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Complement Fragment C5a and Inflammatory Cytokines in Neutrophil Recruitment during Intramammary Infection with *Escherichia coli*

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Generation of inflammatory mediators and leukocyte recruitment to infection at an epithelial surface were studied during *Escherichia coli*-induced mastitis. One uninfected gland of each of eight midlactation cows was challenged with only 30 CFU of *E. coli* McDonald strain 487, a serum-resistant isolate from a cow with mastitis. Bacteria grew logarithmically during the first 10 to 12 h after challenge, reaching concentrations of more than 10^5 CFU/ml with no detectable host response during this time. An intense inflammatory reaction began approximately 12 h after the challenge and was characterized by a breakdown in the blood-milk permeability barrier followed by pyrexia and a pronounced leukocytic influx. Coincident with the onset of mammary inflammation was the appearance of neutrophil chemotactic activity in the milk from infected glands. Factors able to upregulate CD18 expression on peripheral blood neutrophils also appeared in milk at this time. The lack of appearance of chemotactic and CD18-upregulating activities until 12 h after challenge indicated that delays in neutrophil recruitment resulted from an initial lack of bacterial recognition and inflammatory mediator production. Production of complement fragment C5a, tumor necrosis factor, and interleukin-1 (IL-1) occurred earlier than production of IL-6 or IL-8. The early and intense production of C5a indicates that this chemoattractant may be more important than IL-8 during the initial recruitment and activation of neutrophils to a developing *E. coli* infection.

Neutrophils are highly effective phagocytes for clearing an infecting bacterial pathogen from host tissue. Before neutrophils can phagocytose bacteria, they must migrate to the site of infection. The physical process of neutrophil recruitment begins with increased endothelial expression of the adhesion molecules E-selectin and P-selectin which bind weakly to neutrophil ligands (25, 27, 49). This weak interaction and exposure to inflammatory mediators effects an increase in the expression and adhesiveness of CD11b/CD18 on neutrophils (22, 23). Neutrophils can then bind tightly to endothelial ICAM-1 and migrate into the tissues toward a gradient of chemoattractant. The importance of these molecular interactions for host defense is exemplified by individuals of various species lacking CD18 or L-selectin (3, 11, 40, 48). Neutrophil recruitment in such individuals is profoundly impaired, and a high susceptibility to infection is seen even with normally nonpathogenic bacteria.

To elicit neutrophil migration, inflammatory mediators must be produced after bacteria in the infected tissue are recognized. The number of known mediators of inflammation is large and includes complement fragments, arachidonic acid metabolites, vasoactive amines, and cytokines. In an appropriate model, each mediator can be shown to play an important role in the inflammatory process. Several recent studies have shown local production of inflammatory cytokines during mucosal infection, suggesting their involvement in the recruitment of host defenses to an epithelial surface. Cytokines produced during urinary tract infection induced by *Escherichia coli* challenge include tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and IL-8 (2, 15, 37), while another study showed local production of these cytokines during experimental *Actinobacillus pleuropneumoniae* infection of the lung (4). One of these studies also showed a close temporal correlation between IL-8 production and the number of neutrophils migrating to the site of infection (2). Thus, it is clear that cytokines capable of recruiting host defenses are produced in abundance during infection at an epithelial surface.

Naturally occurring infections develop from a low-dose inoculum that is followed by bacterial growth and establishment of infection. In previous studies, inoculum doses as high as 10^8 to 10^9 CFU were used to establish infection. These doses caused a prolonged infection that permitted the study of cytokines during an established infection. However, the high bacterial concentration after challenge made it difficult to study inflammatory cytokine production and the host response during the developing phase of an infection. In addition, some investigators have expressed concern that a large challenge inoculum could provide a misleading cytokine profile in experimental models of sepsis (7), and some of the same concerns could be true for mucosal infections.

The milk compartment of the mammary gland is normally a sterile environment. Experimental infection of the bovine mammary gland can be achieved efficiently by intramammary inoculation of less than 50 CFU of *E. coli*, and repeated samples from the site of infection and inflammation can be obtained simply by milking the gland. Therefore, experimental bovine mastitis provides a useful model to study the early host response and production of inflammatory mediators during *E. coli* infection at an epithelial surface. Furthermore, clinical mastitis caused by intramammary infection with coliform bacteria, including *E. coli*, is common among dairy cows on commercial farms because the mammary gland is susceptible to infection with *E. coli* from the cow's environment (8, 19).

