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Ingestion and Killing of *Listeria monocytogenes* by Blood and Milk Phagocytes from Mastitic and Normal Cattle

CHARLES J. CZUPRYNSKI,[1]* ELISABETH J. NOEL,[1] MICHAEL P. DOYLE,[2] AND RONALD D. SCHULTZ[1]

Department of Pathobiological Sciences, School of Veterinary Medicine,[1] and Food Research Institute,[2] University of Wisconsin—Madison, Madison, Wisconsin 53706

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Human listeriosis resulting from consumption of listeria-contaminated dairy products is emerging as a significant public health concern. There is a need to understand better the processes involved in the pathogenesis of *Listeria monocytogenes*-induced bovine mastitis. In the present report, we describe the results of the in vitro interaction of *L. monocytogenes* with bovine blood and milk leukocytes. Induction of an experimental *L. monocytogenes* mastitis resulted in a rapid and dramatic increase in neutrophils in the milk of infected cows. Blood neutrophils and mononuclear cells and milk leukocytes from listeria-infected and uninfected cows readily ingested *L. monocytogenes* in the presence of serum opsonins. These leukocytes also killed a portion of the ingested listeriae. Ingestion of listeriae evoked a vigorous chemiluminescence response by blood neutrophils and a relatively weak response by blood mononuclear cells. Ingestion, killing, and chemiluminescence by milk leukocytes were directly related to the percentage of neutrophils that were present. Blood neutrophils from healthy donor cattle ingested and killed *L. monocytogenes* when leukocyte-depleted milk and whey from mastitic cows were the sole sources of opsonins, although fewer listeriae were ingested than when normal bovine serum was present. These results indicate that bovine blood and milk phagocytes, like blood and inflammatory phagocytes from other mammalian species, can ingest and kill *L. monocytogenes* in vitro.

Listeriosis has achieved considerable prominence as a serious public health concern as a result of several large outbreaks that were associated with ingestion of listeria-contaminated food (16, 20, 32). Milk and other dairy products were implicated as sources of listeriae in several widely publicized incidents, thus suggesting that the mammary glands of mastitic cattle may be an important reservoir in the pathogenesis of human listeriosis (4, 15). Listeria mastitis presents a particularly insidious problem because the milk obtained from subclinical mastitic cows may be relatively normal in appearance even when significant numbers of listeriae are being shed (13, 15, 18). The well-recognized ability of *Listeria monocytogenes* to multiply at refrigeration temperatures (4 to 10°C) (17) exacerbates the threat posed by storage and consumption of listeria-contaminated dairy products. Awareness and concern about the presence of listeriae in dairy products is very high and is likely to impart a significant economic loss on dairy producers and processors as they attempt to eliminate listeriae from dairy products targeted for human consumption. Although listeriosis is widely studied as a model for the role of cellular immunity in antibacterial resistance, there is surprisingly little information about the pathogenesis of listeriosis in nonrodent mammalian species in general and in cattle in particular (1, 7, 15, 22, 24, 25). We have been unable to locate any reports in the scientific literature describing the outcome of the in vitro interaction of *L. monocytogenes* with bovine phagocytes.

In the present study, we examined the ability of bovine phagocytes to ingest *L. monocytogenes*, produce an oxidative response, and kill the intracellular listeriae. Peripheral blood neutrophils, mononuclear cells, and milk leukocytes were obtained from healthy donor cattle and from cattle experimentally infected with *L. monocytogenes*. The results indicate that bovine phagocytes, like human blood phagocytes (9, 28, 35) and murine inflammatory peritoneal phagocytes (11), are able to ingest opsonized *L. monocytogenes* and inactivate at least a portion of the ingested bacteria.

* Corresponding author.

MATERIALS AND METHODS

Bacteria. A strain of *L. monocytogenes* (Scott A) originally isolated from a patient with listeriosis (14) was used in this study. The bacteria were stored as aliquots in tryptose phosphate broth at −70°C. Before each experiment, an aliquot was thawed and a portion was inoculated into 5.0 ml of tryptose phosphate broth. The listeriae were incubated at 37°C for approximately 18 h, recovered by centrifugation, washed twice in Hanks balanced salts solution that contained 0.25% bovine serum albumin (HBSA), and suspended in their original volume in HBSA. The bacterial concentration of the suspension was estimated by spectrophotometric determination of optical density and confirmed by plate counts on tryptic soy agar (Difco Laboratories, Detroit, Mich.).

