

Roles of Alpha-Toxin and Beta-Toxin in Virulence of *Staphylococcus aureus* for the Mouse Mammary Gland

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Mutants of *Staphylococcus aureus* which fail to express alpha-toxin (Hly), beta-toxin (Hlb), or both have been constructed by site-specific mutagenesis. The virulence of the mutants was compared with that of wild-type toxigenic strains by intramammary inoculation of lactating mice. A bovine strain, M60, and a laboratory strain, 8325-4, caused acute mastitis and death within 48 h for 60% of the mice inoculated. Animals inoculated with Hly mutants also developed acute mastitis, but no deaths occurred. Comparisons of Hly- or Hlb-positive strains with the double mutation Hly Hlb showed that both toxins led to a significantly higher recovery of *S. aureus* from the gland 48 h postinfection. Histopathological examination of mammary glands showed that phagocytosis of bacteria occurred irrespective of toxigenicity, but toxigenic strains, particularly those which were Hly⁺, continued to multiply, invaded the interalveolar tissues, and produced severe lesions. Stimulation of an inflammatory response by inoculation of the mammary gland with endotoxin prior to challenge with *S. aureus* reduced recovery of the bacteria 10- to 100-fold and, under these conditions, neither alpha-toxin nor beta-toxin contributed significantly to growth and survival.

Staphylococcus aureus is an important pathogen that causes a variety of infections in man (11). It is the predominant cause of intramammary infection in dairy cattle, sheep, and goats, which leads to considerable economic loss (7). Staphylococci have many potential virulence factors, including alpha-, beta-, gamma-, and delta-toxins, coagulase, leucocidin, enterotoxins, and protein A. Strains isolated from bovine mastitis express many of these properties (13), but the specific properties which contribute to intramammary virulence have not yet been determined. There are data which indicate that alpha-toxin (2, 14, 21) and coagulase (8, 14) are important, based upon studies with mice and rabbits. Vaccination studies in cattle indicate that protein A may be important in pathogenesis (15), although other studies have given equivocal results (14). Beta-toxin is commonly expressed by bovine strains, but its role in pathogenesis remains uncertain, although the purified protein has been shown to induce inflammatory changes in the mouse mammary gland (21).

Jonsson et al. (14) used chemical mutagenesis to isolate mutants defective in alpha-toxin, coagulase, and protein A. These were studied in an infection model in the mouse mammary gland, and it was concluded that both alpha-toxin and coagulase contributed to virulence. Virulence was drastically reduced in a strain with a double mutation but was regained by a wild-type recombinant strain. Clear evidence for a role of protein A in virulence was lacking. These studies were hampered by the difficulty of ensuring that chemically-induced mutations were unaltered in other characteristics which might influence virulence.

In this study, strains in which the alpha-toxin genes, beta-toxin genes, or both have been inactivated by site-specific mutagenesis were used to infect lactating mice and thus investigate the role of these toxins in bacterial virulence for the mammary gland.

MATERIALS AND METHODS

Nomenclature and abbreviations. Alpha- and beta-toxins are referred to as Hly and Hlb, respectively. Strains with the ability to express these toxins are referred to as Hly⁺ or Hlb⁺, and those with the inability to do so are referred to as Hly and Hlb mutants.

Bacterial strains. The strains used and their properties are listed in Table 1. The mutants were derived from two strains, M60 and 8325-4. M60 was isolated from a case of bovine mastitis and has been used extensively in pathogenesis studies (4, 5). NCTC 8325 is a human strain which has been widely used in genetic studies (16, 18). NCTC 8325-4 is a derivative of NCTC 8325 cured of prophages. The alpha-toxin gene (*hly*) was inactivated by allele-replacement mutagenesis with an in vitro-constructed *hly::Em^r* mutation (17). The beta-toxin gene (*hlb*) was inactivated by lysogenization with a converting bacteriophage (42E) which integrates in the *hlb* gene (9).

