Assessment of Hemolytic and Bactericidal Complement Activities in Normal and Mastitic Bovine Milk

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ABSTRACT

Bactericidal and hemolytic complement activities were investigated in 51 quarter milk samples of 13 cows in late lactation. Hemolytic activity was in all of the samples but one, after accounting for whey inhibitory activity. Mean hemolytic activity and inhibitory activities were .18 and .34 complement hemolytic units. Inflammation, in relation to infection status, increased hemolytic titer and heat-labile bactericidal activities of milk. Correlation coefficient was .76 between albumin content of milk serum and hemolytic titer of samples from infected quarters. Normal milk decreased bactericidal titer of bovine serum against a serum-sensitive Escherichia coli strain. When compared to veronal buffer saline solution, milk did not accelerate decay of hemolytic activity over 7-h incubation at 39°C. Taken together, these results suggest that the adverse effect of milk on both hemolytic and bactericidal activities of complement is limited and might be of significance essentially before full establishment of the inflammatory reaction to bacterial invaders.

INTRODUCTION

Specific and nonspecific mechanisms in the defense of bovine mammary gland have been given interest for a long time (review, 16). Nevertheless, little is known about amounts and activities of complement (Cp) in milk. Conglutinating activity, indicating the presence of Cp components C1, C4, C2, and C3, was detected in colostrum and milk of late lactation (17), and in milk of midlactation (3). Hemolytic activity (HA) was demonstrated only in colostrum (1, 4). Evidence was that milk exerts an inhibitory activity (IA) on Cp-mediated hemolysis (13), which may explain the failure of attempts to detect hemolytic Cp activity in milk from uninflamed glands throughout lactation.

However, at the onset of the inflammatory response following invasion of the mammary gland by microorganisms, milk becomes bactericidal for serum-sensitive microorganisms. This activity is abolished by heating at 56°C for 30 min, suggesting involvement of Cp (9, 6).

This prompted us to reexamine the relationship between inflammation and HA of milk, after allowing for IA activity. Evidence was that inflammation and Cp hemolytic titer of milk are quantitatively related and that IA activity manifests itself not only in the hemolytic test but also in the bactericidal process. Nevertheless, although IA can impair Cp activities in normal milk, even a mild inflammation is able to recruit sufficient Cp to overcome this hindrance.

MATERIALS AND METHODS

Origin of Milk Samples

Fifty-one quarters of 13 cows (1 blind quarter) in late lactation (220 to 360 days) from the experimental herd of our institution were selected by their udder infection status. Infected udder quarters (chronic forms of mastitis) harbored either Staphylococcus aureus, streptococci, Corynebacterium bovis, or coagulase-negative staphylococci. Uninfected quarters were free of bacteria from at least 2 mo as checked by bacteriological controls at 3-wk routine intervals on all cows of the herd. Bacteriological determination were made as in (12).

Processing of Milk Samples

Skim milk samples were obtained by centrifugation at 1000 x g for 20 min to remove cells

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and fat, then at 3000 x g for 30 min to remove bacteria. These samples were assayed within 2 h after preparation for bactericidal activity. For other tests, whey was prepared with rennet (1-h incubation at 30°C) and centrifugation at 3000 x g for 30 min. Whey samples were stored at -70°C until used.

Aseptic Milk

This was obtained from one bacteria-free uninflamed gland, defatted by centrifugation at 4°C, stored at -20°C, and used within 1 wk after preparation. Absence of bacteria was checked by our spreading 25 µl portions of the milk sample on esculin blood agar plates.

Quantitation of Bovine Serum Albumin in Whey

Concentrations of bovine serum albumin (BSA) were measured as in (14). The BSA was purchased from Sigma (Sigma Chemical Co.) and purified further by molecular sieving on Ultrogel Aca 34 (Industrie Biochimique Française). Antiserum for BSA was produced in rabbits, and specificity was checked by double diffusion in agar and immunoelectrophoresis.

Concentration of BSA in whey was determined by the radial immunodiffusion procedure according to the principle of Manchini et al. (10). Every sample was tested in duplicate.

Preparation of Serum Samples

Blood samples were collected by tail vein puncture. They were allowed to clot at 37°C for 1 h, then centrifuged at 2500 x g for 30 min, and sera stored in portions at -70°C until used.

Somatic Cell Counting

Somatic cell count was with a Coulter counter (Model F, Coultronics) according to the procedure recommended by the International Dairy Federation (8).

Bacteria

Two *Escherichia coli* strains were used: the serum resistant strain P4 (032 K?) and the serum sensitive strain B41 (0101 K99). These strains were kindly provided by A. W. Hill (ARC, Compton, England). Strain B41 was chosen because of its sensitivity to Cp in the absence of specific antibodies (7).