Animals. The three cows used as cell donors in the initial experiments (see Fig. 1 to 4) were adult (3- to 6-year-old) lactating Holstein cows producing 22.7 to 27.2 kg of milk per day. All three cows had low titers of serum agglutinins (<1:20) to the strain of *L. monocytogenes* used in this study, suggesting that they had not been recently infected with the organism.

Inoculation of cows. The animals described in this article were part of a study whose purpose was to investigate the resistance to pasteurization of *L. monocytogenes* shed in milk during experimental infection (13). Owing to the lack of published information on establishing experimental *L. monocytogenes* infections in cattle, each animal was inoculated several times with viable *L. monocytogenes*. The first cow (no. 24) was inoculated in the tonsils with 2×10^9 *L.*

monocytogenes, the second cow (no. 193) was similarly inoculated in the tonsils and received an additional intubation of 5×10^{11} *L. monocytogenes* in the rumen. Three weeks later, both cows were inoculated with 1×10^5 to 2×10^5 listeriae in the left front quarter of the udder via the teat canal. The third cow used in these experiments (no. 62) was an uninfected control. The clinical course of the infection was described previously (13).

Bovine blood and milk leukocytes. Bovine blood leukocytes were obtained and separated into granulocyte-rich and mononuclear cell-rich populations by using Ficoll-Hypaque density gradients as described previously (10). Milk leukocytes were recovered from fresh milk by centrifugation, washed several times, and suspended in HBSA (35). Because of poor cell yields, milk leukocytes were not separated on density gradients into granulocyte and mononuclear cell-enriched populations but instead were used as an unseparated cell population. Blood leukocyte numbers were determined by dilution and quantitation in an automated electronic cell counter (Coulter Electronics, Inc., Hialeah, Fla.). Total numbers of milk leukocytes were estimated by dilution and microscopic counting by using a hemacytometer. Cytocentrifuge smears (Shandon Scientific Co., London, England) were prepared for each cell suspension, stained with Diff-Quik (American Scientific Products, McGaw Park, Ill.), and examined microscopically to determine the differential leukocyte count. Viability of leukocyte suspensions was estimated by trypan blue exclusion.

Sera, leukocyte-depleted milk, and whey. Pooled bovine sera from healthy adult cattle were used as an opsonin source in most experiments. These sera were heat inactivated (56°C for 30 min) to remove nonspecific antibacterial activity. After milk leukocytes were harvested by centrifugation as described above, the leukocyte-depleted milk from each animal at each time point was stored separately at −70°C. To obtain whey, thawed milk was acidified to approximately pH 4.6 by the addition of HCl and the precipitated casein was removed by centrifugation; the pH of the whey was adjusted to 6.8 by the addition of NaOH (30).

Phagocytosis and bactericidal activity. Ingestion of *L. monocytogenes* was determined by using previously described methods (9, 11). Briefly, 2.5×10^6 leukocytes and 12.5×10^6 *L. monocytogenes* cells were suspended in 1.0 ml of HBSA that contained 2.5 to 10% pooled normal bovine serum. Duplicate tubes were rotated at 37°C for 30 min. Afterwards, the leukocytes were recovered and washed free of unassociated listeriae by three slow-speed washes ($100 \times g$ for 5 min at 4°C) in cold HBSA. The leukocytes then were resuspended in HBSA, and the suspensions were used to prepare cytocentrifuge smears. At least 200 leukocytes on each smear were scored microscopically (×1,000 magnification) for ingestion of listeriae. Results are expressed as a phagocytic index calculated as follows: phagocytic index = (percent leukocytes that contain bacteria × mean number of bacteria per ingesting leukocyte) × 100. We determined previously that this technique removes uningested bacteria and gives a reasonable estimation of phagocytic activity (9–11).