Growth of organisms for infection studies. Vials containing approximately 10⁹ CFUs of the *S. aureus* strains grown in Todd-Hewitt broth (Oxoid, Basingstoke, England), to which 12% glycerol was subsequently added as cryoprotectant, were stored at -20°C. To prepare a challenge inoculum, the contents of a vial were inoculated into 10 ml of Todd-Hewitt broth and incubated at 37°C for 8 h. Cells were harvested by centrifugation at 10,000 × *g* for 10 min, washed in phosphate-buffered saline (PBS), and diluted in PBS to contain the appropriate challenge dose in 0.1 ml. Viable counts were determined by dilution in PBS and plating on agar containing 5% calf blood incubated at 37°C for 24 to 30 h. This medium also served to confirm the elaboration of alpha- and beta-toxin.

Intramammary inoculation of mice. The method of inoculation has been previously described (4). In summary, female MF1 mice were used within 7 days of littering. Mice were lightly anesthetized by halothane inhalation, and the abdomens were disinfected with 70% ethanol. The teat tip of each mouse was removed aseptically, and a 0.1-ml inoculum was introduced into both the right and left upper abdominal

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TABLE 1. *S. aureus* strains^a

| Strain | Genotype | Properties | Source or reference |
|--------|---|---|---------------------|
| 8325-4 | <i>hly</i> ⁺ <i>hly</i> ⁺ | NCTC 8325 cured of prophage | 16 |
| M60 | <i>hly</i> ⁺ <i>hly</i> ⁺ | Bovine mastitis isolate | 4 |
| DU5725 | <i>hly</i> -421::Em ^{rb} <i>hly</i> ⁺ | 8325-4 defective in alpha-toxin | 17 |
| DU5719 | <i>hly</i> ⁺ <i>hly</i> | 8325-4 lysogenized phage 42E | 18 |
| DU5720 | <i>hly</i> <i>hly</i> | DU5725 lysogenized phage 42E | 18 |
| DU5789 | <i>hly</i> -421::Em ^{rb} <i>hly</i> ⁺ | M60 defective in alpha-toxin production | This study |

^a All strains produce coagulase and protein A.

^b Em^r, Erythromycin resistance.

mammary glands via the teat with a 26-gauge needle. These glands are subsequently referred to as R4 and L4. Mice were examined regularly during the 48 h between inoculation and necropsy, and their behavior and condition were recorded. Any mice in extremis were immediately killed, and the mammary glands were removed. Surviving mice were killed after 48 h except where noted. In some experiments, 25 µg of *Escherichia coli* endotoxin (O26:B6; Difco Laboratories, Detroit, Mich.) was introduced into one of the pair of mammary glands 6 h prior to inoculation with 10⁸ CFU of *S. aureus*.

Removal of mammary glands at necropsy. After killing, the abdomen was thoroughly disinfected and the skin was reflected to reveal the mammary glands which were aseptically removed. The macroscopic appearance of the glands was recorded, and discoloration, swelling, texture, and liquefaction were noted.

Culture and histology of mammary glands. For microbiological examination, the dissected mammary glands were placed in Griffith tubes containing 2 ml of PBS and ground to an even tissue suspension. Dilutions (10-fold) were prepared in PBS, and triplicate 20-µl volumes of each dilution were transferred onto calf blood agar plates which were incubated at 37°C for 24 to 30 h. Colonies were counted, and the numbers of bacteria per gland were calculated.

For histological examination, the mammary glands were fixed in buffered Formalin. After fixation, tissues were dehydrated in increasing concentrations of ethanol and embedded in paraffin wax prior to sectioning and staining with hematoxylin and eosin or by the Gram stain method for tissues.

Statistical analysis. When treatments were compared between animals, the mean number of bacterial recoveries per treatment were compared by using one-way analysis of variance and a pooled standard deviation. The 95%-confidence intervals were used to define statistical significance at *P* < 0.05. When treatments were compared within animals, a paired Student *t* test was used (Minitab Inc., State College, Pa.).

RESULTS

Effect of inactivation of the alpha-toxin gene on survival of mice and bacterial recoveries after intramammary inoculation of *S. aureus*. Eight groups of five mice were inoculated via the intramammary route with 10^{3.5}, 10^{6.3}, or 10^{8.5} CFU of strains M60, 8325-4, or Hly mutants DU5789 and DU5725. Of the 20 mice inoculated with the wild-type strains, 12 died within 48 h or were killed in extremis. Nine of these deaths occurred for the 10 mice inoculated with 10^{8.5} CFU of *S. aureus*. Wild-type strains M60 and 8325-4 appeared to have similar virulence for the mice. No deaths occurred in mice inoculated with Hly mutants, even at the higher doses (Table

2), but animals still showed systemic signs of illness such as lethargy, and a staring coat and severe mastitis were present.

