EXPERIMENTAL COLIFORM (AEROBACTER AEROGENES)
MASTITIS: DISTRIBUTION OF WHEY PROTEINS
DURING THE EARLY ACUTE PHASE

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SUMMARY

Two trials involving inoculation of lactating quarters of one cow with large numbers of Aerobacter aerogenes are described. Only a mild reaction occurred following the first inoculation. Acute mastitis was produced in the second, with agalactia resulting in all quarters by the fourth milking after exposure. All glands returned to full normal production by 2 wk. In both trials, evidence of increased permeability between blood and milk (increased pH, chlorides, serum albumin, and immune globulin) took place within 3 hr. Serum albumin declined rapidly to normal levels, but the immune globulin fraction remained elevated. Following the period of agalactia, a protein here designated X, with a paper electrophoretic mobility intermediate between a-lactalbumin and immune globulin, appeared. The X protein ultimately completely masked a-lactalbumin and comprised as much as 50% of the total whey protein. It disappeared from whey after the glands returned to full milk production. A similar protein was also found in wheys obtained from cows early in the dry phase of lactation. It did not appear to have the paper electrophoretic mobility of any of the major electrophoretic components of blood or milk. It was postulated the X protein was of cellular origin.

In previous papers of this series (10-12) it was shown that acute mastitis was produced following inoculation of normal lactating glands with a culture of Aerobacter aerogenes isolated from a natural case. These glands usually recovered quickly and without treatment, although an occasional gland would remain infected for an extended period of time. Advantage has been taken of these trials in studying several facets of mammary inflammation, among which has been a study of the whey protein distribution in both severe and mild forms of the disease. Some observations on whey protein patterns during these and other trials have been reported (1, 3).

The characteristic feature of the whey protein changes in the mild disease (1) was the early appearance (within 3 hr) of increased serum albumin, followed by a somewhat delayed increase in the immune globulin fraction following udder inoculation. The latter component remained elevated for some time following the return to normal of serum albumin and of the gland to normal secretion. These observations indicated that the transudation of blood proteins is a dynamic process and suggest, in agreement with others (9), that transudation of the two blood-formed proteins into the milk takes place by separate processes.

It is the purpose of this paper to document additional findings from several trials relative to the dynamics of whey protein secretion in severe acute mastitis induced by the inoculation of udder quarters with Aerobacter aerogenes.

MATERIALS AND METHODS

The experimental animals, method of inoculation, and laboratory and clinical observations made during a trial have been described (11). Two trials were conducted with Cow 2414. The first (October 3) was in the seventh month of the third lactation. Two million viable A. aerogenes were injected into the right rear (B), left front (C), and left rear (D) quarters, respectively. One milliliter of saline vehicle was injected into the right front (A) quarter as control. The second trial involved a similar inoculation into the same quarters on October 24.

The cow was milked by hand and the quarter milk weights and volumes recorded. In the October 3 trial, the B quarter was milked at 3 hr, the D at 6 hr following inoculation.

Received for publication July 24, 1963.

1 Supported in part by the Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture.
acidiﬁcation to pH 4.6 with one part N HCl; two parts N acetic acid followed by centrifuga-
tion and ﬁltration. Total whey proteins were
determined by the biuret method previously
described (3). The whey proteins were sepa-
rated by zone electrophoresis according to the
method outlined by Bortree et al. (1). A nor-
mal whey was included in each electrophoresis
run, to aid in localization of the different pro-
teins of the abnormal wheys. Duplicate strips
were run for each whey and results averaged.

Chlorides were titrated from 15 ml of milk
made to 50 ml with distilled water with 3 N
Ag NO₃ and KCr₂O₇ as indicator. Catalase is
reported as the percentage of gas present in
the side-arm of a Smith fermentation tube after
incubation for at least 3 hr at 37 ° of 3 vol of
milk to 1 vol of freshly prepared 1% H₂O₂.

RESULTS AND DISCUSSION

Trial 1 (October 3). Despite the large inocu-
um of ±2 million viable A. aerogenes into
each of three quarters of Cow 2414, the degree
of mastitis was comparatively mild (Tables 1
and 2). These results are markedly similar to
those recorded for Cow 2333 (Table 6) of the
second paper of this series (12). It would
appear that the early leukocytosis into the
inoculated glands was of sufﬁcient magnitude
to inhibit signiﬁcant growth of the organism.
An additional modifying factor may have been
the fact that two quarters were milked out at
3 and 6 hr post-inoculation, thereby serving
to remove some of the irritant. Moderate leuko-
openia resulting from movement of leukocytes
into the glands was evident at the third hour.