Bacterial killing was determined as described previously for *L. monocytogenes* (9, 11) and other bacterial targets (10). Briefly, suspensions of 2.5×10^6 leukocytes and 2.5×10^6 *L. monocytogenes* were rotated (8 rpm; Labquake Shaker; Labindustries, Berkeley, Calif.) at 37°C for 2 h in a total volume of 1.0 ml of HBSA that contained 2.5 to 10% pooled bovine serum. Samples were removed, diluted in sterile distilled water, and plated on tryptic soy agar. The plates were incubated at 37°C for 24 h, and the colonies were counted to determine the number of viable *L. monocytogenes*. Results are expressed as the $\log_{10}$ decrease in the number of viable *L. monocytogenes* remaining after incubation compared with the number present at the beginning of the incubation period.

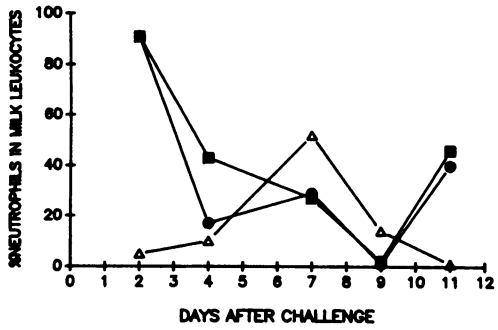

FIG. 1. Percentage of neutrophils in milk leukocytes obtained at various times after intramammary challenge with *L. monocytogenes* (■, cow 193; ●, cow 24). An uninoculated control is also included (△, cow 62).

Luminol-dependent chemiluminescence. Leukocyte oxidative activity in response to ingestion of opsonized listeriae was determined by luminol-dependent chemiluminescence as described previously (10). Briefly, leukocytes (5×10^5), preopsonized *L. monocytogenes* (5×10^7), and luminol (5×10^{-6} M) were incubated in a total volume of 0.3 ml of phenol red–free Hanks balanced salts solution that contained 5% fetal bovine serum at 39°C in a Packard Autopicolite Luminometer (Los Alamos Diagnostic Laboratories, Los Alamos, N. Mex.). The emission of light by each reaction tube was measured for 5 s in each cycle for a total of eight cycles over a 35-min incubation period.

RESULTS

Effects of *L. monocytogenes* infection on percentage of neutrophils in bovine milk. Intramammary challenge with *L. monocytogenes* resulted in a rapid and marked influx of neutrophils into the mammary gland; at 2 days after challenge, more than 90% of the leukocytes in the milk were neutrophils (Fig. 1). The percentage of neutrophils decreased steadily through 9 days after challenge and then unexpectedly rebounded to a relatively high level at 11 days after challenge. The percentage of neutrophils in milk from the control animal was relatively low (<15%) except on day 7, at which point approximately 50% of the leukocytes in the milk from this animal were neutrophils. We have no ready explanation for this anomalous increase in neutrophils unless a spontaneous subclinical mastitis occurred.

Ingestion of *L. monocytogenes* by blood and milk leukocytes. Bovine blood neutrophils from infected and uninfected cattle readily ingested *L. monocytogenes* in vitro (Fig. 2A). Blood mononuclear cells also ingested *L. monocytogenes*, although to a lesser extent than did neutrophils (Fig. 2B). Milk leukocytes obtained from the two listeria-infected cows (nos. 193 and 24) at 2 days after challenge avidly ingested *L. monocytogenes* in vitro (Fig. 2C). Microscopic examination indicated that ingestion of *L. monocytogenes* by milk leukocytes was done almost entirely by neutrophils; few mononuclear cells appeared to contain listeriae. This is also reflected in the decreased ingestion of *L. monocytogenes* by

