows. Milk films were prepared and stained with Levowitz stain (7), to determine the concentration of total milk somatic cells (neutrophils, lymphocytes, monocytes, and epithelial cells) (8). Additional milk films were prepared from an endotoxin-infused quarter and stained with Pyronin Y-methyl green stain (9) for differential milk somatic cell counts.

After obtaining each sample of blood or milk the infused and control quarters were palpated for signs of swelling and rectal temperatures were obtained.

A mixed-model least squares analysis of variance was performed, fitting effects for treatment groups (endotoxin vs control), cows within treatment group, time intervals, and treatment by time interval interaction. Differences between treatment groups were tested against differences among cows within groups.

Results and Discussion. The total and differential leukocyte profiles for the two control cows remained unchanged ($P > 0.05$) throughout the study. Total circulating leukocyte, neutrophil, lymphocyte, eosinophil and monocyte concentrations averaged 8700, 2300, 5100, 540 and 770 cells/mm^3, respectively. Thus, minor disturbances attributable to blood sampling technique and intramammary infusion of saline were not sufficient to cause detectable changes in these blood constituents.

The dynamic aspects of acute coliform mastitis were readily apparent within 1.5 hr following intramammary infusion of endotoxin as evidenced by a significant ($P < 0.01$) reduction in the concentration of segmented neutrophils (Table I). The concentration of circulating neutrophils declined precipitously over the next 3.5 hr, and by 5 hr only 6% of the original concentration of these cells were present in the circulatory system.

Importantly, significant changes in the concentration of milk somatic cells (Table II) was not detected until 3 hr postinfusion at which time concentrations increased from 5.7×10^4 cells/ml milk at 0 hr to 57.5×10^4 cells/ml. Thus, a time lag of 1.5 hr occurred between the noted depression in circulating segmented neutrophils and the extravascular mobilization of these cells into milk. This time lag may not be attributed to a paralysis in the immigration of these cells into milk but rather to lysis which prevented visually counting these cells. This interpretation is supported by the finding that swelling, was apparent within 2 hr after endotoxin infusion (Table II). Swelling was mediated, in part, by the lysis of newly immigrated blood neutrophils, since Jain, Schalm and Lasmanis (10) failed to detect swelling following intramammary infusion of 5 mg of *E. coli* endotoxin into cows made neutropenic by the intravenous administration of equine antibovine leukocyte serum.

The increase in body temperature fol-

TABLE I. Concentration of Plasma Corticosteroids and Total and Differential Circulating Leukocytes and Erythrocytes Before and After Infusion of *E. coli* Endotoxin into One Quarter of the Bovine Mammary Gland.[a]

Hours relative to infusion of endotoxin	Corticosteroid conc (ng/ml of plasma)	(per mm³ in blood)							Erythrocytes (× 10⁴/mm³ in blood)
		Circulating leukocytes	Neutrophils			Lymphocytes	Monocytes	Eosinophils	
			Juvenile[b]	Band	Segmented				
−1	1.0	6486	30	65	2594	2821	359	515	603
0	6.4	6126	0	61	2389	2757	429	460	604
0.5	3.5	5950	0	60	2410	2618	476	357	530
1	2.8	5558	0	30	2445	2446	333	278	547
1.5	1.8	5470	0	30	1793	2926	410	300	535
2	10.6	4513	0	23	1625	2460	113	248	589
2.5	17.5	3852	0	135	1445	2003	39	250	625
3	17.4	3182	64	159	811	1909	30	207	612
4	25.4	2295	103	115	356	1526	69	126	603
5	22.9	2170	239	163	162	1345	119	108	532
6	8.8	1958	176	235	284	1165	29	68	532
8	8.1	2704	406	379	662	1041	176	41	554
10	8.6	3669	532	807	660	1412	165	55	566
12	1.1	5226	967	653	993	2038	418	157	526
18	4.6	6434	1287	804	1544	2316	290	193	576
24	2.9	7382	812	1107	2325	2510	295	332	565
72	2.0	8342	167	417	3503	2502	1043	500	616
120	4.3	6632	0	332	2653	2288	895	497	571
168	2.0	6463							576
Significance level	—*	—*	—*	—*	—*	—*	—*	—*	NS[c]
Coefficient of variation (%)	73	19	64	73	29	19	57	47	6

[a] Each value represents the mean of 4 cows.

