Adhesive proteins of haemagglutinating *Staphylococcus aureus* isolated from bovine mastitis

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Two proteins derived from the cell wall of *Staphylococcus aureus*, exhibiting apparent molecular masses of 116 kDa and 145 kDa, were found to bind to human buccal and bovine lactiferous sinus epithelial cells. By using antibodies specific for fibronectin-binding protein of *S. aureus* of human origin, the 116 kDa protein, but not the 145 kDa protein, was identified as a fibronectin-binding protein. The 145 kDa protein bound to bovine fat globule membranes, human buccal epithelial cells, bovine lactiferous sinus epithelial cells and sheep erythrocytes. The properties of the 145 kDa protein suggest that it is an adhesin with a possible role in the early stages of the development of bovine mastitis.

Introduction

Bovine mastitis, i.e. inflammation within the mammary glands of dairy cattle, is a serious disease causing heavy economic losses worldwide. *Staphylococcus aureus*, the most frequent pathogen, gives rise to both acute and chronic forms of mastitis.

Although it is generally believed that bacteria bind to and colonize mucosal surfaces in a specific manner (Christensen *et al.*, 1985) and that *S. aureus* adheres to bovine mammary gland epithelial cells *in vitro* (Frost *et al.*, 1977), the pathogenesis of *S. aureus* in the mammary gland is still not fully understood. After transmission of bacteria to the teat orifice and penetration through the teat canal, *S. aureus* cells have to resist the wash-out effect of milking. Experimental infections using low numbers (500 c.f.u.) of *S. aureus* to inoculate teat cisterns 1 h prior to machine milking give rise to clinical mastitis (P. Jonsson, unpublished data). Such experiments strongly suggest an *in vivo* adhesion of bacteria to udder epithelial cells.

Another interesting phenomenon of relevance to the pathogenesis of *S. aureus* in the mammary gland has been suggested by Sandholm *et al.* (1989). They proposed that binding of bacteria to fat globules enables *S. aureus*

to resist milk flow and distributes bacteria towards the upper regions of the ductular system and of the mammary gland by flotation of the fat globules.

Haemagglutinating properties of *S. aureus* isolated from bovine mastitis have recently been found to be correlated with the ability to adhere to epithelial cells (Lindahl *et al.*, 1989).

The aim of this work was to examine the cell wall of *S. aureus* to establish whether the bacterium produced adhesive factors relevant to pathogenesis.

Methods

Strain and cultivation. *S. aureus* strain 14391 was a clinical isolate from a case of acute bovine mastitis in 1985. It was preserved at –70 °C in Tryptic soy broth (TSB) containing 15% (v/v) glycerol.

Cultivation was done at 37 °C on nutrient agar supplemented with lactose and trace elements. One litre of medium contained: 3 g Bacto beef extract (Difco), 5 g Bacto peptone (Difco), 5 g NaCl, 1 g lactose, 0.3 g Fe(NH₄)₂(SO₄)₂, 0.015 g MnSO₄·4H₂O, 0.020 g MgSO₄·7H₂O and 15 g Bacto agar (Difco).

Haemagglutination. Bacterial cells grown on nutrient agar were collected with sterile cotton swabs and suspended in 0.11 M-sodium phosphate buffer (PB), pH 7.4. The optical density at 550 nm was adjusted to 1.0, which corresponded to 5×10^8 c.f.u. ml⁻¹. Twenty-five microlitres of bacterial suspension was diluted in a twofold series in U-shaped wells of a polystyrene microtitre plate (Flow Laboratories) to a dilution of 1/64 in 0.11 M-PB, pH 7.4. An equal volume of 0.11 M-PB, pH 7.4, was added to each well, as was 25 µl of erythrocyte suspension (see below).

As a negative control, 25 µl of an erythrocyte suspension was

Abbreviations: PB, phosphate buffer; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gradient gel electrophoresis; TBS, Tris-buffered saline.

incubated with 2 vols 0.11 M-PB, pH 7.4. After briefly rocking the microtitre plate it was incubated without shaking for 3 h at 4 °C. Haemagglutination appeared as a mat of erythrocytes covering the bottom of the wells, while in the absence of haemagglutination a red button of sedimented erythrocytes formed in the bottom of the wells. The reciprocal of the highest bacterial dilution giving an unequivocally positive reaction was recorded.

Erythrocytes. Blood from sheep was collected in 1% (w/v) sodium citrate and washed three times with 10 vols 0.11 M-PB, pH 7.4. Erythrocytes were suspended and diluted to a concentration of 5×10^7 cells ml⁻¹.