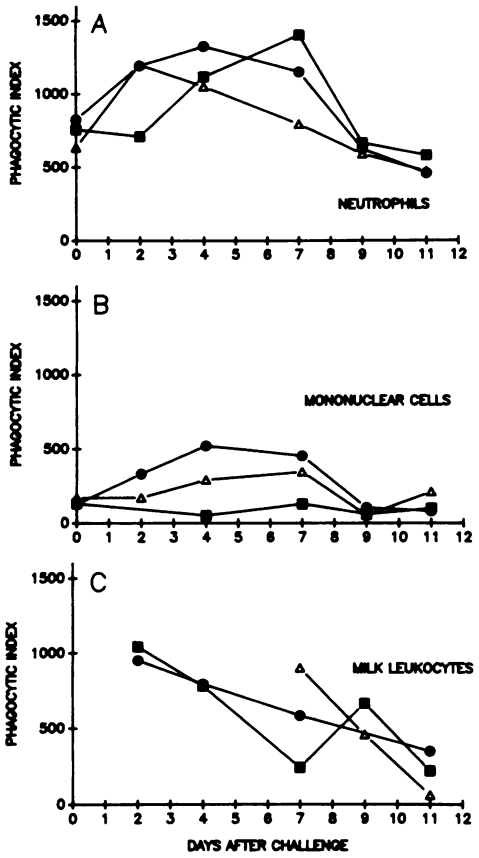

FIG. 2. Ingestion of *L. monocytogenes* in vitro by bovine blood neutrophils (A), blood mononuclear cells (B), and milk leukocytes (C) at various times after intramammary *L. monocytogenes* challenge (■, cow 193; ●, cow 24). An uninoculated control is included (△, cow 62). Results are expressed as the phagocytic index (see Materials and Methods).

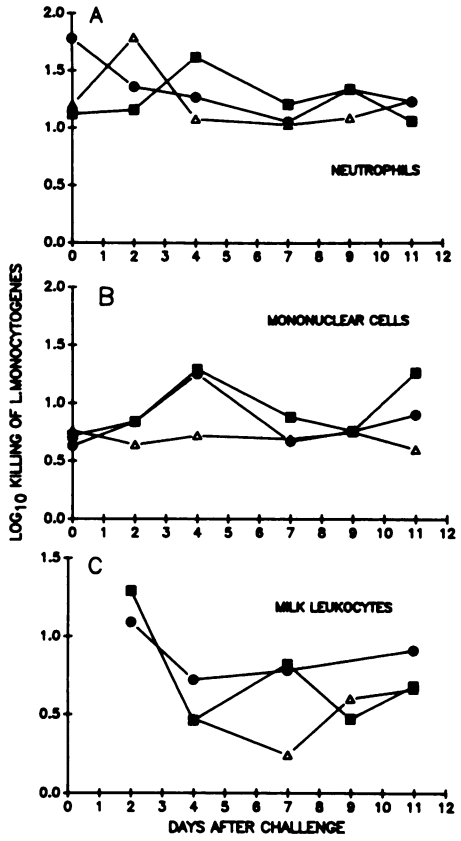

FIG. 3. Killing of *L. monocytogenes* in vitro by blood neutrophils (A), blood mononuclear cells (B), and milk leukocytes (C) at various times after intramammary *L. monocytogenes* challenge (■, cow 193; ●, cow 24). An uninoculated control is included (△, cow 62). Results are expressed as the $\log_{10}$ reduction in viable listeriae during a 2-h incubation period.

milk leukocytes with time after challenge (Fig. 2C) as the number of neutrophils in the milk coordinately declined (Fig. 1). Because of the low numbers of neutrophils initially present in the milk of the uninfected control cow (no. 62), we were unable to assess ingestion of listeriae; and it was only after this animal displayed an anomalous increase in milk neutrophils that ingestion of listeriae comparable to that demonstrated by the milk leukocytes of the infected animals was observed (Fig. 2C).

Killing of *L. monocytogenes* by blood and milk leukocytes. Bovine blood neutrophils taken at various times from both infected and uninfected animals exhibited moderate listericidal activity, killing 1.0 to 1.75 $\log_{10}$ *L. monocytogenes* (Fig. 3A). Bovine blood mononuclear cells demonstrated somewhat less listericidal activity. Although mononuclear cells from the two infected cattle killed somewhat better at 4 days after challenge, the general levels of killing by cells from infected and uninfected cattle were similar (Fig. 3B). The level of killing by milk leukocytes was similar to that of blood mononuclear cells except at 2 days after challenge, at which time the high percentage of neutrophils (>90%) in the milk leukocyte population was associated with a somewhat greater killing of listeriae (Fig. 3C).

