Calcium- and mucin-binding proteins of staphylococci

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The association of staphylococci with the mucus gel that overlays the mucosa of the respiratory tract may lead to clearance of cocci or, in certain conditions such as cystic fibrosis (CF), to colonization. In the present study, a quantitative radioassay was used to study the effect of Ca^{2+} , which is elevated in CF sputa, on the adhesion of ^3H -labelled *Staphylococcus aureus* to submaxillary gland mucin immobilized in MaxiSorp 96-well, break-apart modules. Ca^{2+} significantly enhanced the adhesion of *S. aureus* (five strains) and *Staphylococcus epidermidis* (four strains). The reaction was specific because adhesion was not enhanced in the presence of Mg^{2+} , Ca^{2+} + EGTA (a Ca^{2+} chelator) or protamine and was not attributable to hydrophobicity of the test strains. Staphylococcal adhesion was significantly ($P \leq 0.005$) blocked in the presence of highly sialated and sulphated reagents, which suggests that Ca^{2+} binds to the sialic acid and sulphate residues of immobilized mucin. The Ca^{2+} -binding sites on the surface of *S. aureus* were trypsin-sensitive; in addition, ^{125}I -labelled solubilized *S. aureus* surface proteins reacted with immobilized mucin in a direct binding assay, and the reaction was significantly enhanced by Ca^{2+} . Autoradiography demonstrated that ^{45}Ca bound directly to two polypeptides (M_r 170 000 and 150 000) of solubilized staphylococcal surface proteins separated by SDS-PAGE, and that ^{125}I -labelled mucin bound directly to three staphylococcal polypeptides (M_r 40 000, 35 000, and 29 000). These results suggest that *S. aureus* adhesion to mucin is mediated by at least two mechanisms: via Ca^{2+} -binding surface proteins in the presence of Ca^{2+} and via mucin-binding surface proteins unrelated to Ca^{2+} .

Introduction

It is well known that *Staphylococcus aureus* is an initial and chronic colonizer of the mucus gel present in the airways of patients with cystic fibrosis (CF) (Burns & May, 1968; Goering *et al.*, 1990; Marks, 1990). Bauernfeind *et al.* (1987) reported a longitudinal study of sputa from 102 patients with CF in which the incidence of *S. aureus* and *S. epidermidis* was approximately 36%. Kilbourn (1978) hypothesized that the increased concentration of Ca^{2+} which is present in sputa of CF patients makes the sputum susceptible to colonization by *S. aureus*. Subsequent *in vivo* studies have shown that *S. aureus*, *S. epidermidis* and *S. saprophyticus* have the capacity to associate with the mucus gel coating the upper respiratory tract of ferrets, and *in vitro* these staphylococci bind specifically to immobilized purified mucin in a quantitative solid-phase radioassay (Sanford *et al.*, 1989). Growth conditions of the staphylococci

affected their capacity to bind to mucin *in vitro*; growth in the presence of insoluble complexes of calcium magnesium phosphate significantly enhanced adhesion to mucin. The present study examines the role of calcium in the adhesion of staphylococci to immobilized mucin by use of a modification of the original radioassay and also partially characterizes the staphylococcal adhesins and mucin receptors.

Methods

Media and reagents. The following media and chemical reagents were used: M199, a chemically defined medium modified with Earle's salts and containing L-glutamine, pH 7.2 (Grand Island Biological Co.); Hanks' balanced salt solution (HBSS; Gibco); phosphate-buffered saline, pH 7.2 (PBS); FTA haemagglutination buffer (BBL); Tween 20 (Fisher Scientific Co.); heparin (from bovine lung, 1000 USP units ml^{-1} ; Organon Inc.); recombinant lysostaphin (Applied Microbiology); [*methyl*- ^3H]thymidine (specific activity 15 Ci mmol^{-1} , 555 GBq mmol^{-1} ; ICN); ^{125}I (specific activity 17.0 mCi per μg iodine; Amersham); sodium phosphate (dibasic heptahydrate; Aldrich); sodium iodide (Aldrich); Bio-Rad protein assay (Bio-Rad); ^{45}Ca (calcium chloride in water; specific activity 17.96 mCi mg^{-1} , 665 MBq mg^{-1} ; Du Pont); Liquiscint scintillation cocktail (National Diagnostics); phenylmethylsulphonyl fluoride (PMSF; United States Biochemical); Rainbow protein molecular mass markers [myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa),

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Abbreviation: CF, cystic fibrosis.

ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa); Amersham]. The remaining reagents were purchased from Sigma: purified mucin type 1-S (from bovine submaxillary glands), which is qualitatively similar in chemical composition to human respiratory mucin (Nisizawa & Pigman, 1959) and asialomucin; fetuin (from foetal calf serum) and asialofetuin (type I); glycophorin A (from human blood) and asialoglycophorin A; acid hydrolysate of casein (amcase, from bovine milk); CaCl_2 ; MgCl_2 ; assay kits for colorimetric determination of the concentrations of calcium and of magnesium, which were used according to the manufacturer's instructions; EGTA; trypsin type 1; sodium metaperiodate; dextran sulphate (M_r 500000); de-*N*-sulphated heparin (from porcine mucosal heparin); troponin (from bovine muscle); sodium dodecyl sulphate (SDS); glycerol; β -mercaptoethanol; bromophenol blue; gelatin; Nonidet P-40; and, Coomassie Brilliant Blue-R250.

Bacteria. Five strains of *S. aureus* were used: strain Ci-1, which was isolated from the lung of a patient who died from post-influenzal staphylococcal pneumonia (Sanford *et al.*, 1986); strains CF837 and CF845, which were both isolated from the sputa of two patients with CF and were obtained from John R. W. Govan, University of Edinburgh, UK; prototype strain 8325-4 and its protein-A-deficient isogenic mutant, DU5723 (Patel *et al.*, 1987), kindly supplied by T. J. Foster, University of Dublin, Ireland. In addition, four strains of *S. epidermidis* were used: the slime-producing strain RP62A (ATCC 35984) and its non-slime-producing mutant strain RP62NA (Schadow *et al.*, 1988) both supplied by G. B. Pier, Channing Laboratory, Boston, MA, USA; and CONS 61w and CONS 52, which were clinical isolates obtained from infected premature neonates in Medical Center Hospital, San Antonio, Tx, USA. Bacteria were labelled by inoculation of each 50 ml volume of M199 with $1-2 \times 10^8$ c.f.u. of staphylococci in a tissue culture flask (150 cm²; Corning) and in the presence of 10 μCi of [*methyl*-³H]thymidine per ml of medium. Bacteria were incubated in stationary culture at 37 °C for 18 h, harvested by centrifugation at $8000 \times g$ for 30 min at 5 °C, suspended in HBSS, and stored at -70 °C. Several aliquots were repeatedly washed with sterile deionized H₂O and freeze-dried for determination of dry weight. C.f.u. were determined by the standard agar overlay procedure.

In vitro radioassay. ³H-labelled staphylococci were assayed for the capacity to bind to immobilized mucin by a modification of an earlier procedure (Sanford *et al.*, 1989). Mucin (100 μl ; 250 μg dry wt), diluted in 0.1 M-carbonate buffer, pH 9.6, was added to individual wells in MaxiSorp 96-well, break-apart modules (Nunc) and incubated at 37 °C for 18 h. Modules were stored at 4 °C until needed. Before a test run, each well of immobilized mucin was treated with 200 μl blocking buffer (0.5% casein/0.05% Tween 20 in PBS) for 60 min at 37 °C and then drained. This step blocked sites not sensitized with mucin. In addition, control wells which did not contain immobilized mucin were treated with blocking buffer. The test bacteria were thawed, washed three times in PBS, and suspended in PBS to a concentration of approximately 60 μg dry wt for *S. aureus* ($\sim 2 \times 10^8$ c.f.u.) and approximately 150 μg dry wt for *S. epidermidis* ($\sim 4.6 \times 10^8$ c.f.u.) per 50 μl volume. Labelled staphylococci (50 μl) were added to each well and incubated at 37 °C for 60 min. Unbound bacteria were removed by aspiration and the wells were gently washed with PBS until all unbound staphylococci had been removed as determined by examination with an inverted microscope. Wells were drained, and each well was submerged in 7 ml scintillation cocktail. Radioactivity was determined in a liquid scintillation spectrometer (Tracor Analytic model 6895; Tracor Instruments). D.p.m. were corrected for background and for quenching by use of external standards and were converted to ng staphylococci, as previously described (Sanford & Ramsay, 1987). Control values were subtracted from test values and results expressed as mean corrected ng bound to immobilized mucin. All assays were done at least in triplicate.