Epithelial cells. Clinically healthy udders of cows assembled for slaughter were chosen. Udders were excised immediately after slaughter, cleaned, and the required quarters dissected out. The epithelial lining of the ductular system from the streak canal to the ductioles was exposed by cutting with a pair of scissors. The epithelial surface was rinsed several times with 0.11 M-PB, pH 7.4, to remove milk.

Epithelial cells were obtained by gentle unidirectional scraping of the surface of the lactiferous sinuses with a soft interdental brush. The brush bearing detached cells was then twirled in 10 ml 0.14 M-NaCl containing 0.01 M-sodium phosphate buffer (PBS), pH 7.4. The same area was not scraped more than eight times to avoid collection of basal membrane cells. Cells were then washed three times in 0.11 M-PB, pH 7.4, at 250 g for 10 min, resuspended and counted in a Bürker chamber. After final washing the cells were resuspended in the same buffer to approximately 1×10^6 cells ml⁻¹.

Human buccal epithelial cells were gently scraped off with sterile cotton swabs and suspended in 0.11 M-PB, pH 7.4. They were washed and diluted as described above for lactiferous sinus epithelial cells.

Periodate treatment. Periodate treatment was performed with 20 mM-periodic acid in cold 0.11 M-PB, pH 6.5, for 20 min at 4 °C. Cells were washed in 10 vols 0.11 M-PB, pH 6.5, and treated with cold 5 mM-NaBH₄ for 30 min. After treatment, cells were washed in 10 vols 0.11 M-PB, pH 7.4, harvested, resuspended and diluted to the initial concentration.

Immunoglobulins. *S. aureus* strain 14391, grown on nutrient agar, was suspended in saline (2×10^8 bacteria ml⁻¹) and killed by heating for 10 min at 80 °C. Bacterial cells (1×10^8) in saline were mixed with an equal volume (0.5 ml) of Freund's complete adjuvant. Following collection of preimmune serum, subcutaneous injections were made on three occasions at 2 week intervals. Blood was drawn 2 weeks after the last injection. The serum was applied to a Protein-A Sepharose gel bed and the retained immunoglobulins were eluted with 1 M-acetic acid.

Solubilization of the bacterial cell wall. *S. aureus* strain 14391 was cultivated in 2.4 litres of TSB on a rotary shaker at 37 °C for 6 h. Bacterial cells were sedimented by centrifugation at 6800 g for 10 min and washed with 0.2 M-potassium phosphate buffer, pH 7.0. Pellets were suspended at 0.1 g (wet weight) bacteria ml⁻¹ in 0.2 M-potassium phosphate buffer, pH 7.0, containing 2 mM-phenylmethylsulphonyl fluoride (PMSF) and 5 mM-benzamidine.

After addition of lysostaphin (5 µg ml⁻¹), DNAase (5 µg ml⁻¹) and 4 mM-MgSO₄, the suspension was incubated for 3 h at 37 °C. Solubilized material was separated from cell debris by centrifugation at 17500 g for 20 min at 4 °C. The supernatant fraction was filtered through a 0.22 µm Millipore filter and dialysed twice against 1 litre of sample buffer (20 mM-sodium phosphate buffer, pH 7.0, containing 0.5 M-NaCl and 1 mM-imidazole) at room temperature for 2 h. Dialysed material was again centrifuged (27000 g, 20 min, at 4 °C) before application onto Chelating Sepharose 6B (Pharmacia).

Immobilized metal ion chromatography. Chelating Sepharose 6B was washed according to the instructions of the manufacturer. A column (18 × 900 mm) containing 15 ml gel was prepared and loaded with 2.5 ml 0.2 M-CuSO₄ in water. The column was washed with 100 ml water followed by 25 ml 20 mM-sodium phosphate buffer, containing 0.5 M-NaCl and 20 mM-imidazole (pH 7.0). The column was finally washed with 100 ml 20 mM-sodium phosphate buffer containing 0.5 M-NaCl and 1 mM-imidazole (pH 7.0). The sample was applied, followed by a 200 ml washing step using sample buffer (see above), and then eluted with a 200 ml imidazole gradient (1–20 mM). Material eluting early (i.e. before 10 mM-imidazole) was pooled and dialysed twice against water and twice against 20 mM Tris/HCl (pH 7.6) containing 0.25 M-taurine.

