Ovine-Associated *Staphylococcus aureus* Protein with Immunochemical Similarity to Toxic Shock Syndrome Toxin 1

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A toxic shock syndrome toxin 1 (TSST-1) antibody-binding protein produced by an ovine-associated strain of *Staphylococcus aureus* was examined. The protein showed total identity to TSST-1 by immunodiffusion analysis. Western blots (immunoblots) of proteins separated by isoelectric focusing revealed that the TSST-1 antibody-binding protein had a pI of 8.6 rather than 7.0, the pI of standard TSST-1.

It has been reported that toxic shock syndrome (TSS) toxin 1 (TSST-1)-producing *Staphylococcus aureus* strains identified by isoelectric focusing (IEF) were not always the same as those identified by immune techniques; in one study, 10% of the strains which bound specific TSST-1 antibodies were negative for a protein of isoelectric point (pI) 7.0 to 7.2 (1, 9), the pI characteristic of TSST-1. TSST-1 antibody-binding proteins of unusual pIs could explain these discrepancies, but such proteins have not yet been reported. While screening animal-associated *S. aureus* for TSST-1 production, we found that the ovine mastitis-associated *S. aureus* FRI1214 strain produced a protein with the strongest visible immune reaction to TSST-1-specific antibodies in the immunodiffusion test but produced no protein of pI 7.0 to 7.2. It was the purpose of this study to determine the pI and molecular size of the TSST-1 antibody-binding protein produced by this strain. For comparative purposes, five animal mastitis-associated *S. aureus* strains and a known TSST-1-positive, TSS-associated strain were also examined for TSST-1 production and when positive, the molecular sizes and pIs of the TSST-1 antibody-binding proteins were determined.

S. aureus FRI1214 and FRI1169 (TSS associated) were obtained from M. S. Bergdoll (University of Wisconsin, Madison); five bovine-associated *S. aureus* strains (Table 1) were isolated from diseased animals and provided by D. Boriley (University of Minnesota, St. Paul); and *S. aureus* ATCC 12598 (Cowan 1) was obtained from the American Type Culture Collection (Rockville, Md.).

Culture filtrates containing extracellular proteins were obtained by growing strains aerobically at 37°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) (25 ml in a 250-ml flask) for 24 h in a Dubnoff metabolic shaking incubator at 120 rpm (Precision Scientific, Chicago, Ill.), removing the bacterial cells by centrifugation (5,000 × *g* for 10 min), and filtering (unit 120-0020; Nalgene Labware Div., Nalgel/Sybron Corp., Rochester, N.Y.).

Protein A was removed from all culture filtrates by immunoglobulin G (IgG) affinity chromatography. Rabbit IgG was prepared by purification on protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.) according to Goding (5), using antisera which had been prepared against nitrate reductase of a higher plant (4). The purified IgG was adjusted to pH 8.5 after addition of sodium bicarbonate to a final concentration of 0.1 M and coupled to CNBr-activated

Sepharose 4B (Pharmacia) according to instructions provided by the manufacturer. A 4-ml sample of IgG-Sepharose was packed into the Pharmacia K9 column, and 1 ml of crude extract of the various bacterial strains was applied with the eluent that absorbed at 280 nm, collected, and used for electrophoresis. The IgG-Sepharose column was regenerated by washing with 0.1 M acetic acid–0.15 M NaCl, followed by equilibration with phosphate-buffered saline prior to the next use. All these procedures were carried out at laboratory temperatures, although the column was stored in the cold room between uses. The effectiveness of this removal was monitored by first demonstrating the presence of protein A in a culture filtrate of *S. aureus* Cowan 1 (ATCC 12598), a TSST-1-negative and prolific producer of protein A (K. Jensen, Ph.D. thesis, University of Copenhagen, Munksgaard, Copenhagen, Denmark, 1959), by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) (as described below), with complete loss of the active band following IgG affinity chromatography. Extracellular proteins were separated by SDS-PAGE. Filtrate proteins were assayed with the Bio-Rad protein assay kit with a bovine globulin plasma albumin protein standard (Bio-Rad Laboratories, Richmond, Calif.).

SDS-PAGE was performed by utilizing a 6% stacking gel, 12.5% resolving gel, and a discontinuous buffer system as described by Laemmli (7). The protein samples (from 5 to 10 µg of extracellular protein) were electrophoresed in a 0.75-mm-thick gel at 15 mA per slab until the bromophenol blue dye reached the gel bottom, which was approximately 4 h. The gels were stained with Coomassie blue to reveal TSST-1 and molecular size standards (Hoefer Scientific Instruments, San Francisco, Calif.). The separated proteins in identical gels were then transferred to nitrocellulose paper prepared according to Towbin et al. (14) and the Trans-Blot cell (Difco) operating instructions. The transfer was carried out in a Transphor apparatus (Hoefer) at 60 V for 2 h. After the transfer, the membrane was incubated with 3% gelatin in Tris-buffered saline at pH 7.5 for 1 h at room temperature to saturate unoccupied protein-binding sites. The membrane was rinsed with Tris-buffered saline-Tween 20 (0.05%) and incubated serially with TSST-1 monoclonal antibodies (MAbs) and horseradish peroxidase-conjugated goat anti-mouse IgG (GAM-HRP) (Bio-Rad) as described by the manufacturer. In some cases, culture filtrates were screened for proteins with epitopes for TSST-1 MAbs by dot blotting GAM-HRP conjugate (6).

