**Staphylococcus aureus** Enterotoxin D Is Secreted in Milk and Stimulates Specific Antibody Responses in Cows in the Course of Experimental Intramammary Infection

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An enterotoxin D (SED)-producing strain of Staphylococcus aureus was used to infect one mammary gland of each of 17 lactating dairy cows. All glands became infected and shed bacteria over a sampling period of 3 weeks. Serum and milk antibodies specific for SED were monitored by an enzyme-linked immunosorbent assay for 12 weeks. Elevated anti-SED antibodies were detected in all cows after infection, and immunoglobulin of the G2 subclass comprised most of the specific serum response. SED was detected in mastitic milk samples from two cows at levels of 5 to 10 ng/ml. An in vitro lymphocyte proliferation assay showed that SED at levels below 10 pg/ml induced proliferation of bovine lymphocytes and that sheep antiserum specific for SED neutralized this proliferative response. Sera obtained from the cows pre- and postinfection inhibited lymphocyte proliferation at SED concentrations of 10 and 50 ng/ml, respectively. The addition of SED to whole blood or to isolated neutrophils had no significant effect on neutrophil function in vitro. The results show that SED is secreted during mammary gland infection, is mitogenic for bovine lymphocytes, and stimulates the production of specific antibodies.

Staphylococcus aureus is a major cause of intramammary infection in ruminants and is a causative agent of a range of human and animal diseases. **S. aureus** mastitis tends to commence with an acute clinical episode which subsequently develops to become a chronic infection (1). The cure rate after antibiotic therapy is low (42). The chronic nature of bovine staphylococcal mastitis and the ability of the bacteria to withstand strong inflammatory responses may be associated with an impairment of the immune response mediated by factors secreted by **S. aureus** (39).

**S. aureus** produces a family of related superantigens (SAgs) that includes several staphylococcal enterotoxins (SE) and toxic-shock-syndrome toxin (TSST) variants (6). Staphylococcal SAgs are prototypical microbial superantigens, characterized by their ability to bind to major histocompatibility complex class II molecules and specific αβ T-cell receptors (32). SAgs bypass the antigenic specificities of T-cell receptors and stimulate abnormally large numbers of T cells. At extremely low concentrations, these molecules can induce profound disturbances in the homeostasis of the immune system (17, 44). These toxins play a critical role in human toxic shock syndrome and food poisoning, but their possible role in the onsets or maintenance of other diseases is not well understood (41).

Geographical differences exist in the occurrence of SAg-producing strains causing mastitis (22). Kenny et al. (19) found that **S. aureus** strains producing enterotoxin D (SED) alone or in combination with **S. aureus** enterotoxin C (SEC) and TSST-1 accounted for 22% of the isolates from New York State. In Norway, a previous study showed that 58% of **S. aureus** isolates expressed SAgs and that the production of SEC and TSST-1 in combination predominated (40). Some reports have suggested that **S. aureus** strains that express SEC and TSST-1 cause severe clinical mastitis unresponsive to therapy (12, 27), whereas other investigations have failed to find a significant correlation between SAg production and clinical manifestations of mastitis (23, 40).

Although the in vitro effect of some staphylococcal SAgs on bovine cells has been studied in detail (5, 8, 9, 45), evidence of in vivo production and the effect of these toxins on clinical disease is scarce. Niskanen et al. detected SEC, but not TSST-1, had an impact on the response after the intramammary infusion of these toxins (21). Kuroishi et al. measured antibodies to SEC and TSST-1 in experimentally infected cows and showed that the infusion of SEA caused inflammatory reactions in the udder (31). In a recent study, Kuroishi et al. measured antibodies to SEC and TSST-1 in mammary gland secretions and observed the inflammatory response after the intramammary infusion of these toxins (21). They found that SEC, but not TSST-1, had an impact on the severity of mastitis.

Studies on the effect of SED on bovine lymphocytes are lacking, as is information on the ability of specific bovine antibodies to modulate the effect of SED. The recruitment of neutrophils from blood to milk and their ability to take up and kill bacteria are important factors in the outcome of intramammary infections. An inhibitory effect of SEA on bovine neutrophils in an in vitro bactericidal assay has been reported (29), but there are few other reports on the effect of staphylococcal SAgs on neutrophil function.