Ion-exchange chromatography. A Mono Q (HR 5/5) column was used with Beckman System Gold equipment to further purify the material obtained by Chelating Sepharose 6B chromatography. A 5 ml sample in 20 mM-Tris/HCl buffer (pH 7.6) containing 0.25 M-taurine, was applied and eluted with a 50 ml NaCl gradient (0.2–0.5 M). The purity of eluted material was analysed by SDS-PAGE, using 10–15% polyacrylamide gradient gels, in a Pharmacia Phast system. Gels were silver-stained according to the method supplied by the manufacturer.

Agglutination of *S. aureus* with specific IgG. Haemagglutination-positive *S. aureus* (strain 14391) cells (5×10^8 cells ml⁻¹) in PBS, pH 7.4, were mixed on a glass slide with an equal volume of rabbit IgG specific for purified staphylococcal protein (0.7 mg ml⁻¹) in PBS, pH 7.4. The reaction was recorded after 2 min in comparison with a similar mixture lacking IgG. Specific IgG were produced as described above, by using 9 µg purified staphylococcal protein in Freund's complete adjuvant for each injection.

Binding of solubilized *S. aureus* proteins to erythrocytes and epithelial cells. Sterile-filtered and dialysed lysostaphin lysate of *S. aureus* in PBS, pH 7.4 (100 µl), was incubated for 1 h at room temperature with an equal volume of sheep erythrocytes (5×10^9 cells ml⁻¹), human buccal epithelial cells or bovine lactiferous sinus epithelial cells (1×10^6 cells ml⁻¹). Cells were separated from unbound material by centrifugation at 1800 g for 20 min on a 1.5 ml cushion of 10% (v/v) Percoll in PBS, pH 7.4, solubilized with 2% (w/v) sodium dodecyl sulphate and prepared for SDS-PAGE.

Fat globule membranes. Fat globule membranes were prepared as previously described (Lindahl, 1989). Briefly, fresh milk was skimmed by centrifugation at 4000 g and 4 °C for 15 min. The collected cream was repeatedly washed by suspension in warm (40 °C) buffer and centrifugation as above. The washed cream was churned and the released fat was removed after centrifugation as above. The remaining fluid was centrifuged at 39000 g at 4 °C for 1 h and the resulting pellet containing fat globule membranes was suspended in PBS, pH 7.4.

SDS-PAGE. SDS-PAGE, with 10–15% gradient gels, was performed according to Blobel & Dobberstein (1975). Polypeptides separated by SDS-PAGE were transferred onto nitrocellulose membranes as described by Kijimoto-Ochiai *et al.* (1985).

Transblots were developed as follows. After quenching for 2 min in 20% (v/v) Tween 20, 10 mM-Tris, pH 10.2, followed by a 45 min incubation in PBS, pH 6.8, containing 5% (w/v) bovine serum albumin, the nitrocellulose filters were washed in 0.14 M-NaCl containing 10 mM-Tris/HCl buffer, pH 7.6 (TBS). Preimmune serum (diluted 1/400), rabbit IgG raised against heat-killed *S. aureus*, or rabbit IgG Fab fragments specific for *S. aureus* fibronectin-binding protein (supplied by Dr G. Fröman, University of Uppsala, Uppsala, Sweden) (10 µg ml⁻¹) in TBS was used as the primary antibody. Incubation was performed for 1 h at room temperature, followed by washing in TBS, twice, for 10 min. Transblots were then incubated for 1 h with a biotin-conjugated secondary antibody (goat anti-rabbit IgG, Sigma) diluted

1/400 in TBS. After washing, filters were incubated for 1 h with peroxidase-conjugated avidin-biotin complex (Dakopatts) followed by washing. Staining was done with a mixture containing 3-aminoethyl-9-carbazole.

Binding of radiolabelled protein purified from lysostaphin lysates. Purified proteins were radiolabelled using Enzymobeads (Bio-Rad). Protein (1 µg) was labelled with 0.5 mCi (18.5 MBq) Na¹²⁵I for 15 min by the procedure supplied by the manufacturer.

Binding of iodinated proteins to human buccal epithelial cells was done in Minisorp tubes (Nunc). The incubation mixture contained 5×10^7 cells and labelled protein corresponding to between 10^4 and 3×10^4 c.p.m. After incubation, 50% (150 µl) of the mixture was transferred to a new tube with 200 µl PBS (pH 7.4), layered on top of 10% (v/v) Percoll in PBS (1.5 ml) and centrifuged at 1800 g for 30 min at 4 °C. The supernatant fraction was discarded and the radioactivity of the resulting pellet was measured in a gamma-counter. Background values were obtained from tubes without cells, but otherwise treated as above. All determinations were made in duplicate.