There was no evidence that, in the presence of Hlb, Hly increased recoveries of *S. aureus* from the mammary gland. Indeed, there was a consistent trend towards higher recoveries of the Hly mutants, compared with recoveries of the parent strains, although this was not statistically significant.

Examination of mammary glands 48 h postinfection with Hly⁺ Hlb⁺ or Hly⁻ Hlb⁺ strains revealed extensive but similar pathological changes. Macroscopically, the glands were swollen, reddened or discolored, and had a friable texture. Histologically, there was a severe coagulative necrosis involving most of the gland, alveolar structure had largely disappeared, remaining secretory epithelial cells were extensively vacuolated, and there were large amounts of serous exudate throughout the gland. Few intact neutrophils were seen, particularly with Hly⁺ strains.

Role of alpha- or beta-toxin in the recovery of *S. aureus* after intramammary inoculation of mice. To examine the effects of alpha- and beta-toxins in isolation, mutants of 8325-4 were selected. Two groups of five (or in one case six) mice were inoculated in the R4 and L4 glands with approximately 10⁸ CFU of DU5720 (Hly Hlb mutant), DU5719 (Hly⁺ Hlb), DU5725 (Hly Hlb⁺), or 8325-4 (Hly⁺ Hlb⁺). Six mice died or were killed (Table 3), and the surviving mice were killed after 48 h; the glands were removed from all mice, and the bacteria were counted. Glands inoculated with *S. aureus* that produced either alpha- or beta-toxin alone showed significantly higher recoveries than did those inoculated with the Hly Hlb mutant DU5720 (Table 3). Six mice inoculated with the toxigenic strains died, and the remainder showed more severe signs of illness than did the nontoxigenic strain, DU5720. Mice inoculated with DU5719 often showed a blue coloration around the teat and, as for DU5725, swelling was obvious. Both the toxigenic strains

TABLE 2. Effect of inactivation of the alpha-toxin gene on mice and recoveries of *S. aureus* 48 h after intramammary inoculation

| Strain | Properties | Inoculum (log CFU) | Mean (SEM) log CFU recovered per gland | No. of deaths/total no. inoculated |
|--------|-----------------------------------|--------------------|--|------------------------------------|
| M60 | Hly ⁺ Hlb ⁺ | 8.4 | 10.06 (0.03) | 5/5 |
| M60 | Hly ⁺ Hlb ⁺ | 6.3 | 8.44 (0.43) | 1/5 |
| M60 | Hly ⁺ Hlb ⁺ | 3.4 | 8.09 (0.66) | 2/5 |
| DU5789 | Hly ⁻ Hlb ⁺ | 8.7 | 10.27 (0.05) | 0/5 |
| DU5789 | Hly ⁻ Hlb ⁺ | 6.3 | 9.01 (0.09) | 0/5 |
| DU5789 | Hly ⁻ Hlb ⁺ | 3.6 | 8.35 (0.63) | 0/5 |
| 8325-4 | Hly ⁺ Hlb ⁺ | 8.4 | 9.47 (0.02) | 4/5 |
| DU5725 | Hly ⁻ Hlb ⁺ | 8.4 | 10.25 (0.12) | 0/5 |

TABLE 3. Effect of alpha- and beta-toxins on the recoveries of *S. aureus* from the mouse mammary gland 48 h postinoculation with 10^8 CFU^a

| Strain | Properties | No. of mice/no. of glands inoculated | No. of mice dead/ill/well | Mean log (SD) CFU bacteria recovered/gland |
|--------|-----------------------------------|--------------------------------------|---------------------------|--|
| 8325-4 | Hly ⁺ Hlb ⁺ | 10/20 | 3/7/0 | 8.44 (0.83) ^b |
| DU5719 | Hly ⁺ Hlb ⁻ | 10/20 | 2/8/0 | 8.90 (0.30) ^{b,c} |
| DU5725 | Hly ⁻ Hlb ⁺ | 10/18 ^d | 1 ^e /9/0 | 9.51 (0.21) ^c |
| DU5720 | Hly ⁻ Hlb ⁻ | 11/21 ^f | 0/2/8 | 7.37 (0.79) |

^a Analysis of variance was as follows for the indicated sources. Factor: df = 3, SS = 48.012, MS = 16.004, *F* = 42.95. Error: df = 75, SS = 27.947, MS = 0.373.