[b] Includes metamyelocyte and myelocyte neutrophils.

[c] NS = not significant ($P > 0.05$).

* $P < 0.01$.

TABLE II. Rectal Temperature, Udder Swelling and Total Milk Somatic Cell Counts Following Intramammary Infusion of *E. coli* Endotoxin.[a]

Hours relative to infusion of endotoxin	Rectal temp (°)	Mammary swelling[b]		Somatic cells[d] ($\times 10^6$/ml in milk)	
		Uninoculated quarter	Inoculated quarter	Uninoculated quarter[c]	Inoculated quarter
0	38.8	—	—	0.124	0.057
0.5	38.8	—	—		0.050
1	38.9	—	—	0.156	0.046
1.5	38.9	—	—	0.158	0.064
2	39.0	—	+	0.156	0.022
2.5	39.3	—	++	0.155	0.022
3	39.6	—	+++	0.136	0.375
4	40.2	—	+++	0.107	1.721
5	40.8		+++		1.740
6	40.9	—	++	0.034	8.490
8	39.9	—	++	0.130	37.261
10	39.1		+		28.943
12	38.7	—	+	0.019	28.311
18		—	—	0.019	53.776
24	38.6	—	—	0.120	38.351
72	38.6	—	—	0.326	19.714
120	38.7	—	—	0.183	1.979
168		—	—	0.120	0.153
216		—	—	0.030	0.052
Significance level	—*			NS[e]	—*
Coefficient of variation (%)	1			200	94

[a] Each value represents the mean of 4 cows.

[b] — = no swelling; + = slight swelling; ++ = moderate swelling; +++ = extreme swelling.

[c] Within cow control quarter.

[d] Includes granulocytes (neutrophils, eosinophils and basophils), agranulocytes (lymphocytes and monocytes) and epithelial cells.

[e] NS = not significant ($P > 0.05$).

* ($P < 0.01$).

lowing endotoxin infusion coincided with the degree of mammary swelling of the infused quarter (Table II). At 2.5 hr postinfusion, body temperatures rapidly increased reaching critical temperatures between 4–6 hr postinfusion. Following this crisis, temperatures quickly returned to normal, with the infused quarter regaining its normal texture shortly thereafter.

A shift left in the differential circulating leukocyte count was initiated 2.5 hr following endotoxin infusion with the appearance of band neutrophils (Table I), normally rare in bovine blood (11). This emphasis toward immature neutrophils, became more pronounced with the appearance of myelocyte and metamyelocytes at 3 hr. With time, the shift left continued to become more pronounced and reached maximum proportions between 18–24 hr. Moreover at this time, the concentration of total milk somatic cells reached enormous proportions averaging at least 1000-fold above control values (Table II). On the basis of these results it is readily apparent that the overwhelming response initiated by endotoxin was one of massive mobilization of immature neutrophils into circulating blood and rapid extravascular migration of these cells into milk as a result of intensive

chemotactic activity in the udder.