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Experimental *E. coli* mastitis is essentially identical to these natural cases. Previous research has shown that the severity of *E. coli* mastitis may be increased in some cows because of an inability to recruit neutrophils into the milk compartment (16, 17). Therefore, a better understanding of the inflammatory mediators involved in neutrophil recruitment could lead to improved preventive programs.

Objectives of this experiment were to study neutrophil recruitment during the early phase of an *E. coli* infection and to better define the early cascade of inflammatory mediators involved in this response. This research demonstrated that a delay in neutrophil recruitment occurs after intramammary *E. coli* challenge despite intense bacterial multiplication soon after the challenge. The results also showed that the complement fragment C5a may be an important chemoattractant in the early recruitment of neutrophils across an epithelium as its generation was abundant and preceded that of IL-8.

MATERIALS AND METHODS

Animals. Thirteen clinically healthy Holstein cows in midlactation were used for this experiment. Nine of these cows were experimentally infected in one randomly selected mammary gland that was free of bacterial infection prior to challenge. An intramammary infection did not develop in one challenged cow, as indicated by the failure to culture *E. coli* from the milk of this cow at any subsequent time. This challenged gland also failed to exhibit any sign of inflammation; therefore, all data from this cow were excluded from the results. Four other cows served as nonchallenged controls. All cows were milked by machine every 12 h.

Bacterial challenge. *E. coli* McDonald strain 487, a natural bovine mastitis isolate, was used for the intramammary challenge (26). This isolate was found to be serum resistant (18, 20). Within 2 days of an experiment, *E. coli* cultures were grown for approximately 24 h on blood agar. One isolated colony was used to inoculate 10 ml of Todd-Hewlett broth. This culture was then incubated for 6 h at 37°C and refrigerated before dilution plating to determine the bacterial concentration. Two hours before infusion, the broth culture was diluted in low-endotoxin Earle's balanced salt solution to a bacterial concentration of 15 CFU/ml. Challenged glands were infused with 2 ml (~30 CFU) of diluted *E. coli* immediately following the morning milking.

Sample collection. Prior to each milking, samples for analysis were collected by hand-milking approximately 50 ml of milk from the teat of each experimental gland. The first portion of milk from infected glands was collected aseptically for bacteriological analysis. Milk samples were also collected at 2-h intervals for the first 18 h after the challenge and then at 6-h intervals for the next 18 h. Rectal temperatures and blood samples from the coccygeal vein or artery were also taken at these times. A portion of each milk sample was preserved with bronopol for cell count analysis. The remaining foremilk was ultracentrifuged (48,000 × *g* for 40 min) to remove fat and casein, and the whey was harvested and stored frozen (−20°C). Serum was harvested from coagulated blood by centrifugation and stored frozen (−20°C).

Analyses. Somatic cell counts (SCC) in milk samples were determined electronically (Fossamatic 360; A/S N. Foss Electric, Hillerød, Denmark) at a commercial milk analysis laboratory (Wisconsin DHI Cooperative, Menomonie, Wis.). Based on experience with experimentally induced *E. coli* mastitis, samples from infected glands that were anticipated to have high cell numbers were diluted 1:10 in phosphate-buffered saline (PBS) before analysis. The bovine serum albumin concentration in whey samples was measured by an enzyme immunoassay as described previously (38). Concentrations of C5a/C5a-des-Arg in whey samples were measured by an antigen capture enzyme-linked immunosorbent assay. Briefly, monoclonal antibody to bovine C5a/C5a-des-Arg (33) was used for antigen capture and rabbit anti-bovine C5a antiserum was used for detection. A calibration curve obtained with dilutions of purified C5a-des-Arg was used to calculate concentrations. Milk whey samples were diluted 1/2 or 1/5 prior to analysis.