Hydrophobicity. The surface hydrophobicity of *S. aureus* test strains, grown in M199 as described above, was measured by the procedure of Rosenberg *et al.* (1980). Hydrophobicity is expressed as percentage adsorption to hexadecane.

Pretreatment of staphylococci. ³H-labelled staphylococci were rinsed in PBS and suspended at a concentration of 500 μg (dry wt) of staphylococci ml⁻¹ in PBS (pH 6.1) only, or in PBS that contained trypsin type 1, sodium metaperiodate or PMSF. Bacterial suspensions were incubated at 37 °C for 90 min; bacteria suspended in the periodate solution were incubated in the dark. Pretreated bacteria were washed in PBS and used in the *in vitro* radioassay.

Preparation of surface-labelled proteins of S. aureus. Surface proteins of staphylococci were labelled as follows. A fresh culture of *S. aureus* (200–300 mg wet wt), grown in M199 as described, was suspended in 0.5 ml 50 mM- Na_2HPO_4 buffer, pH 7.4, and transferred to a vial that contained five activated Iodo-beads (Pierce) and 1 mCi of ¹²⁵I. Beads were activated according to the manufacturer's instructions. The vial was placed in ice and gently agitated for 15 min. The reaction was terminated by separation of the bacterial suspension from the activated Iodo-beads. Labelled bacteria were washed repeatedly in buffer containing 1 mg of NaI ml⁻¹ with a final wash in 10 mM-Tris/HCl, 15 mM-NaCl, pH 7.4. Bacteria were pelleted by centrifugation, suspended in 5 ml 25% (w/v) sucrose in PBS containing 200 μg lysostaphin ml⁻¹, and incubated at 37 °C for 30 min with agitation. The suspension was centrifuged (10000 g for 30 min at 5 °C), and the supernatant fluid, which contained solubilized surface-labelled proteins, was filtered (0.45 μm), heated at 60 °C for 30 min, and dialyzed (dialysis membrane tubing, 3500 M_r cutoff, Spectrum Medical Industries) in repeated changes of deionized H₂O at 4 °C. A colorimetric assay, utilizing Coomassie Brilliant Blue G-250, was used to determine total protein concentrations according to the instructions of the manufacturer (Bio-Rad). Surface-labelled proteins were tested for direct binding to immobilized mucin in the radioassay.

Separation of surface proteins. The surface proteins of *S. aureus*, grown in M199, were solubilized by treatment with lysostaphin, dialysed, and total protein was determined. Specimens were freeze-dried and stored at -20 °C until needed. The solubilized surface proteins were dissolved in buffer [0.0625 M-Tris HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (w/v) β -mercaptoethanol, 0.02% bromophenol blue] without heating, and subjected to electrophoresis (120 V constant current) on a 5% (w/v) stacking and 10% (w/v) resolving SDS-polyacrylamide electrophoresis gel according to the method of Laemmli (1970). Separated polypeptides were electro-eluted out of the gel on to untreated nitrocellulose paper (NCP) by electrophoresis at a constant current of 20 mA for 24 h in buffer (14% (w/v) glycine, 0.3% Tris, 20% (v/v) methanol). NCP strips were used in direct binding assays with ⁴⁵Ca and ¹²⁵I-labelled mucin. Duplicate gels were stained for proteins using the Bio-Rad silver stain according to manufacturer's instructions (Bio-Rad) and counterstained with Coomassie blue as described by Dzandu *et al.* (1984).