Bactericidal Assay

Bacteria were cultured overnight in the semidefined liquid medium used by Hill (5), harvested by centrifugation (2500 x g for 20 min), washed once in saline, and adjusted to appropriate suspension by serial 10-fold dilutions in saline solution except the last one in veronal buffer (VB), plus .1% human serum albumin. Twenty microliters (containing about 5 x 10² cfu) were added to round-bottom microtiter wells (Nunc) along with 80 µl of skim milk. Trays were incubated 2 h at 37°C. After they were mixed on an orbital mixer, 20-µl samples were taken from each well and viable counts determined by the poured plate method. Viable counts at zero time were determined from two wells treated in the same way but sampled before incubation. Every milk sample was submitted to a set of tests consisting of two wells of fresh skim milk inoculated with either B41 or P4 and one well of heat-treated milk (56°C for 30 min) inoculated with B41.

Heat-labile bactericidal activity was defined: 1) less than 50% of the B41 viable count at zero time was recovered after incubation; 2) 80% or more of the initial count was recovered in the heated sample and in the fresh sample inoculated with the strain P4. The latter requisite confirmed that reduction of colony forming units was inhibited by heat treatment, not agglutination, and was ineffective on a serum resistant strain.

Hemolytic Assay

Details of the method are in (13). Briefly, ⁵¹Cr-labeled guinea-pig erythrocytes (GPRBC) sensitized with a subagglutinating amount of rabbit anti-GPRBC were placed in microtiter wells. Pooled bovine sera as a source of complement to achieve about 50% of ⁵¹Cr release were added to each well before addition of the samples on the test to overcome the milk IA. After incubation (37°C, 90 min) and centrifugation, a 50-µl portion of the supernatant fluid was added to 5 ml of a liquid scintillation cocktail (ACS, Amersham) and ⁵¹Cr released activity measured in a betacounter (Rackbeta 1215, LKB). The Cp hemolytic unit 100% (CH 100) was defined as the smallest amount of Cp that lysed 100% of sensitized red cells. Determination of CH100 titer was obtained by difference of counting between heated (56°C, 30 min) and unheated whey samples from standard linear regression.

RESULTS

Bactericidal Activity of Bovine Serum Against E. coli B41

Sera from 11 of the 13 cows under experiment killed more than 50% of the bacterial inoculum up to 1:160 dilution in VB. Two sera were bactericidal up to 1:320 dilution. This activity was abolished by heating at 56°C for 30 min (results not shown). The rate of killing appeared to be dose-dependent (Figure 1) in relation to a prolonged lag phase in more diluted serum. Nevertheless, killing was completed after 2 h of incubation, and accordingly this time was retained for testing of samples of unknown Cp activity.

Bactericidal Activity of Milk

Twenty-six of the 51 milk samples were bactericidal for E. coli B41. Of these bactericidal samples, only two were able to kill the serum resistant strain P4 and retained their bactericidal activity against the serum sensitive strain B41 after heat-treatment. Data from these two quarters (the only two quarters with clinical mastitis) were not taken into account thereafter. Activity of the other 24 bactericidal samples was abolished by heating and was attributed consequently to Cp action.

Table 1 shows that indicators of inflammation (BSA and somatic cells) were more elevated in bactericidal samples. The HA was in all of the tested samples but one. This result was obtained only after correction for IA, which on an average reached .34 CH100. The HA was higher in bactericidal samples.

Influence of chronic infections on HA and bactericidal activity of milk.

Somatic-cell count and milk BSA content were significantly higher in samples from infected quarters. The mean HA and percentage of bactericidal samples also were augmented (Table 2).

<table>
<thead>
<tr>
<th>No. infected quarters</th>
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</table>

TABLE 1. Somatic cell-count, bovine serum albumin (BSA) concentration, and hemolytic titer in whey samples in relation to heat-labile bactericidal activity.

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Somatic cell-count</th>
<th>BSA concentration</th>
<th>Hemolytic titer</th>
<th>No. infected quarters</th>
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<tr>
<td></td>
<td>(X 10^-3/ml)</td>
<td>(µg/ml)</td>
<td>(CH100^2/ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>SD</td>
<td>X</td>
<td>SD</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bactericidal 24</td>
<td>2,530</td>
<td>5,500</td>
<td>365</td>
<td>380</td>
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<tr>
<td>Nonbactericidal 25</td>
<td>440</td>
<td>470</td>
<td>220</td>
<td>85</td>
</tr>
<tr>
<td>Significance</td>
<td>P&lt;.07</td>
<td>P&lt;.07</td>
<td>P&lt;.001</td>
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1 Complement hemolytic unit 100%.
TABLE 2. Hemolytic and heat-labile bactericidal activities in relation to infection status and resulting inflammation (estimated through somatic cell-count and bovine serum albumin (BSA) concentration).