^{b,c} Values followed by the same letter are not significantly different (*P* < 0.05).

^d Two glands were excluded because of contamination.

^e One mouse was destroyed after 24 h.

^f One gland was excluded because of contamination (11 mice inoculated).

produced macroscopic changes to the gland, a hemorrhagic discoloration, swelling, and were friable when dissected. DU5720 induced less severe changes, and the glands remained cream colored (with some areas of reddening) and elastic.

Histopathology of mammary infection with *S. aureus*. To examine the histopathological changes during infection, three groups of 10 mice were inoculated with 10^8 CFU of strain DU5719, DU5720, or DU5725. Pairs of mice were killed at 20 min or 2, 6, 8, or 24 h postinoculation. Glands were aseptically removed and halved. One half was used for bacteriological culture, and the other half was fixed and stained by the Gram stain method or with hemotoxylin and eosin.

Between 20 min and 2 h after inoculation, all strains could be seen closely associated with the secretory epithelium of the alveoli. Bacteria were also noted in the lumen of the alveoli, but these were generally associated with stained material, possibly cell debris or milk components. After 2 h, there was evidence of polymorphonuclear leukocytes (PMN) marginating in blood vessels and being present in the interalveolar tissue. After 6 to 8 h, PMN were accumulating in the alveoli and there were also changes to the secretory epithelium, most notably distension and vacuolation, and on occasions, cocci were seen within these vacuoles. At this stage, the majority of organisms in alveoli that contained PMN had been phagocytosed. While some PMN contained many staphylococci, adjacent PMN had apparently been unable to phagocytose. In those alveoli not containing PMN, there was a tendency for the organisms to remain associated with epithelium or debris. Up to 8 h postinoculation, consistent differences between the Hly⁺, Hlb⁺, and Hly Hlb mutant strains were not observed and mean bacterial recoveries (plus or minus standard error of the mean) were similar (DU5720, 8.17 ± 0.32 ; DU5719, 8.03 ± 0.3 ; DU5725, 7.77 ± 0.06). However, by 24 h, large areas of the glands infected with DU5719 (Hly⁺) showed relatively few intact PMN in, or close to, the alveoli, although PMN were present in interalveolar tissues. Rapid multiplication of the staphylococci was occurring (Fig. 1A). There were large areas of necrosis, and abscesses were developing. In glands infected with DU5725 (Hly Hlb⁺), multiplication of bacteria had occurred within the alveoli and the organisms were often present in large clumps, apparently where they had been associated with a neutrophil which had subsequently disrupted (Fig. 1b). More PMN and macrophages were present in DU5725-infected glands than in DU5719-infected glands.

but epithelial hyperplasia and vacuolation were marked and, in some areas, necrosis was advanced. In DU5720 (Hly Hlb mutant)-infected glands, the staphylococci were generally restricted to the alveoli in which there were many intact PMN often containing bacteria. There was less necrosis and greater preservation of cellular architecture than with the toxigenic strains, although a disseminated inflammation was present (Fig. 1C) and some abscesses were developing. At this stage, the mean number of bacteria recovered per gland plus or minus standard error of the mean was 8.58 ± 0.1 for DU5720, 9.05 ± 0.09 for DU5725, and 9.49 ± 0.03 for DU5719.

Effect of endotoxin stimulation prior to inoculation on the recoveries of *S. aureus* from the mammary gland. Inoculation of 25 µg of endotoxin into the R4 gland 6 h prior to infection led to an inflammatory response by the time of inoculation. Strains of *S. aureus* were then introduced into both the R4 and L4 glands.