Direct binding of calcium to staphylococcal surface proteins. NCP strips that contained separated polypeptides of *S. aureus* solubilized surface proteins were assayed for Ca^{2+} binding activity by the method of Maruyama *et al.* (1984). Troponin, a protein that contains a Ca^{2+} -binding subunit, was included as a positive control. Briefly, the strips were soaked in buffer (60 mM-KCl, 5 mM-MgCl₂, 10 mM-imidazole-HCl, pH 6.8), incubated in buffer that contained 1 mCi ⁴⁵Ca for each 100 ml for 18 h at 25 °C, rinsed with 50% (v/v) ethanol for 5 min, dried in air, and exposed to Cronex video imaging film (Du Pont) with a Quanta-III CK intensifying screen (Du Pont) at -70 °C.

Direct binding of mucin to staphylococcal surface proteins. Forty milligrams of mucin were labelled with ¹²⁵I by the chloramine T method of Daugharty *et al.* (1972). Labelled mucin was dialysed against

repeated changes of PBS to remove unbound label and stored at 4 °C [specific activity 143 c.p.m. (µg mucin)⁻¹]. The NCP strips were placed in buffer [50 mM-Tris/HCl (pH 7.5), 0.15 M-NaCl, 0.25% gelatin, 0.15% sodium azide, and 0.1% Nonidet P-40] that contained 5% (w/v) bovine serum albumin (BSA) for 1 h, and then rinsed four times with buffer that did not contain BSA. Strips were soaked for 1 h in multiple changes of buffer (60 mM-KCl, 5 mM-MgCl₂, 10 mM-imidazole/HCl, pH 6.8), overlaid with this same buffer that contained ¹²⁵I-labelled mucin (total activity 2.86 × 10⁶ c.p.m.), and allowed to incubate for 18 h at 25 °C with gentle agitation. Strips were washed four times in 50% ethanol, allowed to set in 50% ethanol for 5 min with agitation, air-dried, and processed for autoradiography as described above.

Statistical analysis. The arithmetic means, standard deviation and standard error of the means were determined for each set of data. Where appropriate, comparisons were made by a single-factor analysis of variance by the Student unpaired *t* test (two tailed) using the StatView 512+ computer package (BrainPower, Calabasas, CA, USA).

Results

Effect of calcium on the adhesion of staphylococci to immobilized mucin.

The adhesion of labelled staphylococci to immobilized mucin in the presence of various concentrations of calcium is shown in Fig. 1. All three test strains of *S. aureus* gave a dose response curve and demonstrated significantly enhanced adhesion in the presence of Ca²⁺.

Specificity of adhesion

Ca²⁺-enhanced adhesion was seen with two additional test strains of *S. aureus*: 8325-4 and its protein A-deficient mutant DU5723 (Fig. 2). The fact that DU5723 was adhesion-positive indicated that protein A was not the adhesin that mediated Ca²⁺-enhanced binding. The

Fig. 1. Mean corrected adhesion of ³H-labelled *S. aureus* to immobilized mucin. Concentration of Ca²⁺ (mmol l⁻¹) is indicated per 50 µl of bacterial suspension per well. Bars represent standard error of the means of eight samples. Specific activity [d.p.m. (µg dry wt of staphylococci)⁻¹] was: 3.1 × 10³, Ci-1; 4.4 × 10³, CF837; and 8.5 × 10³, CF845. □, Strain Ci-1; ■, strain CF837; △, strain CF845.

four strains of *S. epidermidis* were also adhesion-positive in the presence of Ca²⁺. The binding interaction was determined to be specific for Ca²⁺, because enhanced adhesion was blocked in the presence of EGTA, which is a specific Ca²⁺ chelator, and adhesion was not enhanced in the presence of Mg²⁺ or in the presence of highly positively charged protamine. In addition, enhanced adhesion could not be explained by non-specific hydrophobic interactions, because the hydrophobicity of test strains was low (< 26%) as seen in Table 1.