The signiﬁcant feature of the whey protein
distribution was the early increase and the
equally rapid decline in the relative concentra-
tion of serum albumin. The sixfold increase in
serum albumin and twofold increase in im-
mune globulin at 3 hr suggest an almost irra-
tional permeability between blood and milk. The
increased pH and chloride concentrations also
bear this out. The glands appear then to re-
cover quickly, as far as permeability is con-
cerned. However, in agreement with previous
work, the immune globulins tend to remain
elevated. This fraction was above preinocula-
tion levels at the beginning of the next trial
on October 24.

Trial 2 (October 24). This trial was con-
ducted in an effort to establish if any immunity
had developed with regard to the October 3
trial. The inoculum was again ±2,000,000
(actual count 2,3000,000) viable A. aerogenes
given in the same quarters (B-C-D). The
**TABLE 2**

Supplemental observations, Cow 2414, trial of October 3

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Quarter</th>
<th>pH</th>
<th>Chlor-</th>
<th>Catal-</th>
<th>Milk production</th>
<th>CMT</th>
<th>Cell count</th>
<th>Bacteria count</th>
<th>Clinical signs</th>
<th>Rectal temp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rine *</td>
<td>lase</td>
<td>(lb)</td>
<td>(ml)</td>
<td>(× 10⁶)</td>
<td>(× 10⁹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6.65</td>
<td>0.180</td>
<td>0</td>
<td>3.5</td>
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<td>0</td>
</tr>
<tr>
<td>B</td>
<td>6.61</td>
<td>0.169</td>
<td>0</td>
<td>5.7</td>
<td>2125 N</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
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<td>0.174</td>
<td>0</td>
<td>3.5</td>
<td>1375 N</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>D</td>
<td>6.61</td>
<td>0.168</td>
<td>0</td>
<td>4.7</td>
<td>1625 N</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
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<tr>
<td>+ 3 B</td>
<td>6.85</td>
<td>0.225</td>
<td>45</td>
<td>70</td>
<td>3</td>
<td>10.0</td>
<td>270.0 H</td>
<td>102.0</td>
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</tr>
<tr>
<td>+ 6 C</td>
<td>6.90</td>
<td>0.263</td>
<td>70</td>
<td>100</td>
<td>3+ 150.0</td>
<td>534.0</td>
<td>H</td>
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<td></td>
<td>6.60</td>
<td>0.186</td>
<td>0</td>
<td>1.2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
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<tr>
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<td>0.191</td>
<td>45</td>
<td>3.3</td>
<td>1300 3</td>
<td>25.0</td>
<td>5.2 F</td>
<td>104.4</td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>6.65</td>
<td>0.226</td>
<td>55</td>
<td>0.8</td>
<td>350 3+</td>
<td>40.0</td>
<td>4.5 CH</td>
<td>101.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>6.70</td>
<td>0.224</td>
<td>57</td>
<td>2.6</td>
<td>1100 3</td>
<td>20.0</td>
<td>663.8 S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+24 A</td>
<td>6.60</td>
<td>0.173</td>
<td>0</td>
<td>4.3</td>
<td>1750 N</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>101.8</td>
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<tr>
<td>C</td>
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<td>0.218</td>
<td>55</td>
<td>3.3</td>
<td>1300 3</td>
<td>30.0</td>
<td>85.5 C</td>
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<td>0.210</td>
<td>55</td>
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<td>30.0</td>
<td>128.5 F</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.60</td>
<td>0.168</td>
<td>0</td>
<td>2.8</td>
<td>1150 N</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
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<tr>
<td>+36 B</td>
<td>6.62</td>
<td>0.180</td>
<td>10</td>
<td>4.5</td>
<td>1850 2</td>
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<td>0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C</td>
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<td>0.212</td>
<td>30</td>
<td>1.8</td>
<td>760 2</td>
<td>21.0</td>
<td>0 F</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D</td>
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<td>0.206</td>
<td>40</td>
<td>2.8</td>
<td>1100 3</td>
<td>10.0</td>
<td>1 F</td>
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<td></td>
<td></td>
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<tr>
<td>A</td>
<td>6.57</td>
<td>0.163</td>
<td>0</td>
<td>3.6</td>
<td>1540 N</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>+48 B</td>
<td>6.62</td>
<td>0.167</td>
<td>0</td>
<td>5.4</td>
<td>2065 T</td>
<td>0.4</td>
<td>0 F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>6.67</td>
<td>0.211</td>
<td>10</td>
<td>2.6</td>
<td>1100 1</td>
<td>1.0</td>
<td>0 C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>6.67</td>
<td>0.203</td>
<td>35</td>
<td>3.8</td>
<td>1620 2</td>
<td>2.0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The cow is in late lactation, hence the high preinoculation values.
  **Aerobacter aerogenes** in fresh milk; + = present in incubated milk.
  * = quarter firm; F = flakes in the milk; S = swelling of the quarter; C = clots in the milk.