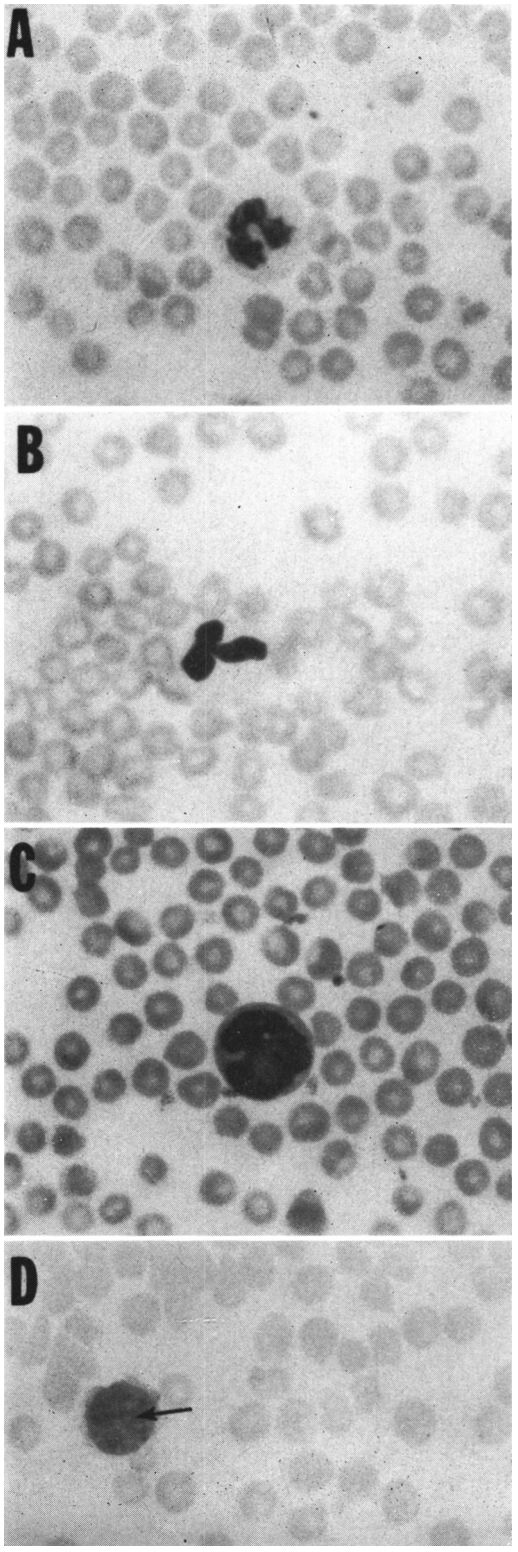
During the left shift, many abnormal neutrophils were observed in peripheral blood smears (Fig. 1). The nuclei of these "toxic" neutrophils (Fig. 1a) were horseshoe-shaped, containing densely clumped chromatin, similar to that found in normal mature segmented neutrophils. Thus it would appear that toxemia expressed itself morphologically by delaying the segmentation of neutrophilic leukocytes. Other signs of toxemia were the appearance of neutrophils with bizarre nuclear patterns (Fig. 1b).

The acute neutrophil response diminished following a peak shift to the left at 18 hr. Attenuation of the shift left began approximately 72 hr (Table I) and by 120 hr, myelocyte and metamyelocytes were no longer present in peripheral blood. However, a significant concentration of band cells was still evident. It appears that the attenuation of the shift left was the result of diminished release of chemotactic factors within the mammary gland, since the massive movement of cells into milk rapidly diminished after 72 hr (Table II). A substantial increase in segmented neutrophils beyond preinfusion levels occurred at 72 hr. This was interpreted as an overshoot by the hemopoietic system in its attempt to restore neutrophil equilibrium.

Further examination of the sequential hemogram (Table I), revealed that lymphocytes and eosinophils were all depressed ($P < 0.01$) as a consequence of the host defense response to endotoxin. The continued decrease in lymphocytes, monocytes and eosinophils was consistent with the findings by Carroll, Schalm and Lasmanis (1) who also reported similar changes in a cow given an intramammary infusion of 20 mg endotoxin. As to the fate of these cells, extravascular mobilization into milk could not account for their decrease since differential milk somatic cell counts of prepared milk films from an endotoxin-infused quarter indicated that milk lymphocyte concentrations were very constant, averaging less than 5.0×10^4 cells/ml of milk throughout the study. Monocyte and eosinophil migration into milk was more difficult to assess because of their low concentrations in peripheral blood and the difficulty in distinguishing these cells once in milk from polymorphonuclear leukocytes.

The initial decline in circulating lymphocytes noted within 1.5 hr after endotoxin infusion was not attributed to the lytic effect known to be produced by corticosteroids (12), because significant depressions in circulating lymphocytes occurred 0.5 hr following the noted increase in plasma corticosteroid concentrations (Table I). Most importantly, definitive studies by this (13) and other laboratories (14) clearly indicate that elevated plasma corticosteroid concentrations seen after adrenocorticotropin (ACTH) stimulation occurs within 15 min following ACTH administration and that significant depressions in the percentage of circulating lymphocytes will not occur until 2 hr post-ACTH injection. Furthermore, the lymphopenia in response to ACTH administration differs markedly from that observed in response to endotoxin. Circulating lymphocyte concentrations following ACTH administration tend to remain unchanged, whereas the percentage of lymphocytes relative to the total leukocyte count is significantly depressed. Such a response was termed "relative lymphopenia" by Smith and Merrill (15) who also observed such changes following intramuscular administration of 250 IU ACTH into mature dairy cows. Thus the decrease in circulating lymphocytes during the first 4 hr following endotoxin infusion may be attributed to either cell lysis as a result of the immune response to endotoxin and/or to their removal by mammary lymph nodes (16).