Characterization of mucin receptor(s)

A series of experiments were done to determine the effect of sulphated and sialated groups on the binding phenomenon. Results in Table 2 show that Ca²⁺-enhanced adhesion was significantly decreased (*P* ≤ 0.005) in the presence of all reagents tested. Substitution of immobilized asialomucin for mucin in the assay wells reduced direct adhesion by a mean of 42%, which suggests that sialic acid is a receptor for Ca²⁺. These results were supported by the data which showed that highly sialated fetuin and glycophorin blocked adhesion by a mean of approximately 93%. The fact that asialofetuin and asialoglycophorin also significantly decreased adhesion (mean of 59%) suggests that components of these glycoproteins other than sialic acid were involved in the blocking phenomenon. The use of heparin and dextran sulphate, which are rich in sulphate groups but do not contain sialic acid groups, significantly blocked adhesion (mean of approx. 65%); not surprisingly, heparin with the *N*-SO₃ groups removed also significantly blocked adhesion but to a lesser extent (mean of 43%). Thus, it appears that sulphate groups are involved in the Ca²⁺ cross-linking of staphylococci to mucin.

Characterization of staphylococcal adhesins

Table 3 summarizes the results obtained when ³H-labelled staphylococci were subjected to various reagents and treatments before use in the *in vitro* radioassay and compared with untreated controls. The adhesion of all three test strains of staphylococci to immobilized mucin was significantly reduced when the bacteria were treated with trypsin, which suggested that a surface protein(s) was involved in the Ca²⁺-enhanced adhesion interaction. Because PMSF, a protease inhibitor that irreversibly inactivates trypsin and other proteases, was added to bacterial suspensions with trypsin to stop enzyme activity, the staphylococci were also pretreated with PMSF only as a control. Results showed that the Ca²⁺-mediated adhesion to mucin was further enhanced in the presence of this enzyme inhibitor; the increase was

Fig. 2. Mean adhesion of ^3H -labelled staphylococcal strains to immobilized mucin. To each well was added a 50 μl of bacteria suspended in PBS diluent only (■) or diluent containing one of the following: Ca^{2+} (10 mM l^{-1}) (□), Ca^{2+} (10 mM l^{-1}) + EGTA (10 mM l^{-1}) (▨), Mg^{2+} (10 mM l^{-1}) (▩), or 5 μg of protamine (▤). Bars represent standard error of the means of three samples. Specific activity [d.p.m. ($\mu\text{g dry wt of staphylococci}^{-1}$)] was: 6.5×10^3 , Ci-1; 3.8×10^3 , CF837; 4.4×10^3 , CF845; 2.2×10^4 , 8325-4; 2.8×10^4 , DU5723; 4.5×10^3 , CONS 61w; 1.1×10^4 , CONS 52; 4.5×10^3 , RP62A; and 5.9×10^3 , RP62NA.

Table 1. *Hydrophobicity of the test strains of staphylococci*

Strain	Hydrophobicity (%)*
<i>S. aureus</i>	
Ci-1	19
CF837	15
CF845	14
8325-4	23
DU5723	20
<i>S. epidermidis</i>	
CONS 61w	14
CONS 52	9
RP62A	25
RP62NA	14

* Expressed as percentage adsorption to hexadecane of strains grown in M199 medium.

statistically significant with strain CF845. Carbohydrate surface components did not appear to contribute to the interaction, because periodate treatment of the staphylococci had no significant effect on adhesion.