The presence of the X protein made quantitation of the wheys quite imprecise for a period following the agalatic phase. However, an estimate of the relative concentration of the immune globulin and X protein is shown in Figure 7 for one quarter. It must be borne in mind that the X protein concentration also included that of a-lactalbumin (Figure 6). In addition, fractions of low concentration were observed with mobility slower than the immune globulin which could not be identified. These were in addition to some light absorption due to a browning reaction that occurred in this area of the paper strips. These small peaks were also present following dialysis of the wheys, after which the browning reaction disappeared.

Of most interest is the total amount of individual proteins appearing in the entire se-
**TABLE 3**

Clinical and laboratory findings, Cow 2414, October 24

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Cells *</th>
<th>Bacteria b</th>
<th>Clinical signs c</th>
<th>WBC d</th>
<th>Rectal temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 6</td>
<td>0.2</td>
<td>35.0</td>
<td>25.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+ 10</td>
<td>0.0</td>
<td>50.0</td>
<td>25.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+ 36</td>
<td>0.3</td>
<td>40.0</td>
<td>25.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+ 48</td>
<td>0.3</td>
<td>50.0</td>
<td>25.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+ 60</td>
<td>0.2</td>
<td>50.0</td>
<td>25.0</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>+ 72</td>
<td>0.2</td>
<td>50.0</td>
<td>25.0</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>+ 84</td>
<td>0.3</td>
<td>40.0</td>
<td>25.0</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>+ 120</td>
<td>0.2</td>
<td>40.0</td>
<td>25.0</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* = Test not done.

* Number of A. aerogenes in thousands/milliliter; + = present in incubated milk only.

S = Swelling; F = flakes in the milk; C = clots in the milk; P = milk purulent.

Total leukocytes/mm blood.

* Cow depressed, with anorexia.

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![Fig. 1](MILK volume (liters). Following inoculation into RR (B), LF (C), and LR (D) quarters.): 2414, October 24.)

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![Fig. 2](PERCENT WHEY PROTEIN (skim).)

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![Fig. 3](MILKING AFTER INOCULATION)

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![Fig. 4](MILKING AFTER INOCULATION)

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Although the relative concentration was still elevated at the third milking, the total amount transudating from blood to milk (Table 4) was higher had it been measured earlier. Despite the return of normal permeability is as a result of depressed milk secretion. Thus,
production and of normal total whey protein levels at the 31st milking, the absolute concentration of udder-formed proteins is still below preinoculation levels. As in Trial 1, the immune globulin content remains elevated. Indeed, this fraction was above preinoculation levels at the 31st and last milking before terminating this lactation. At that time the milk was cell-free and sterile.

The nature and source of the X protein is presently under investigation. It is present in wheys of cows dried off naturally, showing up when milk production reaches a low point and disappearing before colostrum formation. This aspect will be the subject of another report.
TABLE 4
Total amount of whey proteins appearing in the milk following inoculation of the B-C-D quarters of Cow 2414 with two million A. aerogenes (Amount in grams)

<table>
<thead>
<tr>
<th>Time</th>
<th>Serum albumin</th>
<th>Immune globulin</th>
<th>a-Lact-albumin</th>
<th>B-Lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hr</td>
<td>1.26</td>
<td>8.96</td>
<td>6.16</td>
<td>30.14</td>
</tr>
<tr>
<td>+10 Hr, 1st</td>
<td>5.70</td>
<td>7.31</td>
<td>3.76</td>
<td>9.63</td>
</tr>
<tr>
<td>+32 Hr, 2nd</td>
<td>3.43</td>
<td>5.62</td>
<td>1.19</td>
<td>4.33</td>
</tr>
<tr>
<td>+32 Hr, 3rd</td>
<td>0.93</td>
<td>6.32</td>
<td>0.39</td>
<td>1.53</td>
</tr>
<tr>
<td>+1 week, 14th</td>
<td>1.60</td>
<td>20.73</td>
<td>4.13</td>
<td>15.78</td>
</tr>
<tr>
<td>+16 day, 31st</td>
<td>0.60</td>
<td>12.18</td>
<td>6.09</td>
<td>32.63</td>
</tr>
</tbody>
</table>

Although it is extremely hazardous to relate protein mobilities obtained by zone electrophoresis with those obtained by the free moving-boundary method, present evidence suggests the X protein is the same as that observed by Larson (7) from dry-cow whey separated by moving boundary electrophoresis. Larson postulated this protein could represent an accumulation of an a-globulin from blood. Although some a- and b-globulin from blood are undoubtedly present in the wheys obtained early after inoculation, it is felt that X is not a blood protein. Despite repeated attempts, we have never been able to correlate mobility of the X protein on paper with any protein of serum or plasma separated concomitantly. Furthermore, the protein appeared when other evidence of increased permeability (increased pH, chlorides, and serum albumin) was on the wane.