As before, the recoveries of Hly⁺ or Hlb⁺ *S. aureus* were greater than those of the Hly Hlb mutant strain for glands not treated with endotoxin. In endotoxin-treated glands, the recoveries of all strains were similar and significantly lower (10- to 100-fold) than those from their control glands (Table 4).

DISCUSSION

Infection models for the lactating mouse have been extensively used to investigate the pathogenesis of staphylococcal mastitis (4, 5, 14). These models have many advantages over cattle for this work including cost, repeatability, and the opportunities for microbiological or histological analysis of the entire gland. The lesions seen in staphylococcal mastitis in the mouse are similar to those that occur in cattle (5, 6), particularly if endotoxin is used to induce a chronic rather than an acute response.

Although acute staphylococcal mastitis occurs in the cow, it is less prevalent than in other species such as the rabbit (2) or sheep (6). This is despite the fact that the majority of bovine strains elaborate alpha-toxin, which is known to be a lethal and necrotizing toxin (8, 13). In this study, the acute effect of alpha-toxin was confirmed since Hly⁺ Hlb⁺ *S. aureus* often killed mice (12/20) within 48 h of infection while Hly Hlb⁺ mutants did not. However, inactivation of the alpha-toxin gene did not reduce the recovery of the staphylococci from the gland; indeed these mutants, which still elaborated beta-toxin, were recovered in higher numbers than the parent strains. These data might be consistent with reports of in vitro antagonism between alpha- and β-toxins (12).

Comparing the strains which were Hly Hlb mutant (DU5720), Hly⁺ Hlb (DU5719), Hly Hlb⁺ (DU5725), or Hly⁺ Hlb⁺ (8325-4), it was shown that the presence of either alpha- or beta-toxin significantly increased recovery of bacteria from the mammary gland. The demonstration that beta-toxin significantly increases recoveries from the mouse mammary gland and thus contributes to virulence in vivo is a novel finding.

Histological examination showed little difference between Hly⁺ Hlb⁺, and Hly Hlb mutant strains in the first hours postinoculation. Also, bacteriological examination did not reveal marked differences in counts. This may not be surprising since washed bacteria were inoculated and considerable growth might be necessary for biologically significant amounts of toxin to accumulate, particularly since alpha-toxin is elaborated in the late-logarithmic or stationary phase