Results indicated that staphylococcal surface proteins appeared to have a role in Ca^{2+} -enhanced adhesion to mucin. Therefore, ^{125}I -labelled surface proteins from strains Ci-1, CF837 and CF845 were tested in a direct

Table 2. *Adhesion of S. aureus to immobilized mucin in the presence of sulphate or sialic acid groups*

Approximately 458 μg (dry wt) of ^3H -labelled *S. aureus* was suspended in 400 μl Ca^{2+} (10 mM l^{-1}) in PBS alone or PBS containing various concentrations of the test reagents. A 50 μl volume of each bacterial suspension was added to each well of immobilized mucin or control well in the radioassay procedure. Corrected mean ng of staphylococci bound $\pm$ SEM was calculated from data from six samples. Data are expressed as the mean percentage of control bound ($\pm$ SEM) to immobilized mucin, where binding in calcium + PBS alone represented 100 %

Strain	Test reagent	Concn (ml $^{-1}$)	Adhesion (mean % of control $\pm$ SEM)
Ci-1	Fetuin	1.0 mg	4 $\pm$ 1
	Asialofetuin	1.0 mg	42 $\pm$ 1
	Glycophorin	1.0 mg	12 $\pm$ 3
	Asialoglycophorin	1.0 mg	43 $\pm$ 4
	Asialomucin*		50 $\pm$ 7
	Dextran sulphate	0.5 mg	39 $\pm$ 2
	Heparin	100 USP units	34 $\pm$ 8
	De-N-sulphated heparin	1.0 mg	51 $\pm$ 5
CF837	Fetuin	1.0 mg	4 $\pm$ 1
	Asialofetuin	1.0 mg	43 $\pm$ 2
	Glycophorin	1.0 mg	10 $\pm$ 2
	Asialoglycophorin	1.0 mg	46 $\pm$ 7
	Asialomucin*		56 $\pm$ 4
	Dextran sulphate	1.0 mg	40 $\pm$ 0.8
	Heparin	100 USP units	40 $\pm$ 2
CF845	De-N-sulphated heparin	1.0 mg	60 $\pm$ 4
	Fetuin	1.0 mg	7 $\pm$ 0.9
	Asialofetuin	1.0 mg	32 $\pm$ 0.8
	Glycophorin	1.0 mg	11 $\pm$ 1
	Asialoglycophorin	1.0 mg	36 $\pm$ 7
	Asialomucin*		68 $\pm$ 4
	Dextran sulphate	0.1 mg	27 $\pm$ 3
	Heparin	100 USP units	29 $\pm$ 6
	De-N-sulphated heparin	1.0 mg	60 $\pm$ 3

* In these assays immobilized asialomucin was substituted at the same concentration for the immobilized mucin in the wells.

binding assay in the presence and absence of Ca^{2+} . The results showed that the labelled proteins did bind to immobilized mucin, and that the binding was enhanced in the presence of Ca^{2+} (Fig. 3); these results seemed to indicate that different proteins mediated the binding to mucin in the presence and absence of Ca^{2+} . Similar results (not shown) were obtained with labelled surface proteins from two other clinical isolates of *S. aureus*, as well as strain 8325-4 and its protein-A-deficient isogenic mutant, DU5723.

Subsequent experiments were done to determine whether the proteins that bound to Ca^{2+} were, in fact, different from the proteins that bound to mucin. Unlabelled surface proteins of *S. aureus* were solubilized under conditions that prevented lysis of intact cells, separated by SDS-PAGE, blotted on to NCP and reacted with ^{45}Ca and ^{125}I -labelled mucin. Results of autoradiography (Fig. 4) demonstrated that ^{45}Ca bound directly to

Table 3. Adhesion of untreated or pretreated *S. aureus* to immobilized mucin

Pretreatment*	Concn	Adhesion (mean % of control $\pm$ SEM)
<i>PMSF</i> †	0.2 mmol ml ⁻¹	
Ci-1		150 $\pm$ 14
CF837		132 $\pm$ 15
CF845		242 $\pm$ 23‡
<i>Trypsin type 1</i>	2 mg ml ⁻¹	
Ci-1		18 $\pm$ 7‡
CF837		0‡
CF845		0‡
<i>Trypsin type 1</i>	20 μ g ml ⁻¹	
Ci-1		75 $\pm$ 18
CF837		17 $\pm$ 8‡
CF845		0‡
<i>Sodium metaperiodate</i>	10 mmol ml ⁻¹	
Ci-1		130 $\pm$ 36
CF837		66 $\pm$ 16
CF845		119 $\pm$ 6