Cytokine concentrations in whey and serum samples were analyzed for TNF, IL-1, and IL-6 by bioassays as described previously (43) and by enzyme immunoassay for IL-8. Briefly, IL-1 was measured by its ability to induce proliferation of the bovine T_h cell line, 300B1, using recombinant bovine IL-1β as the assay standard. The specificity of the IL-1 activity was verified by blocking with recombinant human IL-1 receptor antagonist. The bioactivity of TNF was measured as the cytotoxic activity for WEHI 164 subclone 13 cells with recombinant murine TNF-α as the assay standard. The specificity of the TNF assay was verified by using rabbit polyclonal antiserum to recombinant bovine TNF-α. IL-6 activity was measured by its ability to induce proliferation of the murine plasmacytoma cell line 7TD1 with recombinant human IL-6 as the assay standard. IL-8 was measured with a human IL-8 enzyme-linked immunosorbent assay kit essentially as described by the manufacturer (R&D Systems, Minneapolis, Minn.). Undiluted whey samples were added directly to the immunoassay wells, and quantification was achieved by comparison to standards of recombinant human IL-8 in plasma as supplied with the kit. The intra-assay and interassay coefficients of variation for all cytokine assays were less than 15%.

Levels of inflammatory mediators in milk are reported as concentrations. No correction was made to account for changes in the volume of milk within the gland at any particular time. Changes in volume would have been particularly large for samples collected just prior to a twice-daily milking versus samples collected within a few hours thereafter.

Chemoattractant assay. The chemotactic activity of milk whey samples was measured as the ability to direct neutrophil migration under agarose (28). Neutrophils from healthy cows were isolated by hypotonic lysis from packed erythrocytes as described previously (36). Neutrophils were placed in the center wells of migration plates with 10 μl of sample in wells on one side of the neutrophils and 10 μl of PBS in wells on the opposite side. The plates were incubated at 39°C for approximately 90 min. The neutrophils were fixed and stained, and the agar was removed. Chemoattractant activity was measured as the chemotactic index, i.e., the distance moved toward the sample divided by the distance moved toward PBS, where a value of 1.0 is equivalent to no chemotactic activity. Each assay was performed with neutrophils from two or three normal cows, and the results were averaged. Zymosan-activated serum, used as a positive control, gave an average chemotactic activity of 1.33. To evaluate the chemotactic activity of IL-8 in this assay, recombinant human IL-8 (R&D Systems) was diluted to test concentrations in PBS and placed in sample wells. Intra-assay and interassay coefficients of variation were 12 and 17%, respectively. All whey samples from the same infected gland were analyzed in the same assay.

Neutrophil CD18 expression. The effect of milk whey samples on CD18 expression by neutrophils was determined by combining 10 μl of sample with 100 μl of anticoagulated (EDTA-treated) blood from a healthy cow. This mixture was incubated at room temperature for 5 min of stimulation. A 10-μl sample of monoclonal antibody (50 μg/ml) to CD18, R15.7 (10) was then added, and the mixture was incubated for 15 min at room temperature. Blood cells were washed twice with Ca^{2+}- and Mg^{2+}-free PBS and resuspended with 100 μl of PBS, and 10 μl of fluorescein-conjugated anti-mouse immunoglobulin G secondary antibody was added for another 15-min incubation at room temperature. Excess secondary antibody was removed by washing, and erythrocytes were removed by hypotonic lysis. Labeled cells were analyzed in a flow cytometer (FACScan; Becton Dickinson, San Jose, Calif.), and neutrophils were identified by forward- and side-scatter characteristics. Expression of CD18 was calculated as the mean channel fluorescence of neutrophils stained with specific antibody minus the fluorescence of neutrophils incubated with the same isotype of irrelevant antibody. Background fluorescence after labeling with the irrelevant antibody was less than 3% of the specific fluorescence. Stimulated CD18 expression was calculated as CD18 expression by neutrophils incubated with samples as a percentage of CD18 expression by neutrophils incubated with PBS, where 100% is equivalent to no stimulation. Within an assay, blood from two or three cows was used and the results were averaged. Platelet-activating factor (1 μg/ml) was used as a positive control and stimulated an average 15% increase in CD18 expression. Intra-assay and interassay coefficients of variation were 2.6 and 10.0%, respectively. All whey samples from the same infected gland were analyzed in the same assay.

Statistical analyses. Statistical comparisons were made by comparing areas of response curves and values of maximum response for each parameter. Rectal temperature, a systemic reaction, was compared between challenged and unchallenged cows in a two-sample *t* test. For other parameters, infected glands were compared to glands of control cows in a two-sample *t* test. Infected glands were compared to contralateral, uninfected glands of the same cow in a paired *t* test. Values for IL-1, IL-6, IL-8, and cell count were logarithmically transformed for analysis.