Chemiluminescence response of blood and milk leukocytes to *L. monocytogenes*. We next determined the oxidative response of bovine blood and milk leukocytes to opsonized *L. monocytogenes*. As might be expected, *L. monocytogenes* elicited vigorous chemiluminescence responses by blood neutrophils that were similar for neutrophils from infected and uninfected cattle (Fig. 4A). In comparison, blood mononuclear cells gave a relatively weak chemiluminescence response (Fig. 4B), an observation that is consistent with results from previous studies that suggested that bovine blood monocytes have little if any chemiluminescence activity (T. Phillips and R. Schultz, unpublished observations). The chemiluminescence response of milk leukocytes (Fig. 4C) appeared to reflect the percentage of neutrophils present in the milk at the various time points, the response being highest for leukocytes obtained from listeria-infected cattle at 2 and 11 days after challenges, times when the greatest percentage of neutrophils was present in the milk leukocyte suspensions (Fig. 1).

Effects of milk and whey on neutrophil ingestion and killing of *L. monocytogenes*. The above-described experiments suggest that bovine neutrophils are capable of ingesting and killing *L. monocytogenes* and that the level of antilisteria activity of milk leukocytes is dependent on the number of neutrophils present in the milk. These results were obtained by using serum rather than cell-free milk and whey as the opsonin source. Because others have suggested that components in normal and mastitic milk influence bovine milk neutrophil phagocytic and bactericidal activities (26, 27, 30), we decided to examine the effects of the fluid-phase compo-

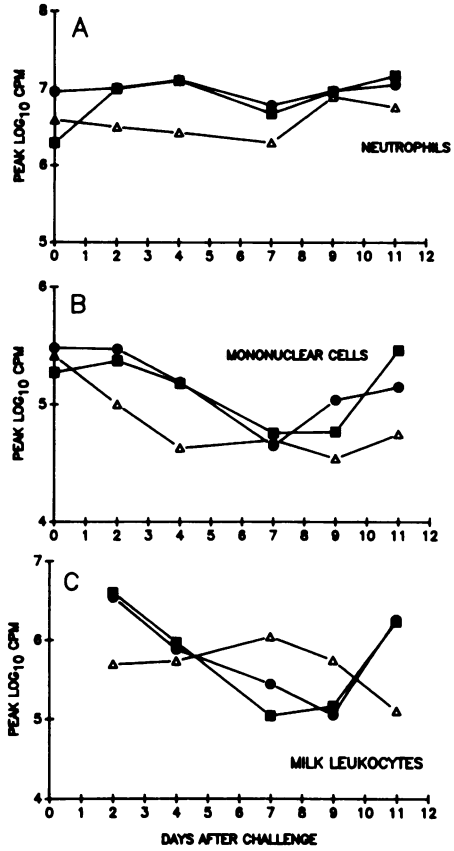

FIG. 4. Chemiluminescence response of bovine blood neutrophils (A), blood mononuclear cells (B), and milk leukocytes (C) at various times after intramammary *L. monocytogenes* challenge (■, cow 193; ●, cow 24). An uninoculated control is included (△, cow 62). Results are expressed as the peak response (log_{10} counts per minute) to opsonized listeriae (100 listeriae per leukocyte). Similar results were obtained when the cumulative chemiluminescence responses (total counts per minute) were compared.

nents of milk on bovine neutrophil antilisteria activity. Blood neutrophils were obtained from healthy uninfected donors and assessed for their ability to kill *L. monocytogenes* in the presence of cell-depleted milk (25% final concentration) or whey (10% final concentration) obtained at various times before and after intramammary challenge of the listeria-infected cattle described in Fig. 1 to 4. As shown in Table 1, neither neutrophils, serum, nor cell-free milk alone was listericidal; rather, 0.3 to 0.8 log_{10} growth of *L. monocytogenes* occurred during 2 h of incubation with any one of these components. As demonstrated above (Fig. 3), the combination of neutrophils and serum resulted in significant killing of listeriae (Table 1). Cell-depleted milk obtained from one listeria-infected donor (no. 24) at 2 to 11 days after *L. monocytogenes* challenge promoted killing of listeriae by blood neutrophils from two normal donors. The ability of cell-depleted milk to serve as an adequate source of opsonins was confirmed in subsequent experiments in which we observed that whey prepared from the milk that was collected from cow 24 at 2 and 4 days after challenge promoted ingestion of *L. monocytogenes* in a dose-dependent manner (Table 2), although to a lesser extent than did 2.5% pooled serum.