The aim of the present study was to investigate the secretion of SED in experimental bovine **S. aureus** mastitis and to observe whether a measurable humoral immune response against

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this enterotoxin was generated during the course of infection. Experiments were performed to ascertain whether purified SED exerted a mitogenic effect on bovine lymphocytes or influenced neutrophil function in vitro.

MATERIALS AND METHODS

**Bacteria.** *S. aureus* strain M60, which secretes SED, was used to establish experimental bovine mammary infections. The bacteria were grown overnight on modified *Staphylococcus* medium 110 agar (Difco Laboratories, Detroit, Mich.). A single colony was transferred to 10 ml of modified *Staphylococcus* medium 110 broth and incubated for 16 h at 37°C with end-over-end rotation. The bacteria were recovered by centrifugation, washed twice in Dulbecco’s modified Eagle’s medium (Gibco, Gaithersburg, Md.), and resuspended in Dulbecco’s modified Eagle’s medium.

**Animals.** Seventeen American Holstein Friesian cows in mid-lactation were used. The cows were in their first to third lactation. None of the animals had a history of clinical mastitis due to *S. aureus*, and all mammary quarters tested negative for *S. aureus*. One mammary gland from each cow was selected for infection on the basis of a somatic cell count (SCC) of less than 200,000 cells/ml and the absence of pathogens. After the cows were milked, the teat of the selected quarter was disinfected, and 1 ml of a bacterial suspension containing *S. aureus* and the absence of pathogens. After the cows were milked, the teat of the selected quarter was disinfected, and 1 ml of a bacterial suspension containing between 50 and 250 CFU of bacteria was infused using a teat cannula. For the in vitro assays, blood samples from clinically healthy Norwegian Red cows were used.

**Sample collection and analysis.** For the first 21 days postinoculation and thereafter at weekly intervals for 9 weeks, milk samples were collected for SCC analysis and bacteriological examination. Somatic cell counts were determined using a Fossomatic cell counter (Foss Electric, Hillerød, Denmark) while bacterial counts were determined by plating milk samples in triplicate on tryptic soy agar containing 5% bovine blood. Milk and blood samples for antibody analysis were collected at weeks 3, 6, 9, and 12. The milk samples were centrifuged, the fat was aspirated, and the cell pellet was discarded before both milk samples and sera were stored at −20°C.

**Serological methods.** The total immunoglobulin G (IgG) anti-SED antibody response in serum was measured using an indirect enzyme-linked immunosorbent assay. A solution of 1.0 μg/ml SED (Toxin Technology, Sarasota, Fla.) was prepared in 50 mM carbonate-bicarbonate buffer (pH 9.6), and 0.1 ml was added to wells of MaxiSorp microtiter plates (Nunc, Roskilde, Denmark). Serum was diluted 1:500 in phosphate-buffered saline supplemented with 0.05% Tween 20 (PBST), and 0.1 ml was added to each well. Protein G conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), diluted 1:300 in PBST, was used as a conjugate. Both test sera and conjugate were incubated at room temperature for 2 h. P-nitrophenyl phosphate tablets (Sigma) dissolved (1 mg/ml) in 1 M diethanolamine buffer (pH 9.8), containing 0.5 mM MgCl₂ and 0.02% sodium azide, were used as a substrate. The absorbance at 405 nm was read, and the reaction was stopped when the optical density (OD) of a pooled positive control serum sample was 1.0. The total IgG anti-SED antibody response in milk was measured as for serum, except that 0.1 ml of milk diluted 1:10 in PBST was added to each well.

An indirect enzyme-linked immunosorbent assay using monoclonal antibodies against bovine IgG1, IgG2, or IgM (18) was used to evaluate the isotype composition of anti-SED antibodies. Sera diluted 1:500 were added to microtiter plates coated with SED as described above, and mouse monoclonal antibodies specific for either IgG1, IgG2, or IgM were added at a 1:100, 1:200, or 1:200 dilution, respectively. Rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma), diluted 1:500 in PBST, was used as a conjugate. All incubations were performed at 37°C for 2 h. The development and reading of the plates was performed as described above.