* Before use in the radioassay, approximately 500 μ g (dry wt) ³H-labelled *S. aureus* was suspended in 1 ml PBS (pH 6.1) only or PBS that contained enzyme or enzyme inhibitor (PMSF). Suspensions were incubated at 37 °C for 1.5 h. Pretreated bacteria were washed with PBS, suspended in PBS that contained Ca²⁺ (10 mmol l⁻¹), and added to wells sensitized with immobilized mucin as described. Corrected mean ng of adherent staphylococci $\pm$ SEM was calculated from data from six samples. Data are expressed as the mean percentage of control bound ($\pm$ SEM) to immobilized mucin, where adhesion with staphylococci pretreated with PBS only represented 100%.

† PMSF (final concn 0.2 mM) was also added at the end of the incubation period to suspensions that contained trypsin, to terminate enzyme activity.

‡ $P < 0.004$.

two polypeptides with approximate molecular masses of 170 and 150 kDa. As expected, ⁴⁵Ca also bound to troponin (molecular mass 17.5 kDa). In contrast, ¹²⁵I-labelled mucin bound to three polypeptides with approximate molecular masses of 40, 35, and 29 kDa. A stained gel of the solubilized surface proteins separated by electrophoresis (Fig. 4, lane A) showed a minimum of 29 polypeptides, most of which were not reactive with either radiolabelled probe. In addition, when unlabelled CaCl₂ (5 mM and 10 mM) was used in place of MgCl₂ in the diluent for ¹²⁵I-labelled mucin, binding to the three polypeptides was not blocked (data not shown). These data support the conclusion that the surface proteins of *S. aureus* that bind Ca⁴⁵ are different from the surface proteins that bind mucin in the absence of calcium.

Discussion

The interaction of bacterial pathogens with mucus gel present on respiratory mucosa may lead to ciliary clearance of bacteria and thereby help keep the lower respiratory tract free of potential pathogens (Florey, 1955; McNabb, 1981). However, the interaction may

Fig. 3. Binding of *S. aureus* ¹²⁵I-labelled solubilized surface proteins from Ci-1 ($\diamond$, $\blacklozenge$), CF837 ($\triangle$, $\blacktriangle$), and CF845 ($\square$, $\blacksquare$) in PBS only (open symbols) or PBS containing 10 mM Ca²⁺ l⁻¹ (filled symbols) to twofold serial dilutions of unlabelled mucin (concentration of undiluted mucin, 10 mg ml⁻¹) immobilized in wells as described in the text. Results of triplicate determinations are expressed as the corrected mean c.p.m. for bound labelled proteins; bars represent standard error of the means. Specific activity [c.p.m. (μ g of total protein)⁻¹] was: 1954, Ci-1; 2338, CF837; and 2649, CF845.

Fig. 4. Solubilized surface proteins (SSP) of *S. aureus* strain DU5723 [180 μ g total protein (well A), 360 μ g total protein (wells B and D)] and 100 μ g troponin (well C) were subjected to SDS-PAGE. Lane A, polypeptide profile of SSP after staining with silver and Coomassie Brilliant Blue R. Lanes B–D, autoradiographs demonstrating the direct binding of ⁴⁵Ca to SSP (lane B) and the troponin control (lane C), and the direct binding of ¹²⁵I-labelled mucin to SSP (lane D). The positions of molecular mass standards are indicated on the left. Similar results were seen with SSP of strains 8325-4 and CF837 (not shown).