TABLE 1. Killing of *L. monocytogenes* by normal blood neutrophils incubated in cell-depleted mastitic milk in vitro

Neutrophils	2.5% Serum[a]	25% Milk[a,b]	Log_{10} killing of *L. monocytogenes*[c]	
			Expt 1	Expt 2
+	−	Day 2	1.44	0.89
+	−	Day 4	1.84	0.80
+	−	Day 7	0.89	0.91
+	−	Day 9	1.83	0.89
+	−	Day 11	0.84	0.90
+	+	−	1.16	0.74
+	−	−	−0.34	−0.13
−	−	Day 2	−0.78	−0.47
−	+	−	−0.50	−0.26

[a] *L. monocytogenes* (2.5×10^6) was incubated in the presence (+) or absence (−) of the indicated components for 2 h at 37°C as indicated in Materials and Methods.

[b] Neutrophils and *L. monocytogenes* were incubated with cell-depleted milk (25% final volume) obtained from experimentally infected cow 24 at the indicated times after intramammary *L. monocytogenes* challenge. Similar results were obtained in other experiments that used cell-depleted milk from listeria-infected cow 193.

[c] Log_{10} killing of *L. monocytogenes* during a 2-h incubation period in vitro by using blood neutrophils from two healthy donor cattle (experiments 1 and 2) that were not inoculated with *L. monocytogenes*. The negative sign signifies that the listeriae increased by the indicated amount during the 2-h incubation period.

DISCUSSION

The results of this study provide unique information on the in vitro interaction of *L. monocytogenes* with bovine phagocytes. It was surprising to find that there are no previous reports on this subject, especially since there is well-documented evidence for the pathogenicity of *L. monocytogenes* for cattle. Because listeria contamination of cow's milk has been linked to human cases of listeriosis (4, 14), elucidation of bovine antilisteria defense mechanisms has important implications for both human and veterinary medicine.

Although the small number of animals examined in this study limits the inferences that can be made from our data,

TABLE 2. Dose-dependent effects of whey as an opsonin source for ingestion of *L. monocytogenes* by blood neutrophils from healthy donor cattle

Expt	Opsonin source (%)	% Infected neutrophils	No. of bacteria/ neutrophil	Phagocytic index
1[a]	Whey			
	20	76	4.2	319
	10	50	3.6	180
	5	43	3.3	142
	1	3	1.7	5
	Serum (2.5)	90	7.5	675
2[b]	Whey			
	25	50	4.8	240
	20	38	4.8	182
	10	17	5.3	90
	5	5	4.9	25
	1	1	2.0	2
	Serum (2.5)	52	7.2	374

[a] Whey obtained from cell-depleted milk of experimentally infected cow 24 at 2 days after *Listeria* challenge.

[b] Whey obtained from cell-depleted milk of experimentally infected cow 24 at 4 days after *Listeria* challenge.

several points seem clear. The first is that bovine neutrophils and mononuclear cells can readily ingest virulent *L. monocytogenes*. Because ingestion required only a small amount (2.5%) of pooled normal bovine serum, it seems likely that serum opsonins in uninfected cattle should be adequate for host defense. Blood neutrophils from infected and uninfected cattle were able to kill >1.0 $\log_{10}$ *L. monocytogenes*; blood mononuclear cells exhibited somewhat less listericidal activity. These results are similar to previously reported killing of *L. monocytogenes* by human blood neutrophils and mononuclear cells (9, 28, 36) and by murine inflammatory peritoneal phagocytes (11). Milk leukocytes exhibited a variable ability to kill *L. monocytogenes*, the magnitude of which appeared to be directly related to the numbers of neutrophils in the milk leukocyte population at a given time. Previous studies have suggested that variability in bovine milk neutrophil phagocytic and bactericidal activities may be related to in vivo ingestion of lipid (26) or casein (30) that is present in the milk. The vigorous chemiluminescence response elicited from bovine leukocytes, particularly neutrophils, suggests that oxygen-dependent microbicidal mechanisms were operative during ingestion of *L. monocytogenes* by bovine neutrophils. Although this observation is consistent with the participation of reactive oxygen intermediates in the antilisteria activity of bovine phagocytes, there is controversy regarding the importance of oxygen-dependent mechanisms in the killing of *L. monocytogenes* by phagocytic cells (16, 40). The possible contributions of oxygen-independent killing mechanisms, such as cationic peptides, to phagocyte killing of *L. monocytogenes* (33) may also be worthy of consideration because of the large amounts of these microbicidal peptides that are present within bovine neutrophils (31).