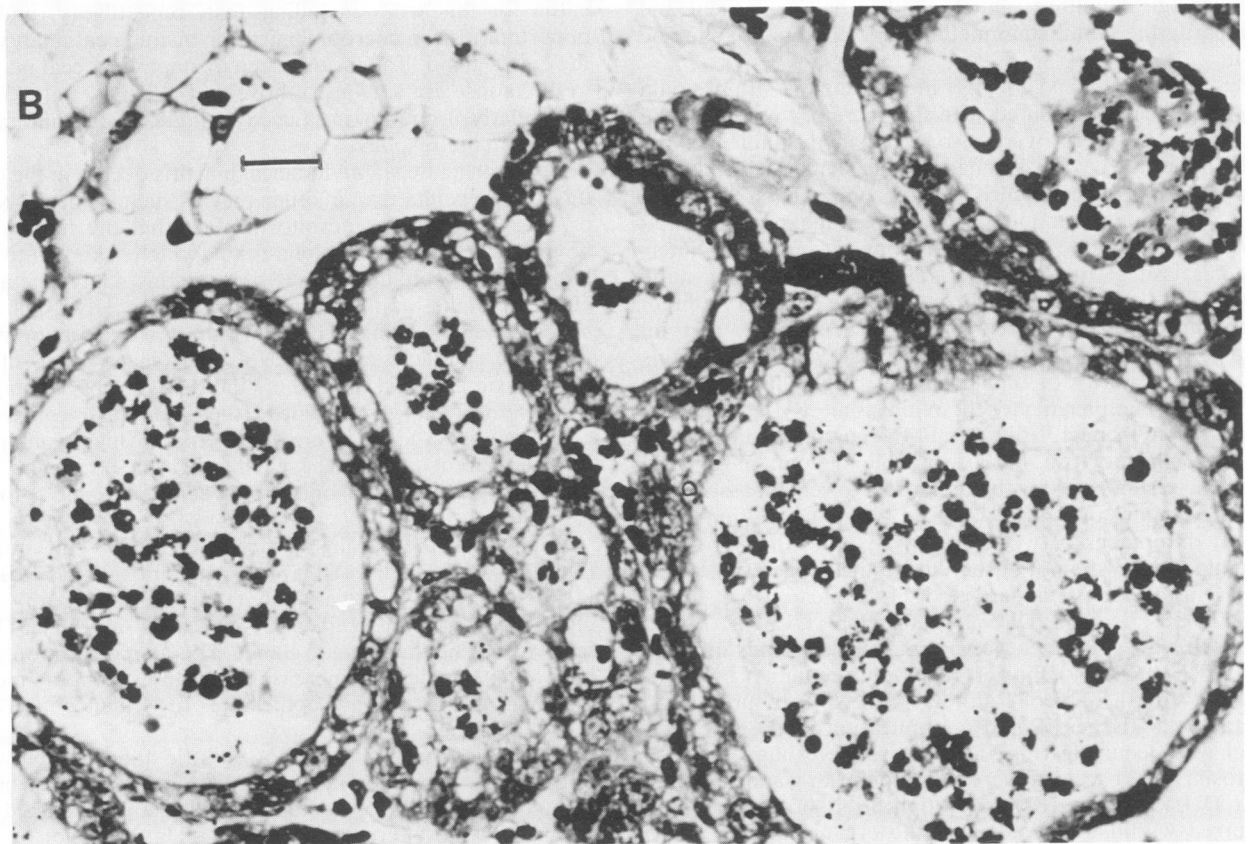
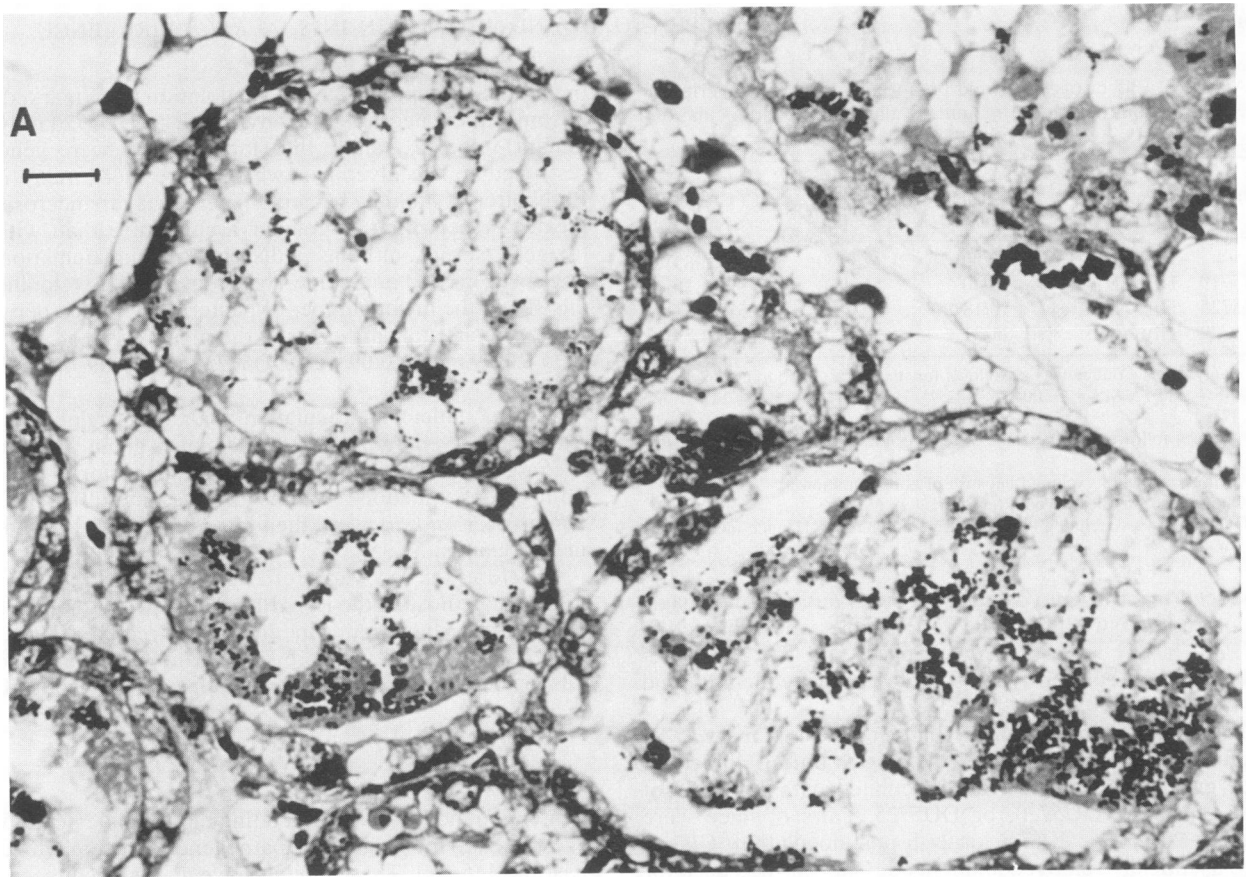