To identify the pIs of *S. aureus* proteins which bind TSST-1 MAbs, the culture filtrates were subjected to IEF

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FIG. 1. Western blot analyses of extracellular proteins of animal-associated strains of *S. aureus*. Purified TSST-1 was applied to lane 1. Lanes 2 through 6 contained proteins produced by *S. aureus* G23, 16619, FRI1214, 5 New, and FRI1169 (TSS-associated control strain), respectively. (A) After separation by SDS-PAGE, proteins were electro-blotted to nitrocellulose and incubated with a 1:500 dilution of TSST-1 MAb 5T. Bound antibodies were detected with GAM-HRP conjugate. Culture filtrate from all strains examined here produced proteins of 24 kDa which bound TSST-1 MAb. *S. aureus* G23 and FRI1169 (lanes 2 and 6, respectively) also produced an additional protein of higher molecular size (26 kDa) which bound the MAb. Identical gels run simultaneously that were stained with Coomassie blue revealed protein molecular size standards (carbonic anhydrase and chicken egg albumin indicated on the left in kilodaltons). (B) Proteins were electro-blotted to nitrocellulose and incubated with 1:500 dilution of TSST-1 MAb 5T after separation by IEF. *S. aureus* G23, 16619, 5 New, and FRI1169 (lanes 2, 3, 5, and 6, respectively) produced proteins of pI 7.0 which bound TSST-1 MAb. Strains *S. aureus* G23 and FRI1169 also produced a minor band of protein which bound the TSST-1 MAb at pI 6.5. *S. aureus* FRI1214 (lane 4) produced a protein which bound TSST-1 MAb at pI 8.6. Identical gels, run simultaneously, were stained with Coomassie blue to reveal protein pI standards (porcine myoglobin, equine myoglobin, and sperm whale myoglobin with pIs of 6.45, 7.3, and 8.3, respectively, indicated on left).

and the resulting polyacrylamide gels were electro-blotted to nitrocellulose and incubated with TSST-1 MAbs as described above. Filtrate proteins were subjected to IEF (Isobox; Hoefer) according to the specifications of the manufacturers. Approximately 10 µg of extracellular protein was applied to polyacrylamide gels of pH 3.5 to 9.5 and run at 15 W of constant power at 5°C for 2 h. Following focusing, the gels were removed, electro-blotted to nitrocellulose, and incubated with TSST-1 MAbs and GAM-HRP as described above. Duplicate analytical IEF gels of the filtrates were run and stained with Coomassie blue. Commercial pI standards of purified proteins (pI range, 4.7 to 10.6; LKB Instruments, Inc., Rockville, Md.) and TSST-1 were also run, and the pIs of culture filtrate proteins were determined with a standard curve prepared with the pI standards.

Production by the *S. aureus* strains of proteins having total identity with TSST-1 was determined by double Ouchterlony immunodiffusion test (8) with a modification published elsewhere (11). TSST-1 produced by *S. aureus* FRI1169 and purified by previously published methods (9) was obtained from Toxin Technology, Inc. (Madison, Wis.), and polyvalent antibodies to this purified TSST-1 were raised in sheep and were a kind gift of H. Pickrum (Procter and Gamble, Cincinnati, Ohio). Murine MAbs 1-3-1, 6-6-4, 6-6-9, 7-2-9, 8-7-5, 10-5-2, 10-7-1, 13-2-1, 13-2-6, and 15-3-4, raised against purified TSST-1 and a kind gift of P. Bonventure (University of Cincinnati Medical Center, Cincinnati, Ohio), were purified and characterized by previously published methods (3). Murine MAbs 5T, 6T, and 8T were a kind gift of M. Bergdoll and were raised against TSST-1 produced by *S. aureus* FRI1169 by previously published methods (13).

In the immunodiffusion analysis, three of the six animal-associated *S. aureus* strains produced a protein with a pattern of total identity with the TSST-1 standard. *S. aureus* FRI1214 produced the strongest visible reaction in the immunodiffusion test. After removal of protein A, extracellular proteins were analyzed by Western blotting using SDS-PAGE and 13 MAbs raised against TSST-1. With this more sensitive test, four of the six animal-associated *S. aureus* strains were found to produce proteins which bound the TSST-1 MAb. In a Western blot done with TSST-1 MAb 5T, *S. aureus* 5 New, G23, FRI1214, and 11619 produced a

protein with a molecular size identical to that of purified TSST-1 (Fig. 1A). *S. aureus* G23 also produced an additional minor protein band of 26 kilodaltons (kDa) with epitopes to TSST-1 MAb. These two TSST-1 MAb-binding proteins (24 and 26 kDa) were also produced by the TSST-1-positive control strain *S. aureus* FRI1169. *S. aureus* 17112 and Brenda did not produce proteins which bound TSST-1 MAb (results not shown).