**Detection of SED.** Milk samples from 10 cows at day 0 and day 2 after inoculation were tested for the presence of SED. SED was detected using Ridascreen Set A, B, C, D, E (R-Biopharm, Darmstadt, Germany) according to the manufacturer’s recommendations.

**Lymphocyte proliferation assay.** The lymphocyte proliferation assay was performed according to the method of Larsen (24). Three repeated experiments were performed, using blood samples from four cows. Briefly, 200 μl of heparinized whole blood diluted 1:5 in growth medium (RPMI 1640; Life Technologies, Rockville, Md.) containing 10 μg/ml concanavalin A (Sigma), growth medium, or purified SED (Toxin Technology). The plates were incubated for 3 days at 37°C in a 5% CO₂ atmosphere before methyl-[³H]thymidine (Amersham Pharmacia Biotec, Buckinghamshire, England) was added. The cells were harvested 24 h later, and the incorporation of methyl-[³H]thymidine was measured in an automatic liquid scintillation counter (United Technologies Packard, Downers Grove, Ill.). The proliferative response was converted to median counts per minute (CPM) of triplicate samples. In order to evaluate the inhibitory effect of anti-SED antibodies on lymphocyte proliferation, 10 μl of a pooled serum sample taken from three cows prior to and 6 weeks after inoculation or 10 μl of SED antiserum from an immunized sheep was added to the wells. Fetal calf serum was used as a negative control.

**Sheep anti-SED antisera.** An adult female sheep was immunized subcutaneously twice weekly with 1 to 5 μg of SED in saline for a period of 4 weeks. In weeks 5 to 8, the sheep received 25 to 50 μg of SED emulsified in incomplete Freund’s adjuvant, and after a 2-week rest period, the sheep was bled.

**Measurement of bovine gamma interferon (IFN-γ).** Heparinized blood samples from five clinically healthy cows were incubated with SED concentrations ranging from 10 ng/ml to 500 ng/ml in a cell culture plate at 37°C in an atmosphere containing 5% CO₂. After incubation, the plate was centrifuged, and the amount of IFN-γ in the supernatant was determined using a BioSource Bovine IFN-γ kit (BioSource, Nivelles, Belgium). The level of IFN-γ was found by relating the OD values to those of a standard curve made using known amounts of recombinant bovine IFN-γ.

**Neutrophil function assays.** Neutrophils from peripheral blood samples were isolated as described previously (4). Isolated neutrophils and heat-inactivated whole-blood samples from 8 to 13 cows were preincubated with various concentrations of SED for 60 min at 37°C and 38.5°C, respectively. Thereafter, a bactericidal assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was performed essentially as described by Stevens et al. (16, 37). Pretreated neutrophils were incubated with opsonized, live bacteria at a bacteria-to-cell ratio of 50:1, and the absorbance at 550 nm was read. The proportion of bacteria killed was determined by comparing the OD with that of a standard curve, and the results are reported as percentages of bacteria killed.

**Phagocytic Burst Assay.** A Phagotest kit (Orpegen Pharma, Heidelberg, Germany), containing unopsonized fluorescein isothiocyanate-labeled Escherichia coli isolates, was used according to the manufacturer’s instructions, except that the temperature was kept at 38.5°C. The results are reported as percentages of phagocytosing granulocytes, measured by flow cytometry (14). A whole-blood flow-cytometric respiratory burst assay was performed as described by Kampen et al. (15), except that S. aureus strain M60 was used. The results are reported as increases in the geometric means of the fluorescence intensity of the granulocytes.

**Statistical analysis.** The results are presented as means with standard errors of the means (SEMs). Student’s *t* test was used for comparison of antibody levels. The effects of different concentrations of SED on lymphocyte proliferation and neutrophil function in vitro were assessed using the Tukey-Kramer honestly significant difference test. All tests were performed with a significance level of 5%.

**RESULTS**

**Infection of cows.** All cows became infected with *S. aureus* strain M60, and signs of clinical mastitis were evident in most cows 48 to 96 h after inoculation of bacteria. The animals shed the bacteria over a sampling period of 3 weeks and remained infected for the duration of the study. There was variation both over time and between cows in the bacterial shedding and the SCC of milk samples from the infected glands (Fig. 1).