Iodinated staphylococcal protein was allowed to bind to proteins from fat globule membranes, human buccal epithelial cells and bovine lactiferous sinus epithelial cells, which had been separated on an SDS-polyacrylamide gradient gel and transferred onto nitrocellulose membranes. Transblots were quenched and incubated with a solution containing 4×10^5 c.p.m. iodinated protein ml⁻¹ for 2 h at room temperature.

Autoradiography was done using Kodak X-AR and an intensifying screen for two weeks.

Results

Agglutination of sheep erythrocytes by *S. aureus* strain 14391 was detectable with 1.3×10^6 c.f.u. ml⁻¹, or more. Periodate-treated erythrocytes were not agglutinated, even with a bacterial concentration of 1.7×10^8 c.f.u. ml⁻¹. Adhesion of strain 14391 to bovine lactiferous sinus epithelial cells varied between 1 and 5 bacteria per cell, a result that made it difficult to obtain reliable data for further studies using this assay. Adhesion of *S. aureus* to human buccal epithelial cells was usually low (5–10 bacteria per cell), but could occasionally exceed 20 bacteria per cell. Periodate treatment of human buccal epithelial cells did not significantly affect adhesion of strain 14391.

Human buccal epithelial cells and sheep erythrocytes incubated for 60 min with a lysostaphin lysate of *S. aureus* strain 14391 retained staphylococcal proteins. This was demonstrated by using antibodies against heat-treated staphylococcal cells to probe for staphylococcal proteins on transblots of the electrophoretically separated polypeptides (Fig. 1). Human buccal epithelial cells and bovine lactiferous sinus epithelial cells showed no difference with respect to binding of staphylococcal proteins (data not shown). Accordingly, for convenience, human buccal epithelial cells were used in most experiments instead of bovine lactiferous sinus epithelial cells.

Staphylococcal proteins which bound to both human

Fig. 1. Western blotting of solubilized human buccal epithelial cells and sheep erythrocytes to which solubilized staphylococcal cell proteins, obtained by lysostaphin treatment, had been allowed to bind. SDS-PAGE was performed and the separated proteins were transferred to nitrocellulose filters. Rabbit IgG raised against heat-killed staphylococcal cells was used as primary antibody to detect staphylococcal polypeptides bound to cells. Lane 1, immunoblot of human buccal epithelial cell proteins; lane 2, immunoblot of sheep erythrocyte proteins. Molecular masses are indicated by the kDa scale.

buccal and bovine lactiferous sinus epithelial cells exhibited apparent molecular masses of 116 kDa and 145 kDa. A staphylococcal protein having an apparent molecular mass of 67 kDa was also detected (Fig. 1). This polypeptide does not, however, bind to epithelial cells, but is present due to its ability to form complexes with other staphylococcal proteins such as the 116 kDa component (data not shown). In a similar experiment, it was shown that the 145 kDa component bound to sheep erythrocytes, whereas the 116 kDa component did not (Fig. 1). Diffuse polypeptide bands below 100 kDa (Fig. 1, lane 2) originated from proteins of sheep erythrocytes, and were visualized with pre-immune serum or even in the absence of primary antibodies.

Both the 116 kDa and 145 kDa components were purified from lysostaphin lysates of *S. aureus* strain 14391 using immobilized metal ion chromatography and anion-exchange chromatography. The purified 145 and 116 kDa proteins, labelled with ¹²⁵I, were allowed to bind to human buccal epithelial cells to confirm the existence of binding properties. Significant fractions of

Fig. 2. Western blotting of purified 145 kDa *S. aureus* cell wall component (lane 1), purified 116 kDa *S. aureus* cell wall component (lane 2), solubilized human buccal epithelial cells to which the purified 145 kDa component was allowed to bind (lane 3), solubilized human buccal epithelial cells to which the purified 116 kDa component was allowed to bind (lane 4), and solubilized human buccal epithelial cell proteins as a control (lane 5). SDS-PAGE was performed and the separated proteins were transferred to nitrocellulose membranes. Fab fragments of rabbit IgG specific for staphylococcal fibronectin-binding protein (lanes 1 and 2), or rabbit IgG raised against heat-killed staphylococcal cells (lanes 3–5), were used as primary antibodies. Molecular masses are indicated by the kDa scale.

the radioactivity added, 9.6% (116 kDa component) and 13.3% (145 kDa component), were found with the collected cells. Periodate treatment of the human buccal epithelial cells did not significantly affect binding.

The purified staphylococcal components separated by SDS-PAGE and transblotted to nitrocellulose filters were incubated with Fab fragments of rabbit anti-fibronectin-binding protein (Fröman *et al.*, 1987). Heavy staining of the 116 kDa component was observed, as well as the presence of degradation products, whereas the 145 kDa component was only very weakly stained (Fig. 2). The purified staphylococcal proteins were found to retain their ability to bind to epithelial cells during purification (Fig. 2).