When culture filtrates from *S. aureus* 5 New, G23, 16619, FRI1214, and FRI1169 were subjected to IEF and stained, proteins of pI 7.0 matching that of purified TSST-1 were clearly visible for all these strains, except for *S. aureus* FRI1214 (results not shown). When a duplicate gel was immunoblotted, proteins of pI 7.0 which bound TSST-1 MAb 5T were found in culture filtrates of *S. aureus* 5 New, G23, 16619, and FRI1169, whereas in the filtrate of *S. aureus* FRI1214, the TSST-1 MAb-binding protein was of pI 8.6 (Fig. 1B). Faint bands of TSST-1 MAb-binding proteins were also seen at pI 6.5 (*S. aureus* G23 and control strain FRI1169). All 13 MAbs in this study reacted with the proteins in the various culture filtrates in an identical manner. Results shown with MAb 5T are representative. The Western blot studies of the IEF- and SDS-PAGE-separated proteins of the seven *S. aureus* strains reported above were repeated several times with the 13 MAbs used in this study with total reproducibility. A summary of the immunochemical characteristics of the animal-associated *S. aureus* strains studied is shown in Table 1.

Although TSST-1 proteins produced by a single strain of *S. aureus* have previously been reported to vary slightly in pI (about 0.1 U [2]), this is the first report of an *S. aureus* strain which produced a protein of complete identity to standard TSST-1 by the Ouchterlony test but differed substantially in pI (1.6 U). Since TSST-1 identification by immune and IEF methods do not always agree (1), TSST-1 proteins of unusual pIs may occasionally account for this discrepancy; the difficulty of the IEF immunoblot technique could be the reason they have not been previously reported. Of the five bovine mastitis-associated *S. aureus* strains examined, three produced a protein identical to standard TSST-1 in pI and molecular size. The ovine mastitis strain, *S. aureus* FRI1214, produced a TSST-1 antibody-binding

TABLE 1. Summary of immunodiffusion and immunoblot analysis of animal-associated *S. aureus* culture filtrates

<i>S. aureus</i> strain	TSST-1 immunodiffusion test	TSST-1 MAb-binding protein(s)	
		Molecular size (kDa)	Isoelectric point (pI)
FRI1214	Positive	24	8.6
5 New	Negative	24	7.0
11619	Positive	24	7.0
G23	Positive	24, ^a 26	6.5, 7.0 ^a
17112	Negative	— ^b	—
Brenda	Negative	—	—
FRI1169 ^c	Positive	24, ^a 26	6.5, 7.0 ^a

^a Represents the major band when a minor band was also visible.

^b —, No TSST-1 MAb-binding protein produced.

^c FRI1169, a strain associated with human TSS, was the source of TSST-1 and was also used as a control in the immunodiffusion and Western blot studies.

protein which exhibited complete identity to TSST-1 in molecular size and in the immunodiffusion reaction but exhibited a pI of 8.6 instead of 7.0, characteristic of TSST-1. This study demonstrates that it cannot be assumed that TSST-1 identified by the techniques of immunodiffusion, Western blot, or enzyme-linked immunosorbent assay necessarily share all other chemical characteristics of the standard TSST-1.

The major MAb-binding protein band produced by the three TSST-1-positive bovine strains had the same molecular size and pI as bovine TSST-1 (24 kDa, pI 7.0), whereas the additional minor MAb-binding protein band produced by *S. aureus* G23 had a molecular size of 26 kDa and a pI of 6.5. The TSS-associated control strain, FRI1169 also produced the minor band (26 kDa, pI 6.5) of TSST-1 MAb-binding protein. This minor MAb-binding protein has been previously described (12, 15), and its exact relationship to TSST-1 remains unknown. It has been suggested that the 26-kDa protein with a pI of 6.5 may represent TSST-1 with its signal peptide still attached (15).

The TSST-1 antibody-binding proteins of 24 kDa and pI 7.0 produced by three of the bovine strains appear to be homologous with the standard TSST-1. The TSST-1 antibody-binding protein of pI 8.6 produced by *S. aureus* FRI1214 is probably closely related to the standard TSST-1, as indicated by its ability to bind all MABs tested and its total identity pattern in the Ouchterlony test. A similar situation exists for staphylococcal enterotoxins C₁ and C₂; these toxins differed by more than one pI unit but reacted with a common antibody (10). Work examining the biological activity of the purified TSST-1 MAb-binding protein of pI 8.6 is currently in progress.

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