FIG. 1. Hematoxylin- and eosin-stained sections of mouse mammary gland 24 h postinoculation with *S. aureus* strain DU5719 (Hly⁺ Hlb mutant) (A), DU5725 (Hly Hlb⁺) (B), or DU5720 (Hly Hlb mutant) (C). In Fig. 1A through C, phagocytic cells are shown in increasing order and pathological changes such as necrosis, liquefaction, and loss of alveolar structure are shown in decreasing order. Bars, 20 μ m.

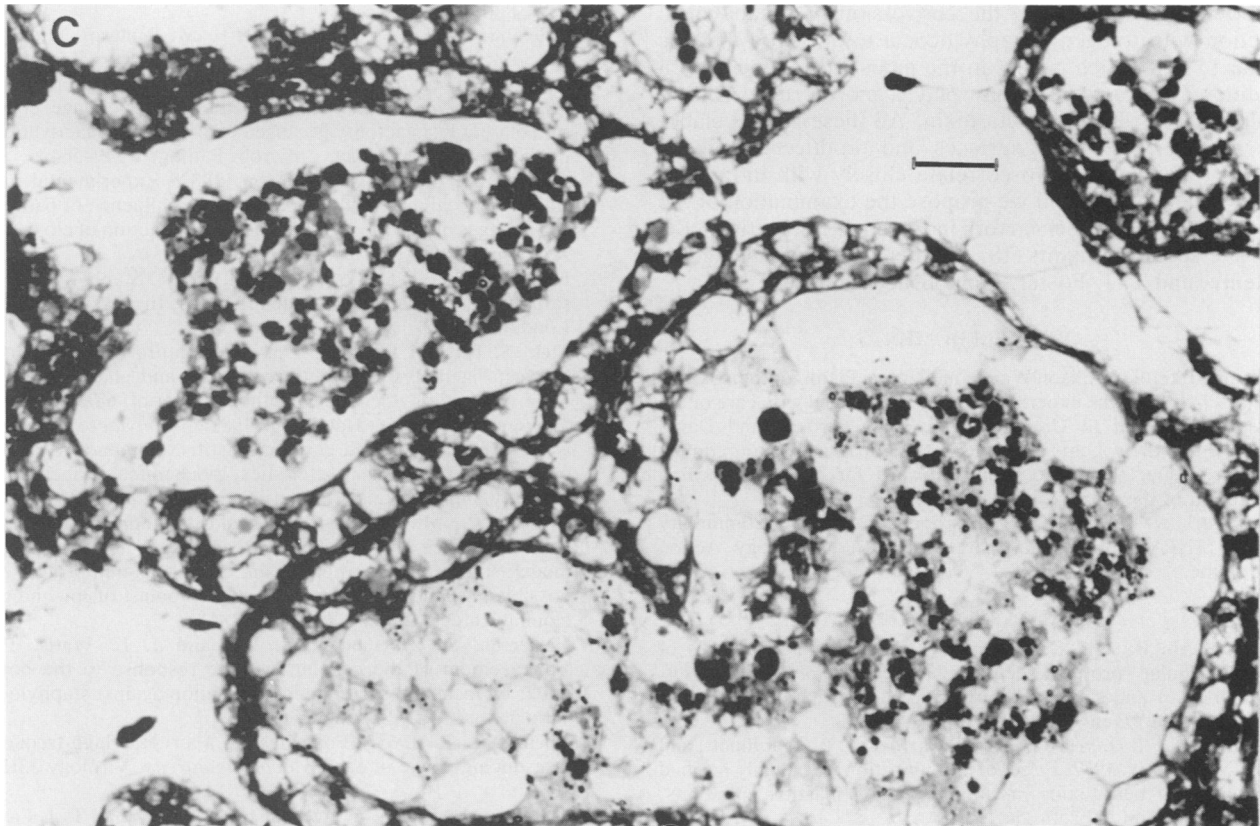


FIG. 1—Continued

of growth in vitro (1). We have detected alpha-toxin in homogenates 24 h postinfection (A. J. Bramley and R. J. Foster, unpublished data).

After 6 to 8 h postinfection, an inflammatory response was present in the alveoli of infected glands and the majority of organisms appeared to be phagocytosed. This has been observed before, and the intracellular location of these bacteria is implicated in the resistance of staphylococcal mastitis to antibiotic therapy (5, 10). Some PMN contained many phagocytosed staphylococci while others were free of bacteria. It is unclear whether this reflects arrival of the PMN in the gland at different times or different phagocytic potential of neutrophil subpopulations.

By 24 h postinfection, neutrophils in glands infected with the Hly⁺ strain became degenerate and fresh neutrophils

were sparse. This may be due to the cytolytic activity of the alpha-toxin, perhaps combined with toxin-induced depression of chemotaxis (19), although some PMN were marginating in blood vessels on the periphery of lesions and were present in the interalveolar tissues. In contrast, purified beta-toxin has been shown to promote a neutrophil response (21). In the glands infected with the Hly H1b⁺ strain, some lysis of neutrophils had occurred, but, in contrast to DU5719 glands, neutrophils were still present in large numbers. This had not prevented bacterial multiplication and invasion of interalveolar tissue by toxigenic staphylococci. In contrast, at 24 h postinoculation, the Hly H1b⁺ staphylococci were associated with neutrophils which were predominately within alveoli. While the secretory alveolar cells were vacuolated, the alveoli were still distinguishable.