Lymphocytes with unusual morphological characteristics were found in peripheral blood smears 24–120 hr following endotoxin infusion (Fig. 1). Some of these lymphocytes contained an irregular nucleus whose staining characteristics tended to be more basophilic than normal (Fig. 1c), while in others the nuclear chromatin material was condensed into numerous nuclear whirls (Fig. 1d).



Plasma corticosteroid concentrations ($P < 0.01$) 2 hr following infusion, reached a maximum at 4 hr, and returned to pre-infusion concentrations at 12 hr (Table I). The magnitude and duration of the plasma corticosteroid response to endotoxin noted between 2 and 12 hr was similar to that observed folowing intravenous administration of 100 IU ACTH (13). The elevation in plasma corticosteroids at 2 hr appeared to have little effect at restoring equilibrium in the number of total and differential circulating leukocytes, since the concentration of neutrophils and total leukocytes did not increase until 6 hr after the noted elevation in plasma corticosteroids. Thus it would appear that immigration of neutrophils to the mammary gland caused by endotoxin infusion, overshadowed any neutrophil potentiating effect characteristically associated with corticosteroids.

Serum glucose determinations performed on samples collected from one of the endotoxin-infused cows averaged 62, 72, 73, 140, 126, 95, 102, 75 and 70 mg % at −1, 0, 1, 4, 5, 6, 8, and 10 hr postinfusion, respectively. Peak serum glucose concentrations coincided with peak plasma corticosteroid concentrations. Others (1) observed 2-fold increases in serum glucose 5 hr following intramuscular infusion of endotoxin in cows, suggesting a cause-and-effect relationship. Thus it would appear that one of the major physiological effects derived from elevated plasma corticosteroid concentrations was not related to the neutrophil potentiating effect, but to its gluconeogenic effect. Further evidence supporting this conclusion stems from the finding that increased survival of mice given a lethal injection of endotoxin and a therapeutic dose of cortisone was related primarily to

FIG. 1. Abnormal cells commonly found in peripheral blood smears following intramammary infusion of *E. coli* endotoxin: (A) Neutrophil showing evidence of delayed maturation; Wright's stain, ×1250; (B) neutrophil with bizarre nuclear pattern; Wright's stain, ×1250; (C) lymphocyte with an irregular shaped nucleus; Wright's stain, ×1250; (D) lymphocyte containing numerous nuclear whirls (arrow).

carbohydrate metabolism (3).

Summary. A mammary quarter in each of 4 cows was infused with 0.5 mg *E. coli* endotoxin to determine its effect on plasma corticosteroids, circulating leukocytes and milk somatic cells. At 1.5 hr following endotoxin infusion, segmented neutrophils decreased 23%, and by 5 hr dropped 93%. A shift left in the differential circulating leukocyte count began at 2.5 hr and reached maximum proportions between 18–24 hr postendotoxin infusion. Circulating lymphocytes, monocytes and eosinophils were all depressed in response to endotoxin. Plasma corticosteroid concentrations increased ($P < 0.01$) 2 hr following endotoxin infusion, reached maximum concentrations at 4 hr, then returned to preinfusion concentrations by 12 hr. The initial decrease in circulating lymphocytes at 2.5 hr was not attributed to the lytic effect of the plasma corticosteroids, since depressions in circulating lymphocytes occurred too soon (0.5 hr) after the increase in plasma corticosteroids. The high plasma corticosteroid concentration appeared to have little effect at restoring leukocyte equilibrium, since the concentration of neutrophils and total leukocytes continued to decline up to 4 hr past the initiation of the plasma corticosteroid response. Serum glucose concentrations coincided with peak plasma corticosteroid concentration and appeared to be one of the primary physiological effects mediated by elevated plasma corticosteroid concentrations. Three hours following endotoxin infusion or 1.5 hr after the noted depression in segmented neutrophils, the concentration of milk somatic cells increased from 5.7×10^4 cells/ml milk at 0 hr to 37.5×10^4 cells/ml. However swelling of infused quarters was detected as early as 2 hr postinfusion and was attributed to diapedesis and lysis of neutrophils. Total milk somatic cell concentrations reached maximum proportions at 18 hr, averaging 54×10^6 cells/ml milk.

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