RESULTS

Bacterial growth and the inflammatory response. Following intramammary inoculation of approximately 30 CFU, *E. coli* grew rapidly in milk with roughly a 10-fold increase every 2 h (Fig. 1). The peak bacterial concentrations occurred within 48 h after challenge and ranged from 10^4 to 10^9 CFU/ml of milk among individual glands. Coincident with the onset of mammary inflammation at roughly 12 h after the challenge (see below), *E. coli* numbers began to decrease. In some glands, this early decline was rapid, with as many as 99% of the bacteria being cleared within 2 h. This early decrease was less dramatic in other glands and did not occur at the same time in all glands, so that the average decrease between 10 and 16 h after the challenge was less marked. The number of *E. coli* organisms often increased again between 2 and 8 h after the initial decrease; in five of eight cows, the bacterial concentration during a relapse was more than 30-fold higher than the

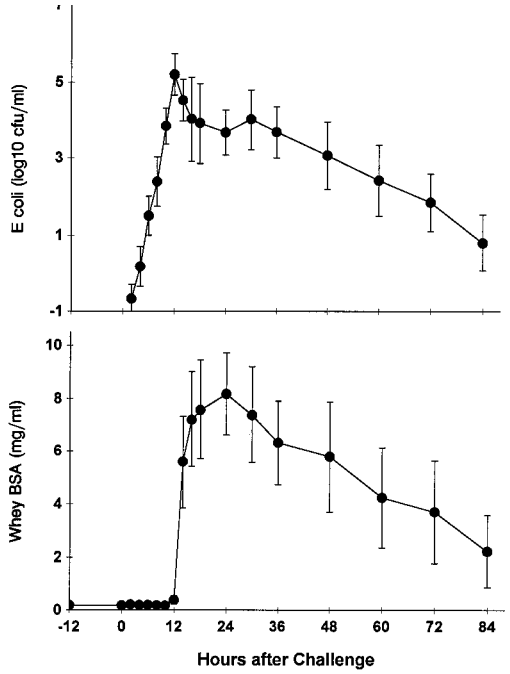

FIG. 1. Bacterial numbers and bovine serum albumin (BSA) concentration in milk during *E. coli* mastitis. Each gland was challenged after milking (time = 0 h) with approximately 30 CFU of *E. coli*. Data are the means ± standard error of the mean (SEM) from eight infected glands.

lowest concentration that was measured before the relapse. Despite these relapses, host defenses eventually controlled the infection, as most bacteria were cleared from all glands by day 6 after the challenge. This recovery occurred without any therapeutic intervention.

Despite the rapid growth of bacteria to high concentrations, there was little host response to the infection during the first 12 h after the challenge. The first detectable response to infection was mammary edema, which developed approximately 12 h after the challenge. The concentration of BSA in milk, a measure of vascular and epithelial permeability, increased shortly thereafter (Fig. 1). This increase in BSA concentration was highly significant compared to the trivial change in BSA concentration among uninfected glands of the same cow and glands of unchallenged cows ($P < 0.001$). Leukocyte numbers in milk, as reflected by SCC, increased shortly after the challenge (Fig. 2). However, this initial increase apparently resulted from normal variations in SCC between milkings, because a similar increase also occurred in unchallenged glands. Leukocyte accumulation in milk in response to intramammary infection did not appear until approximately 16 h after the challenge, when the SCC of infected glands was first significantly elevated relative to uninfected glands ($P < 0.01$). Thus, *E. coli* was apparently free to grow in milk for 16 h before phagocytic defenses had been recruited to the site of infection. Once begun, leukocyte recruitment persisted until most bacteria had been cleared, as the SCC was elevated through 6 days after the challenge.

A systemic reaction occurred in association with the infection, as evidenced by a significant pyrexia among challenged cows compared to controls ($P < 0.001$). Rectal temperatures increased approximately 2°C beginning 12 to 14 h after the challenge, with a maximal elevation at 16 h (Fig. 2). Lethargy and dyspnea were also commonly observed in challenged cows at this time. None of the cows became completely recumbent, and systemic signs had almost completely resolved by 24 to 30 h. Endocrinological changes in similarly challenged cows were reported previously (42).