Purified 145 kDa component (Fig. 2) was used to obtain specific antibodies in a rabbit. A strong agglutination of haemagglutination-positive *S. aureus* (strain 14391), using specific IgG, demonstrated the presence of the 145 kDa component on the bacterial cell wall.

Polypeptides of bovine fat globule membranes, human

Fig. 3. Separation by SDS-PAGE of 20 µg bovine fat globule membrane proteins (lanes 1 and 4), solubilized proteins from 2.8×10^4 human buccal epithelial cells (lanes 2 and 5) and solubilized proteins from 2.5×10^4 bovine lactiferous sinus epithelial cells (lanes 3 and 6). A transblot (lanes 1–3) was incubated with ^{125}I -labelled 145 kDa component (4×10^5 c.p.m. ml^{-1}). Binding was visualized by autoradiography. Coomassie brilliant blue was used to stain the gel for comparison (lanes 4–6). Molecular masses are indicated by the kDa scale.

buccal epithelial cells and bovine lactiferous sinus epithelial cells were separated by SDS-PAGE. Two sets of samples were run on the same gel. One part of the gel was electrophoretically transblotted to a nitrocellulose membrane, while the other part, with the same set of samples, was stained with Coomassie brilliant blue (Fig. 3). After autoradiography, the transblot, to which the radiolabelled 145 kDa component had been allowed to bind, displayed a few bands. The 145 kDa staphylococcal protein bound to components of fat globule membranes (65 kDa and 45 kDa), human buccal epithelial cells (52 kDa) and bovine lactiferous sinus epithelial cells (15 kDa and 13 kDa) (Fig. 3).

Discussion

Current theories concerning the development of mastitis caused by *S. aureus* suggest colonization of the teat canal or skin as a prerequisite for the infection of the

mammary gland. Adhesion of *S. aureus*, which has been a matter of dispute for more than a decade (Anderson, 1978; Frost *et al.*, 1977) is likely to occur, at least in the teat canal. The events following entry of bacteria into the teat cistern, which lead to the development of clinical symptoms of mastitis, are yet to be explained. Interesting ideas such as 'hitch-hiking' on rising cream particles (Sandholm *et al.*, 1989) and the possibility that fibronectin might serve as an epithelial receptor for a staphylococcal surface protein have been presented (Fröman *et al.*, 1987; Mamo *et al.*, 1988). However, more information is still needed to elucidate the pathogenesis of *S. aureus* in the bovine udder. The recent discovery of haemagglutinating properties of *S. aureus* points to the possibility of an adhesive factor with specificity for components other than fibronectin (Lindahl *et al.*, 1989).

Haemagglutination by *S. aureus* does not occur with periodate-treated erythrocytes, indicating the presence of a carbohydrate-specific haemagglutinin. Moreover, the correlation found between haemagglutinating properties and the ability of *S. aureus* to adhere to epithelial cells suggests that the haemagglutinin might be an additional virulence determinant (Lindahl *et al.*, 1989).

Two proteins present in lysostaphin lysates of *S. aureus* have the capacity to bind to human buccal epithelial cells and bovine lactiferous sinus epithelial cells. Thus, they might both act as adhesins. One of them having an apparent molecular mass of 116 kDa, is recognized by antibodies specific for the fibronectin-binding protein of *S. aureus* (strain Newman) and only binds to epithelial cells. The 145 kDa component does not react with antibodies to *S. aureus* fibronectin-binding protein, but has the capacity to bind to epithelial cells as well as to fat globule membranes and sheep erythrocytes.

Since the only component present in lysostaphin lysates of *S. aureus* having the capacity to bind to sheep erythrocytes is the 145 kDa protein, this component is likely to be the haemagglutinin. Its receptor would be of a carbohydrate nature since periodate-treated erythrocytes were not agglutinated. Binding of the 145 kDa protein occurred to proteins of diverse molecular mass present on fat globule membranes, human buccal epithelial cells and bovine lactiferous sinus epithelial cells; this could be explained if a similar carbohydrate structure is present

on these proteins. The 145 kDa protein may be an adhesin that enables initial colonization of the epithelium of the bovine mammary gland by *S. aureus*. Furthermore, the discovery of this component, which binds to fat globule membrane proteins, supports the hypothesis that *S. aureus* uses fat globules as a vehicle for efficient dissemination in the mammary gland (Sandholm *et al.*, 1989).

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