**Measurement of antibodies.** Antibodies to SED in sera were detected in all tested animals, although there was large individual variation (Fig. 2 A). In all but two animals, specific antibodies in sera increased until week 6 and stayed at that level or declined gradually over the next 6 weeks. Two first-lactation animals showed only weak antibody responses to SED. In the two animals with the highest levels of serum antibodies at the time of inoculation, the antibody levels were lower 3 weeks after inoculation but rose to higher-than-preinoculation levels at weeks 6, 9, and 12. Milk antibody responses were evident in all tested cows (Fig. 2 B). In both serum and milk samples, the mean total antibody levels at all time points were significantly higher than at day 0. The serum antibody responses to SED were predominantly of the IgG2 subclass.
with just trace or very low quantities of IgG1 antibodies found. Serum anti-SED IgM remained at preinoculation levels throughout the study period (Fig. 3).

**Presence of SED in milk.** Milk samples from 10 cows at day 0 and day 2 after inoculation were tested for the presence of SED. SED could be detected in only two cows, at levels of 5 and 10 ng/ml, respectively, 2 days after inoculation.

**Mitogenic activity of SED.** The lymphocyte proliferation assay showed that SED caused a significant, dose-dependent proliferation of bovine lymphocytes after 72 h of incubation starting at doses of 0.1 pg/ml and peaking at a count of 52,218 at a concentration of 100 ng/ml (Fig. 4). The addition of 10 µl of sheep anti-SED serum per well neutralized the lymphoproliferative effect of SED for concentrations of SED up to 100 ng/ml (Fig. 5). The same volume of sera, taken from cows prior to intramammary inoculation, had an inhibitory effect on proliferation for SED concentrations up to 10 ng/ml. Sera taken from animals after infection could reduce the effects of SED concentrations of 50 ng/ml (Fig. 5). Stimulation by SED caused a dose-dependent production of IFN-γ in whole blood when evaluated after 24 h of incubation (Fig. 6).

**Effect of SED on neutrophil function.** Final SED concentrations ranging from 30 pg/ml to 300 ng/ml had no apparent effect on the neutrophil granulocytes in the functional assays. However, SED concentrations of 3 µg/ml to 30 µg/ml seemed...
to reduce bacterial killing in the bactericidal assay, but the difference was not statistically significant (Table 1).

**DISCUSSION**

The results from the present study show that SED is secreted in milk and stimulates specific antibody responses in blood and milk samples from cows during the course of an experimental intramammary *S. aureus* infection. Furthermore, a mitogenic effect of SED was demonstrated on bovine lymphocytes in vitro.

Many studies have reported the secretion of staphylococcal enterotoxins by bovine mammary isolates of *S. aureus* when the bacteria are cultured under laboratory conditions. The detec-
tion of an antibody response to SED in serum and milk samples following intramammary infection with *S. aureus* strain M60 and the finding of toxin in selected milk samples provide evidence that SED is secreted in the bovine mammary gland during infection. The relative importance of clones expressing SED as the cause of mastitis varies geographically and over time. Strains expressing SED were prevalent in New York State in the 1990s (19), and strains carrying the *sed* gene are still prevalent in the United States (22). More recently, clones expressing both SEC and TSST-1, most likely harboring the pathogenicity island SaPIbov (10), seem to predominate among bovine isolates in many areas (13, 36). However, the relative contributions, if any, of the different SAgs in the pathogenesis of mastitis are poorly understood.

IgG2 was the principal immunoglobulin found against SED in serum after the experimental intramammary infection. The finding is in accordance with what has been reported for antibodies against staphylococcal alpha-toxin in cows (18). IgG2 is a major opsonin for the phagocytosis of *S. aureus* by bovine blood neutrophils (2) and has been shown to be cytophilic for bovine blood neutrophils and ovine mammary neutrophils (25, 43). The occurrence of low levels of antibodies in the cows at day 0 suggests prior exposure to SED. Although this did not appear to influence the clinical course of the disease or the total level of induced antibodies, the possibility that the predominance of IgG2 might be attributed to a secondary response to the antigen could not be excluded. Also, because the first blood samples were taken 3 weeks postinfection, an early rise in IgM antibodies against SED might have gone undetected.