Inoculation of the mammary gland with endotoxin led to the presence of neutrophils in the alveoli at the time of infection. This considerably modified the outcome of infection, with staphylococcal growth, survival, or both reduced 10- to 100-fold. Under these conditions, the recoveries of toxigenic and nontoxigenic staphylococci were similar. It has been reported that alpha-toxin is not detectable in the mammary gland by using the chronic infection model probably because the numbers of bacteria present are much lower (3).

Under such circumstances, toxigenicity may not contribute significantly to virulence. The chronic model may better mirror the typical disease of the bovine gland in which the growth of bacteria and the consequent elaboration of toxin is controlled. It has been shown that depletion of neutrophils in

TABLE 4. Effect of endotoxin on the recovery of *S. aureus* from the mouse mammary gland 48 h after inoculation^a

| Strain | Properties | Mean log recoveries of bacteria per gland in the presence (+) or absence (-) of endotoxin ^b | | <i>t</i> Value ^c | <i>P</i> |
|--------|----------------------|--|------|-----------------------------|----------|
| | | - | + | | |
| DU5719 | Hly ⁺ H1b | 8.51 | 6.50 | 4.58 | 0.001 |
| DU5725 | Hly H1b ⁺ | 8.97 | 6.57 | 7.05 | 0.001 |
| DU5720 | Hly H1b | 7.95 | 6.70 | 2.78 | 0.02 |

^a There were no deaths in any group.

^b Nine glands per treatment.

^c From paired *t* test.

the bovine gland leads to the conversion of a subclinical infection with toxigenic staphylococci to an acute mastitis similar to that which occurs in the mouse (20). Under such conditions, other factors may play a more crucial role in virulence than alpha- or beta-toxin. All these strains elaborate protein A on their surfaces and produce coagulase. These properties seem to correlate closely with intramammary virulence (14), and we propose the examination of the roles of these and other factors in the mouse mastitis model with site-specific mutants (18; A. Patel, P. Phonimdaeng, M. O'Reilly, and T. J. Foster, unpublished data).

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