It has been suggested that a component of mastitic milk, perhaps immune complexes, inhibits phagocytosis by bovine neutrophils (37, 38). These inhibitory effects have been observed in the presence of undiluted mastitic milk (38) but not when the final concentration of milk was 25%, as in the present study. In our experiments (Tables 1 and 2), leukocyte-depleted milk and whey from *Listeria*-infected cows promoted killing and ingestion of *L. monocytogenes* by blood neutrophils from healthy donor cattle. These data suggest that milk contains sufficient levels of opsonins for ingestion of listeria to occur, albeit somewhat lower than in the presence of 2.5% pooled bovine serum. Bovine whey has been shown to opsonize other unencapsulated bacteria for phagocytosis by bovine neutrophils (2, 8, 19).

There is a paucity of literature on bovine listeriosis with which to compare the results of the present study. Although *L. monocytogenes* is well recognized as the causative agent of circling disease (meningoencephalitis) of ruminants (1) and has been reported to cause mastitis in cattle (14, 15), the host-pathogen interactions that occur in bovine listeriosis remain largely unidentified. Limited information is available regarding the pathogenesis of listeriosis in other ruminant species (e.g., sheep). During a natural outbreak of ovine listeriosis, some protection was transferred by whole blood, but not serum, from immune sheep (24). This observation may indicate that immune T lymphocytes from previously infected sheep can transfer cellular resistance to *L. monocytogenes* in a manner similar to what has been thoroughly described in experimental studies of murine listeriosis (21). Peritoneal exudate macrophages and sera from immune sheep were able to inhibit the growth of *L. monocytogenes* in vitro, whereas growth of listeriae occurred when immune macrophages were incubated with listeriae and normal sera or nonimmune macrophages were incubated with immune or normal serum (23). Ingestions of listeriae by sheep macrophages in that study were equivalent regardless of the immune status of the macrophages or sera. These results suggest that immunoregulation of the pathogenesis of listeriosis in ruminants is generally similar to what has been reported for experimental listeriosis in laboratory rodents.

The results of the present study should be interpreted in reference to the recent outbreaks of listeriosis that resulted from ingestion of listeria-contaminated dairy products. Mastitis likely results from mechanical implantation of *L. monocytogenes*, which is common in silage and feces (39). *Listeria* mastitis can be chronic or acute, and it may be subclinical or clinical. A subclinical mastitis would not result in obvious changes in milk quality (13, 15). The reported invasive ability of *L. monocytogenes* (3, 7, 22, 29, 34) and the phagocytic ability of the bovine mammary epithelium (5) suggest that invasion of mammary epithelial cells by listeriae may play some part in the pathogenesis of listeria mastitis. The results of the present study and of a preceding report (13) indicate that neutrophils are the predominant cell type in bovine milk during the acute response to *Listeria* infection and that these neutrophils ingest and kill a portion of the listeriae. Some of the listeriae avoid destruction, however, and may be protected by their intracellular location against thermal inactivation during subsequent pasteurization (6, 13).

Although prevention of *Listeria* mastitis, as for most forms of mastitis, might be best achieved by proper management and hygiene, the potential efficacy of vaccination might also be considered. Immunization has been shown to enhance systemic antilisteria resistance and to increase the local accumulation of listericidal neutrophils in mice (12). The bovine mammary gland is a unique organ that might be used to study the local effects of systemic immunization on antilisteria resistance, in the hope of obtaining information that might be beneficial in better understanding the pathogenesis of this important ruminant and human pathogen.

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