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Somatic cell-count</th>
<th>BSA concentration</th>
<th>Hemolytic titer</th>
<th>No. infected quarters</th>
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<tr>
<td></td>
<td>× 10^{-3} / ml</td>
<td>(μg/ml)</td>
<td>(CH100 / ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>SD</td>
<td>X</td>
<td>SD</td>
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Infection status

<table>
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<tr>
<th></th>
<th>Somatic cell-count</th>
<th>BSA concentration</th>
<th>Hemolytic titer</th>
<th>No. infected quarters</th>
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<tr>
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<tr>
<td></td>
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<td>16</td>
</tr>
<tr>
<td>Uninfected</td>
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<td>160</td>
<td>230</td>
</tr>
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<td>8</td>
</tr>
<tr>
<td>Significance</td>
<td>P&lt;.05</td>
<td>P&lt;.10</td>
<td>P&lt;.01</td>
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</tbody>
</table>

1 Complement hemolytic unit 100%.

Correlation coefficient was .76 between milk BSA content and hemolytic titer in samples from infected quarters (Figure 2). For the uninfected whey samples, correlation was small (r = .29).

Effect of Normal Milk on Bactericidal Activity

Capacity of milk to hinder the Cp-dependent bactericidal activity was investigated. Double dilutions of bovine serum (50 CH100) were in either VB, fresh milk (aseptic milk), or heat-treated milk. Every dilution was tested for bactericidal activity. Milk protected bacteria to some extent (Figure 3). Hindrance of Cp activity by heat-treated milk was higher than by fresh milk. Whether inactivation of Cp in the milk accounted for all this difference remains unknown.

Effect of Preincubation at 39°C in Milk on Hemolytic Activity

Because milk impaired Cp activities, question arose about the fate of Cp after it has passed into the gland lumen. To appraise the influence of duration of contact with milk, two dilutions of bovine serum containing .7 and .3 CH100 were made in VB and fresh milk. These dilutions were preincubated at 39°C, the temperature of the mammary gland, before measurement of HA at 1-h intervals up to 7 h of preincubation. There were not great differences between VB or milk as diluents (Figure 4). In contrast, patterns were different for the two dilutions. At the higher dilution, HA faded rapidly, whereas at the lower one only 15.2 and 8.7%, of the
activity disappeared in milk and VB, respectively, during the 7 h of preincubation.

DISCUSSION

Hemolytic assay enabled us to demonstrate HA in all whey samples but one. This assay could be interpreted as a measure of the presence in milk of a limiting complement component in diluted bovine serum. Nevertheless, that 76% of whey samples from infected quarters were bactericidal in the absence of added Cp demonstrates that at least one of the two sets of elements belonging to the classical or alternate pathways of Cp activation was in milk. Consequently, the correlation between bactericidal effect and HA of milk tends to support the view that the hemolytic test measures essentially total HA.

Inflammation caused comparable increases in both HA and bactericidal activity (Table 2). The correlation ($r = .76$) between hemolytic titers and BSA in whey from infected quarters is in keeping with results of Mueller et al. (11), who found a correlation of .80 between C3 and BSA in milk from infected quarters. This supports the idea that Cp passes into the milk during an inflammatory response essentially in a passive manner like BSA.

By contrast, the weak correlation between hemolytic titers and BSA ($r = .29$) in our study or between C3 and BSA ($r = .41$) found by Mueller et al. (11) in milk from uninfected quarters suggests that Cp components and BSA may be transferred separately in the absence of inflammation. Nevertheless, that, on an average, HA as well as BSA represent about 1:200 of blood quantities (13) does not support this view. An alternative explanation is that hemolytic titers in normal milk were not determined precisely, because they were too low in regard to the precision of the hemolytic assay.

In (13) milk exerted what was named an "anticomplementary" activity. The term refers to substances that initiate Cp activation in the absence of any specific antigen-antibody reaction. In fact, no data in this work or (13) demonstrated that milk activates the Cp cascade. Thus, we preferred the use of "inhibitory", a more engulfing term, instead of "anticomplementary", to describe the effect of milk activity on Cp action.

In average skim milk, IA was higher than HA. Work showed that precipitation of casein removed the main part of IA, although a noticeable effect remained in whey (13). This activity, defined from the hemolytic assay, impaired also the Cp-dependent bactericidal activity. Nevertheless, this impairment was overcome by relatively reduced amounts of Cp (Figure 3), which correlates with the well-known observation that milk becomes bactericidal for serum sensitive strains in the course of clinical mastitis (2) and, in particular, for the test strain used in this study (6, 15).

Investigation of the effect of prolonged incubation in milk at $39^\circ$C on HA, as it may occur in the mammary gland in the course of an inflammatory reaction, yielded unexpected results: total inactivation or near total conservation, according to the amount of Cp added to fresh milk (Figure 4). Whatever the cause may be, the most significant result with respect to mastitis is that milk behaved like veronal buffer as a diluent, indicating milk did not accelerate the decay of Cp activity. This suggests that, provided sufficient amount of Cp passes into the milk, it may play a part in udder defenses for several hours between two milkings.
Milk IA impairs both HA and bactericidal activity, but is limited, so that it is overcome by even a mild inflammation. The milk IA seems to be of significance essentially in normal milk (from uninflamed gland). In particular, it might reduce the responsiveness of the mammary gland to invading bacteria by interfering with anaphylatoxic and chemotactic functions of Cp that are likely to take part in the early steps of the inflammatory response. This speculative assumption deserves further investigation.

ACKNOWLEDGMENTS

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REFERENCES