SED was detected in mastitic milk samples from two cows at concentrations of 5 to 10 ng/ml, and these were the two animals that displayed the highest anti-SED titers in milk samples after infection. It is possible that in vivo, SED secreted by bacteria binds to a major histocompatibility complex class II expressed by cells found in mammary epithelium and connective tissue so that little becomes free in milk (11). Niskanen et al. (31) detected SEC in mastitic bovine milk samples containing 100 to 1,000 CFU of *S. aureus* per milliliter. They could not, however, detect SEA in infected mammary secretions when using an assay with a sensitivity of 1 µg/ml, although the in vitro production of this toxin was higher than that of SEC. This might reflect in vivo production levels of toxins or various absorption levels of secreted toxin in the gland. Differences in the abilities of SAgs to cross mucosal barriers have been described (35), but these aspects have not been investigated in the bovine udder.

Concentrations of SED in picograms per milliliter caused proliferation of bovine lymphocytes in a whole-blood assay. The potencies of staphylococcal SAgs differ with toxin type and the origin of the cells used in the proliferation assay, but generally concentrations as low as 0.1 to 100 ng/ml have mitogenic effects (3, 45). In addition to causing proliferation of T cells and an uncontrolled release of cytokines like tumor necrosis factor alpha, interleukin-2, and IFN-γ (45), SAgs can induce populations of suppressor cells and reduce humoral immune responses (7, 34). In the present study, the rise in IFN-γ production with increasing SED concentrations corresponded with the findings in the lymphocyte proliferation assay. Lymphocytes from the mammary glands of *S. aureus*-infected cows exhibit decreased responses to lectins and staphylococcal antigens compared to T cells from uninfected cows (33).

SED concentrations up to 300 ng/ml did not influence neutrophil function in vitro, while in the bactericidal assay the two highest concentrations tested appeared to inhibit bacterial killing. In a previous report (29), high SEA concentrations resulted in reduced bacterial killing in an MTT-based bactericidal assay. The SED concentrations required to exert an effect in the MTT bactericidal assay were more than a millionfold higher than the concentrations needed to induce proliferation of bovine lymphocytes. Studies reporting an influence of SAgs on human or bovine neutrophils postulate an indirect effect mediated through proinflammatory cytokine production by mononuclear cells (20, 28). As none of the SED concentrations tested seemed to have any effect in the whole-blood assays in the present study, the apparent effect of high concentrations of SED in the bactericidal assay remains unclear.

In the present study, serum antibodies were able to reduce the mitogenic effect of SED in vitro. The finding that anti-serum from a sheep immunized with SED inhibited the proliferative effect of SED in vitro raises the question of a possible role of SAgs as antigens in vaccines against *S. aureus* diseases. Vaccines based on SE toxoids have been evaluated in models of SE-induced toxicosis (26, 38) and recently in murine models of *S. aureus* infection (30). Although the contribution of staphylococcal SAgs to the pathogenesis of mastitis remains ambiguous, the finding that SED is secreted in vivo and can be detected in mastitic secretions suggests that SAgs may be involved in the disease. Further research is needed to determine whether SAgs play an active role in the pathogenesis of *S.

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**TABLE 1. Results for neutrophil function assays used in this study**

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<thead>
<tr>
<th>Assay</th>
<th>Value at indicated SED concn*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µg/ml</td>
</tr>
<tr>
<td>MTT</td>
<td>55</td>
</tr>
<tr>
<td>Phagotest</td>
<td>54</td>
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<tr>
<td>Respiratory burst</td>
<td>57</td>
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</tbody>
</table>

* Values for the MTT bactericidal assay are percentages of bacteria killed. Values for the Phagotest assay are percentages of phagocytosing cells in the granulocyte gate. Values for the respiratory burst assay are increases in the geometric means of the fluorescence intensity in the granulocyte gate. The results are the means for 8 to 13 animals.
S. aureus intramammary infections in cattle and merit inclusion as antigens in a future S. aureus mastitis vaccine.

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