Chemoattractant activity. The 16-h delay before leukocytes migrated into milk could have been a consequence of the time actually needed for these cells to move from the vasculature through the endothelium, interstitial space, and mammary epithelium. Alternatively, this prolonged period could have been caused by delays in recognition of the presence of bacteria and in the production of inflammatory mediators. To investigate these hypotheses, whey samples were analyzed for the presence of mediators that could induce the responses necessary for neutrophil recruitment, i.e., chemotaxis and upregulation of CD18 expression. Whey samples from infected glands contained strong chemotactic activity (Fig. 3). This activity started approximately 14 h after the challenge, with maximal activity occurring at 16 to 24 h. The chemotactic activity was significantly greater among infected glands than uninfected glands ($P < 0.05$). Milk whey samples from three cows were assayed for their ability to upregulate CD18 expression by neutrophils (Fig. 3). In these cows, the ability to upregulate CD18 expression developed over the same period as chemotactic activity. The appearance of both activities just preceded the leukocyte influx.

Inflammatory mediators. Inflammatory cytokines and complement fragments have been reported to be mediators of leukocyte recruitment. To determine whether these mediators may be involved in neutrophil recruitment to the infected mammary gland, milk whey samples were assayed for the presence of inflammatory cytokines and the C5a/C5a-des-Arg fragment of complement. The first cytokine to exhibit high concentrations in milk was TNF (Fig. 4). Maximal TNF activity was present in infected glands at 12 to 16 h after challenge and thus coincided with the time when mammary edema was most severe. The amount of TNF in milk varied considerably, with

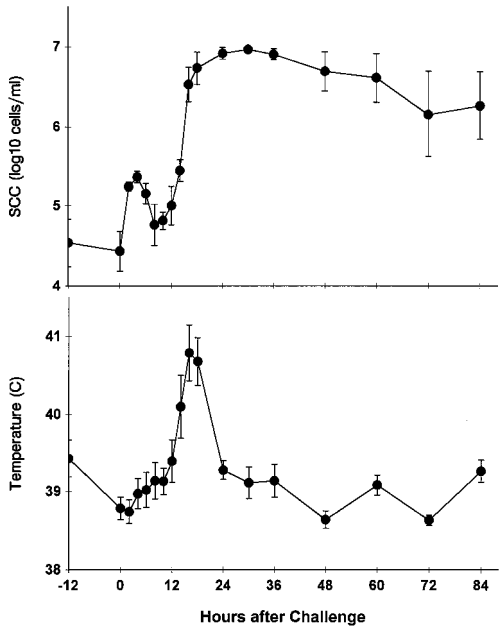

FIG. 2. Milk SCC in infected glands and rectal temperature of cows during *E. coli* mastitis. One gland of each cow was challenged after milking (time = 0 h) with approximately 30 CFU of *E. coli*. Data are the means ± SEM from eight challenged cows.

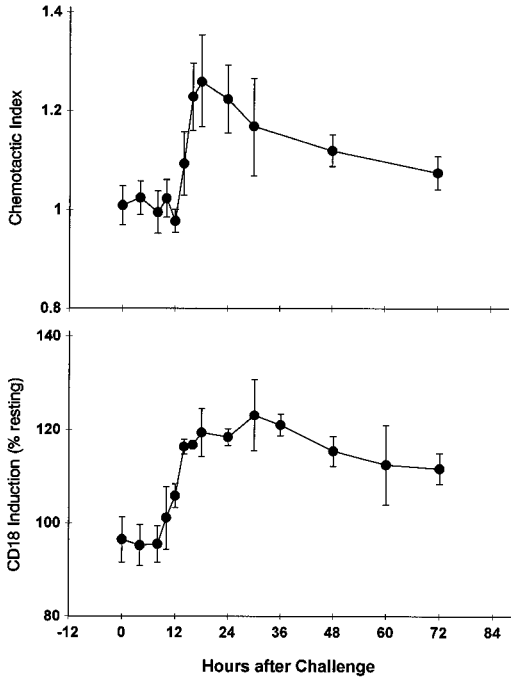

FIG. 3. Chemotactic and CD18-inducing activity of milk whey from glands infected with *E. coli*. Each gland was challenged after milking (time = 0 h) with approximately 30 CFU of *E. coli*. Data are the means ± SEM from eight infected glands for chemotactic activity and from three infected glands for CD18-inducing activity.