The findings in another trial are significant in this respect. Cow 2365 was inoculated in the B-C-D quarters with five million viable A. aerogenes each. The quarters became permanently agalactic at 36 hr. At 24 hr, secretion from the A and B quarters appeared normal, that from the C quarter was watery, and but a small amount of yellow serum could be expressed from the D quarter. Little to no casein or buffer capacity was found in the secretion from the C quarter on acidification to remove casein. But a small albumin and immune globulin peak were the only whey proteins in evidence, suggesting little to no synthesis of protein was taking place in this quarter. Secretion from the D quarter on electrophoresis resembled blood serum qualitatively and nearly quantitatively, suggesting a complete breakdown in permeability between blood and milk in this quarter. In neither case could a fraction with the mobility of the X protein be seen in secretion from the two quarters.

As a working hypothesis, we suggest that the X protein represents a soluble protein released into the milk following tissue breakdown (involution in the dry cow), breakdown that had not time to proceed in the quarters of Cow 2365 and proceeded but to a slight extent in the quarters of Cow 2414, for milk production eventually returned to near-normal limits in the latter instance.

Knowledge concerning the X protein affords explanation for several observations reported in the literature. Smith (13), for example, observed a peak with a mobility of $-4.1$ to $-4.7 \times 10^{-5}$ sq cm/v/sec, in what he considered to be a most abnormal whey. This is within the range of mobility of the X protein shown by Larson. The report by Weight (14) that a-lactalbumin is increased in acute mastitis, and of Lecce and Legates (8) of a fraction migrating between b-lactoglobulin and a-lactalbumin from mastitic whey, could well be due to confusion arising from the similarity in mobility between the X protein and a-lactalbumin and failure to recognize the existence of this protein. Nilsson (9) also observed a protein fraction (Fraction 5) in wheys from mastitic quarters, which has a position on stained strips similar to the X protein. He suggested that since this fraction was present in mastitic but not normal whey, it was indeed a protein unique to mastitis and probably was a locally produced antibody globulin.

Why the immune globulin fraction remains elevated cannot be stated. Present experiments do not shed any light on this question. It has been demonstrated that circulating antibody can be formed by introduction of antigen into the udder (6). However, for appreciable antibody to appear in the milk, the udder had to be subjected to mastitis. The period when a significant total amount of immune globulin begins to be transudated in present experiments (Table 4) is of the order of magnitude that measurable circulating antibody can usually be demonstrated following an antigenic stimulus. Although we have demonstrated the stimulation in formation of nonspecific circulating antibody following inoculation of A. aerogenes into the udder, we have not been able to find
specific antibody to the organism or its endotoxin in blood or whey (4).

It is unfortunate that the word immune has been appended to that protein fraction designated immune globulin (2). With the exception of colostrum, little is known regarding its immune properties, i.e., this fraction may or may not be entirely the sum total of antibody globulin representative of the antigenic history of the animal. The fact that this fraction remains elevated following acute mastitis does not, of necessity, require an immunological explanation. Dixon et al. (5) have shown that the acinar cells in the udder are responsible for concentrating and transudating immune globulin, whereas serum albumin appears to enter milk via the stroma. These workers comment that this selectivity may depend more upon size, shape, and other general physical-chemical characteristics of the gamma globulin molecule rather than on antigenic determinants.

If circulating antibody plays a part in the local protection of the gland, the two trials with Cow 2414 gave ample opportunity to demonstrate such protection. The data show that the cow was apparently even more susceptible to the second inoculation. It is well known that such reactions are common (Schwartzman phenomenon) when two properly spaced but sublethal amounts of endotoxins of similar organisms are administered to laboratory animals. We have shown that endotoxin from A. aerogenes elicits the same array of signs and symptoms as do viable organisms (4). However, the Schwartzman reaction is characterized by hemorrhagic necrosis. There is little evidence that this extreme pathological condition occurred in the October 24 trial. The possibility exists that there may have been some tissue sensitivity. Also, comparison of cell levels in milk at the 3 hr post-inoculation in the two trials (Tables 2 and 3) reveals a significantly higher cell count in the first trial. It has been shown that leukocytosis into the milk is an effective barrier to the unlimited multiplication of A. aerogenes within the lactating gland (12). Thus, much earlier formation of an inflammatory exudate in Trial 1 could be the explanation for failure to obtain a severe clinical response at that time.

REFERENCES