initiate the infectious process of certain invasive micro-organisms if the bacteria have the capacity to attach to and colonize mucus (Freter, 1980), a mechanism that is potentially important in colonization of the respiratory mucosa by staphylococci and *Pseudomonas aeruginosa* in CF patients (Ramphal, 1990). Kilbourn (1984) reported that the concentration of Ca^{2+} in sputum specimens from CF patients is high (range of 0.1 to 4.4 mmol l^{-1}) and suggested an association between Ca^{2+} and colonization by staphylococci. Results from the present study show that the presence of Ca^{2+} actually enhances the adhesion of *S. aureus*, as well as *S. epidermidis*, to mucin (one of the components of sputum) (Figs 1 and 2) and, further, that the enhanced adhesion is specific for Ca^{2+} (Fig. 2, Table 1). Biesbrock *et al.* (1991) reported that mucin binding to *S. aureus in vitro* may be dependent on the formation of heterotypic complexing between human salivary low-molecular-mass mucin and secretory IgA; these binding studies were done in the absence of Ca^{2+} and represent yet another mechanism for the association of staphylococci with mucin.

Results suggest that Ca^{2+} acts as a bridge to cross-link the surface of the staphylococci to receptors present in immobilized mucin. In the airways, mucin is comprised of sialylated and sulphated glycoproteins which are the major macromolecules of respiratory mucus and comprise a dense number of oligosaccharide units attached to a long polypeptide chain (Roberts, 1978). Sialic acid and sulphate residues confer a strong negative charge on mucin, and it has been suggested that these residues bind Ca^{2+} (Forstner & Forstner, 1975). Results of the present study (Table 2) suggest that sialic acid acts as a mucin receptor because, in the presence of Ca^{2+} , adhesion to immobilized asialomucin was significantly decreased, and adhesion was significantly blocked in the presence of highly sialated reagents. Further, the observation that sulphated reagents also blocked binding suggests that sulphate groups may act as mucin receptors. Results of the blocking experiments leave some unanswered questions because asialated and desulphated forms of the same reagents were also able to block binding but to a lesser extent; it is possible that these reagents may interact with the surface of *S. aureus* in the absence of both Ca^{2+} and mucin. In fact, Liang *et al.* (1992) reported that heparan sulphate, which is closely related to heparin, binds directly to *S. aureus* and suggested that binding was due to ionic interactions between the cell surface amino groups and the sulphate and carboxyl groups of heparin sulphate.

One other study has demonstrated Ca^{2+} -dependent binding of intact bacterial cells to mucin. Cohen *et al.* (1985) reported that the adhesion of *Escherichia coli* F-18 to mouse colonic mucus, immobilized on polystyrene, required Ca^{2+} but not Mg^{2+} ; the role of Ca^{2+} in this

reaction was not defined. In the present study, results suggest that *S. aureus* interacted with mucin by at least two mechanisms: via Ca^{2+} -binding proteins in the presence of Ca^{2+} and via mucin-binding proteins in the absence of Ca^{2+} . Herrmann *et al.* (1991) recently described the Ca^{2+} -dependent binding of *S. aureus* to soluble and immobilized thrombospondin. However, the thrombospondin-binding adhesins of *S. aureus* were not sensitive to pretreatment with trypsin, which suggests that these adhesins do not have a protein component and are somehow different from the Ca^{2+} -binding proteins described here. The expression of protein A, a major surface protein of *S. aureus* that binds the Fc part of immunoglobulins (Forsgren & Sjoquist, 1966), is not required for the Ca^{2+} -dependent or -independent binding to mucin as shown by results obtained with a well-defined protein A-deficient isogenic mutant strain (Fig. 2) and the solubilized surface proteins of this strain (Fig. 4), as well as the results seen with adhesion-positive strains of *S. epidermidis* (Fig. 2) which do not express protein A. Whether the Ca^{2+} -binding or mucin-binding proteins are related to other protein adhesins of *S. aureus* or to any putative adhesins yet to be described for *S. epidermidis* remains to be determined.

In summary, this work represents the first example of specific calcium-binding surface proteins associated with a well-recognized human bacterial pathogen and, furthermore, suggests at least one potential role for these surface proteins, that of the adhesion of *S. aureus* to mucus (mucin). It is important to understand the mechanisms of adhesion of *S. aureus* to mucus and the specific host factors that affect adhesion, particularly in clinical conditions such as CF, in which mucociliary clearance is compromised and the bacteria appear to persist in the respiratory tract by colonization of the mucus.

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