two cows exhibiting no detectable TNF while two other cows had maximal concentrations in excess of 5 U/ml. Increases in IL-1 concentrations among infected glands began slightly after increases in TNF, and maximal concentrations were reached later (Fig. 4). All infected glands demonstrated an obvious increase in IL-1 concentration, with peak levels ranging from 2 to 500 ng/ml, a significant increase compared to uninfected glands ($P < 0.05$). IL-6 concentrations were also significantly increased among infected glands ($P < 0.05$). Increases in IL-6 concentrations began approximately 14 h after challenge and were maintained beyond 60 h (Fig. 4). Thus, IL-6 was produced slightly later and for longer than TNF and IL-1. Samples of blood serum were analyzed for TNF and IL-1, but no more than trace amounts of either cytokine were detected at any time, and no difference was found between challenged and control cows (data not shown).

The chemotactic factors IL-8 and C5a/C5a-des-Arg were also present in high concentrations in the milk of infected glands (Fig. 5). Production of IL-8 began slowly and was maintained over a long period. Peak concentrations of IL-8 were significantly higher in infected glands than in uninfected glands ($P < 0.05$), although three of the eight challenged glands did not exhibit obvious increases in IL-8. In contrast, increases in the concentration of C5a/C5a-des-Arg were early and rapid, occurring simultaneously with increases in the BSA concentration. Large increases in the C5a-des-Arg concentration occurred in all infected glands that were studied, and the concentrations were significantly greater among infected glands than uninfected glands ($P < 0.001$). High concentrations of C5a/C5a-des-Arg persisted through 36 h after challenge and were followed by a slow decline.

Chemotactic activity of IL-8 in vitro. The large increase in IL-8 concentration suggested that this chemoattractant may be involved in leukocyte recruitment to the site of *E. coli* infection. However, the biological significance of concentrations seen in vivo was uncertain. Therefore, various concentrations of human IL-8 were studied for their chemotactic activity for bovine neutrophils in the same under-agarose assay that was used for evaluating the chemotactic activity of the wheys. IL-8 was tested at concentrations ranging from 0.1 to 10,000 pg/ml. Maximal chemotactic activity was observed at IL-8 concentrations of approximately 3 pg/ml. This concentration of IL-8 gave a chemotactic response comparable to that of zymosan-activated serum. Although these data demonstrate the strong chemotactic response of bovine neutrophils to IL-8, the potency of homologous IL-8 for these leukocytes remains unknown.

DISCUSSION

Following intramammary inoculation with a low dose of *E. coli*, the bacteria grew logarithmically for approximately 12 h before a host response occurred. Upon development of an inflammatory response, control of the infection began promptly, with nearly complete eradication of the pathogen in 1 week without therapeutic intervention. Thus, the host was readily able to clear the bacteria once the infection was detected and a defensive response had been initiated. The 16-h delay between challenge and neutrophil recruitment could have been a consequence of the mechanics of neutrophil migration; i.e., neutrophil movement from the vasculature through the endothelium, interstitial space, and mammary epithelium into the milk compartment may simply have taken a long time. Alternatively, the delay could have resulted primarily from a lack of recognition of the presence of bacteria and production of inflammatory mediators. The present experiment showed that inflammatory mediators were not produced until approxi-

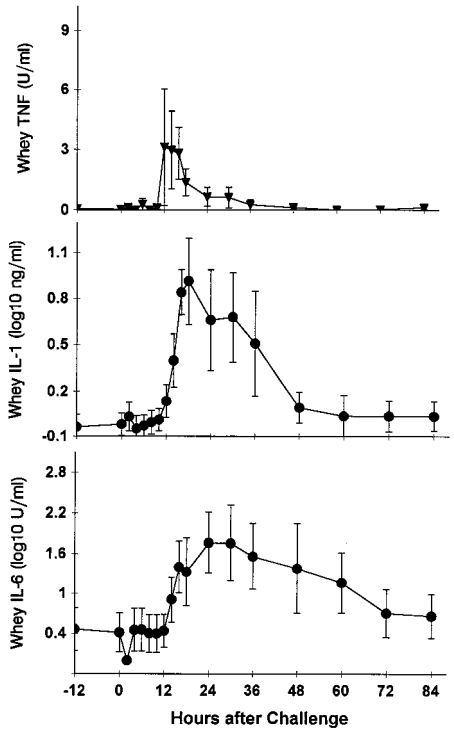

FIG. 4. TNF, IL-1, and IL-6 concentrations in milk whey from glands infected with *E. coli*. Each gland was challenged after milking (time = 0 h) with approximately 30 CFU of *E. coli*. Data are the means ± SEM from eight infected glands for TNF and IL-1 and from six infected glands for IL-6.

mately 12 h after challenge, providing strong support for the latter hypothesis. This conclusion is consistent with earlier work, which has shown that IL-1 production and neutrophil movement from the vasculature into milk can occur within only 4 h after intramammary infusion of *E. coli* endotoxin (43). In addition, other work has demonstrated that neutrophils begin to cross the epithelium within only 2 h following infusion of endotoxin, IL-1, or TNF into the bovine teat cistern (29, 30).

The mechanisms by which the host recognizes bacteria at an epithelial surface are unknown. The first detectable reactions to the infection were vascular changes, which led to local edema and leakage of serum proteins into milk; a leukocytic influx followed within 2 to 4 h. Because bacteria and their components would be unable to cross the initially impermeable blood-milk barrier to stimulate host defenses directly, initial recognition probably occurs within the milk compartment. Although macrophages can produce many inflammatory mediators upon interaction with *E. coli* and its biochemical constituents, e.g., lipopolysaccharide, mediators produced by mammary macrophages would need to cross the impermeable epithelium to recruit neutrophils from the vasculature. In this case, the epithelium would need to permit or facilitate the passage of inflammatory mediators from the milk compartment to the interstitium. Several recent studies have demonstrated that epithelial cells can generate a variety of inflammatory mediators upon interaction with the bacteria (1, 9, 24). One of these studies even showed that the release of inflammatory cytokines by epithelial cells is directed toward the basolateral surface, the direction from which host defenses would be recruited (9). Therefore, the epithelium may have played an important role in the initial recognition of the infection and subsequent production of inflammatory mediators.

The appearance of C5a/C5a-des-Arg in milk closely paralleled increases in the BSA concentration. This observation is not surprising, since levels of complement in milk of uninfected glands are low (32, 47), and so complement fragments could not be generated in large amounts until more complement entered milk coincident with breakdown of the blood-milk permeability barrier. Recent work has shown that milk from normal, noninflamed glands contains only low levels of complement but that this small amount can be activated through the alternate activation pathway (31). Therefore, complement activation could be part of the mechanism by which the bacteria are first recognized.

Multiple inflammatory mediators, including TNF, several ILs, and C5a, were produced in response to intramammary infection with *E. coli*. These mediators were probably generated locally, because TNF and IL-1 were undetectable in serum. Previous work has demonstrated TNF activity in serum during severe episodes of *E. coli*-induced mastitis, i.e., fatal or near-fatal cases (39, 45). In these cases, the circulating TNF could have been absorbed following the intense local production or the TNF could have been generated systemically, because endotoxin and bacteria are often detected in the blood in cases of this severity (14). Local production of inflammatory mediators during intramammary infection is consistent with studies of mucosal infection, where local production of TNF, IL-1, IL-6, IL-8 and other cytokines was found during *E. coli* urinary tract infection (2, 15, 37) and pleuropneumonia caused by the gram-negative bacterium *Actinobacillus pleuropneumoniae* (4). These earlier studies were able to demonstrate TNF production only by quantitation of mRNA levels, because TNF bioactivity was undetectable in inflammatory fluid. During our study of intramammary infection with *E. coli*, TNF bioactivity was found in milk, but only among glands with severe infections and only for a short period during the onset of inflammation.

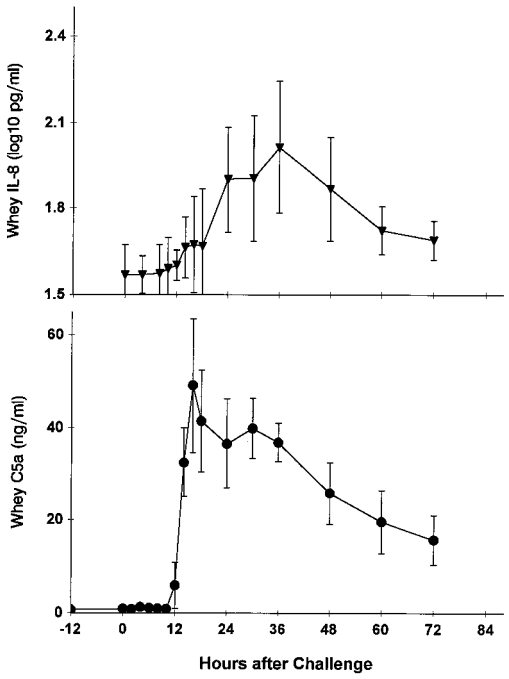

FIG. 5. IL-8 and C5a concentrations in milk whey from glands infected with *E. coli*. Each gland was challenged after milking (time = 0 h) with approximately 30 CFU of *E. coli*. Data are the means ± SEM from eight infected glands for IL-8 and from six infected glands for C5a.

The exact site of inflammatory mediator production is not known. If the cytokines were produced by the epithelium, their concentrations may have been greater on the basolateral surface and their biological effects might have been induced in the interstitium. In this case, their concentrations in milk might not have been an accurate reflection of their production and potential biological activities. In contrast, complement is activated directly or indirectly through contact with bacteria, and generation of complement fragments would have occurred at the site of infection (milk in the case of mastitis). Therefore, complement fragments including C5a would have exhibited their greatest concentrations in milk.

Low concentrations of inflammatory mediators or their activity were sometimes found in milk before challenge or in the milk of uninfected glands. Although some of this activity may have been nonspecific, other investigators have also noted low concentrations of TNF, IL-6, and IL-8 in secretions of noninflamed mammary glands or unstimulated mammary epithelial cultures (5, 34, 46). The physiological significance of basal levels of these mediators is unknown. Although they do not induce obvious inflammation, these mediators may contribute to the small influx of leukocytes that occurs even in uninfected glands, particularly during physiological transitions of the mammary gland (34).

The identity of the chemotactic and CD18-upregulating activity during mammary infection is not definitely known. Because many chemotactic factors are also able to stimulate enhanced CD18 expression (23), the same mediator(s) may have been responsible for both activities. Previous work has shown that the bovine C5a and C5a-des-Arg are equally potent stimulants for bovine neutrophils in vitro, and chemotaxis was induced at concentrations of 1 to 50 ng/ml (12, 13, 29, 44).

Similarly, we and others (29) have demonstrated strong chemotactic activity of IL-8 at concentrations of ≤50 pg/ml. Thus, the peak concentrations of C5a/C5a-des-Arg and IL-8 that were found in mastitic milk, i.e., 50 ng/ml and 100 pg/ml, respectively, would seem to be sufficient to achieve a biological response. The importance of C5a appears to be greater than that of IL-8 during the early inflammatory response, because C5a production was maximal at this time while IL-8 production was low. Peak concentrations of IL-8 did not occur until 24 to 48 h after the challenge. Nonetheless, IL-8 seems to play an important role in neutrophil recruitment because animals lacking its receptor exhibit impaired neutrophil recruitment and decreased resistance to infectious disease (6, 41). Many other inflammatory mediators, including the potent chemoattractant leukotriene B_4 (LTB_4) are generated during coliform mastitis (35). Because increases in LTB_4 concentration lagged behind those of the milk cell count, LTB_4 probably does not mediate the initial neutrophil recruitment. Neither C5a, IL-8, nor LTB_4 was able to induce more than a small neutrophil influx when infused alone into the bovine teat cistern, whereas endotoxin, IL-1, and TNF induced strong responses (29). Thus, these chemoattractants probably do not act alone in the recruitment of neutrophils to an infected epithelial surface. Studies of mice lacking the C5a receptor demonstrated that C5a plays an important role in neutrophil activation and resistance to intrapulmonary challenge with *Pseudomonas aeruginosa*, but the recruitment of neutrophils was normal (21). These results suggest that the role of C5a may be most important for the early activation of neutrophils, while its chemoattractant role may be redundant because of the presence of other inflammatory mediators. Further work is needed to determine the precise roles of C5a and IL-8 in neutrophil recruitment and activation during *E. coli*-induced mastitis.

In summary, this experiment demonstrates that neutrophil recruitment to bacterial infection at an epithelial surface is delayed because inflammatory mediators are not produced until the infection has become established. Once inflammatory mediators are generated, neutrophils begin to cross the epithelium within a few hours. The earlier appearance of complement fragment C5a indicates that this chemoattractant may be more important than IL-8 for the initial recruitment and activation of neutrophils during an *E. coli* infection.

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