The role of extracellular slime in opsonophagocytosis of *Staphylococcus epidermidis*

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Summary. Infections caused by coagulase-negative staphylococci (CNS) are a major problem in immunocompromised patients. It has been claimed that extracellular slime production by CNS predicts pathogenicity and inhibits host defences. Luminol-enhanced neutrophil chemiluminescence (CL) and bacterial killing assays were used to assess the effect of slime production on opsonophagocytosis and killing by polymorphonuclear leucocytes *in vitro*. There was wide variation in CL induction amongst the 43 strains of *Staphylococcus epidermidis* examined. The presence of slime had no influence either on the requirement or on the efficiency of opsonisation. Slime-producing and non-slime-producing strains showed a stepwise increase in induced CL up to a serum concentration of 10%, and were dependent on complement for efficient phagocytosis. The bacterial killing assays confirmed the CL results. Our data suggest that extracellular staphylococcal slime has no specific anti-opsonic property *in vitro*. Opsonophagocytosis may still be hampered *in vivo* by the physical presence of slime.

Introduction

Infections caused by coagulase-negative staphylococci (CNS) have increased in recent years and are amongst the commonest nosocomial infections (Center for Disease Control, 1986); most are associated with implanted foreign bodies. The increase is probably the result of the increasing number of immunocompromised patients with indwelling prosthetic biomaterials rather than any change in virulence of the organisms. CNS are now the commonest cause of foreign body infections (Fitzgerald *et al.*, 1977; Karchmer *et al.*, 1983; Press *et al.*, 1984; Price, 1984).

Because CNS comprise a major part of the normal skin flora of man, assessing the significance of their isolation from infected patients may be difficult. Attempts to find a laboratory marker that correlates with pathogenic potential have been largely unsuccessful. Recently, extracellular slime production by CNS has been claimed to predict pathogenicity (Ishak *et al.*, 1985; Davenport *et al.*, 1986). This slime enhances adherence to plastic materials (Christensen *et al.*, 1982; Peters *et al.*, 1982), inhibits the cellular immune response (Gray

et al., 1984), interferes with granulocyte function (Johnson *et al.*, 1986) and is associated with recurrences of peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (CAPD) (Kristinsson *et al.*, 1986). It has also been postulated that slime may protect the bacteria against opsonophagocytosis (Peters and Pulverer, 1984).

To assess the effect of slime production on opsonisation, phagocytosis and killing by human polymorphonuclear leucocytes (PMNL), we have studied the effects of slime production by strains of *Staphylococcus epidermidis* on opsonophagocytosis as assessed *in vitro* by neutrophil chemiluminescence (CL) and on killing, as determined by standard bacterial killing assays.

Materials and methods

Bacterial strains

A collection of 118 isolates of CNS was available for study. All were from peritoneal effluents of patients who developed peritonitis whilst undergoing CAPD and have been described in detail by Kristinsson *et al.* (1986). The strains were identified according to the classification scheme of Kloos and Schleifer (1975) by the API Staph system (API Laboratory Products Ltd, Basingstoke). For the chemiluminescence experiments, 20 slime-producing and 23 non-slime-producing strains of *S. epidermidis* were selected at random for study. Each strain was tested at

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least twice and results were expressed as the mean. Bacteria were grown for 24 h at 37°C (i.e., to the stationary phase of growth), in either Tryptone Soya Broth (TSB; Oxoid), which facilitates slime production by CNS (Ludwicka *et al.*, 1984), or Nutrient Broth (NB; Oxoid Nutrient Broth No. 2), which does not. The bacteria were harvested by centrifugation, washed once with saline, and then dispersed and resuspended by vortex mixing in Hanks's Buffered Salts Solution (HBSS) to a final concentration of $(0.5-1) \times 10^9$ cfu/ml.

Slime production

Slime production was detected by the method of Christensen *et al.* (1982). Briefly, five or six colonies from a purity plate of the test strain were inoculated into 10 ml of TSB in glass test tubes (1 cm internal diameter). The cultures were incubated for 24 h at 35°C, and then poured out of the test tubes. The inside of each tube was stained with an aqueous solution of safranin (0.25%). Adherent growth was considered to be present if a stained film lined the inner surface of the tube and this was taken as an indication of slime production. The mere presence of a ring at the liquid-air interface was not considered to be a positive result.

Serum

Serum was collected from four healthy human donors, pooled and stored at -70°C until used. Complement was inactivated by heating the serum to 56°C and holding for 30 min. For serum antibody absorption, bacteria were harvested in 1 L of TSB and pelleted by centrifugation. Five volumes of serum were then mixed with one volume of bacteria and incubated for 1 h at 4°C. Complement activation was blocked by 10 mM ethylenediamine tetraacetate (EDTA; Sigma).

PMNL preparation

Two volumes of fresh whole blood from healthy human donors, anticoagulated with preservative-free heparin 10 U/ml, were mixed with one volume of dextran 6% in sodium chloride 0.9% and the red cells were allowed to sediment for 1 h. The leucocyte-rich supernate was drawn off, centrifuged for 10 min at 2000 rpm (350 g), and the residual red cells lysed by osmotic shock. After washing twice with HBSS, the leucocytes were resuspended in HBSS supplemented with heat-inactivated fetal calf serum 1% to a PMNL concentration of 10^6 /ml.

Opsonisation

Bacteria (10^9 cfu/ml) were mixed with whole serum, heated serum or absorbed serum, at a final concentration of 10%, for 20 min at 37°C. In some experiments, serum at different concentrations was used. After centrifugation the pellet was washed twice and resuspended to the

original cell concentration in HBSS. Zymosan 10 mg/ml was opsonised by the same procedure with 10% serum.

Chemiluminescence assay

The method has previously been described by Easmon *et al.* (1980). PMNL chemiluminescence (CL) was enhanced with luminol (5-amino-2, 3-dihydro-1, 4 phthalazinedione; Sigma) and measured at 37°C in a photometer (Luminometer 1250, LKB Wallac). The reaction mixture consisted of PMNL suspension (10^6 cells/ml) 0.5 ml, luminol (2×10^{-6} M) 0.9 ml and bacterial suspension 0.2 ml. The light generated was recorded in mV and the readings were taken every 5 min for 45 min. To minimise day-to-day variation in the readings, opsonised zymosan (Sigma) was used as a control and the data are presented as the CL reading (mV) relative to that induced by opsonised zymosan.

Killing assays

A 400- μ l volume of PMNL suspension (10^6 cells/ml), prepared as described above, was mixed with 200 μ l of bacteria (10^9 cfu/ml) and 200 μ l of serum. A 100- μ l sample was immediately transferred to 10 ml of ice-cold sterile water, and a viable count performed (t_0) by a spread-plate technique (0.1 ml of serial tenfold dilutions was spread on blood agar). The remaining mixture was incubated at 37°C for 45 min on a roller tray and a second 100- μ l sample was then transferred to 10 ml of ice-cold sterile water and a second viable count (t_{45}) was made. PMNL-free and serum-free controls were included for each strain and the relative reduction of viable bacteria in the mixtures containing PMNL was expressed as percentage killing.

Statistics

The mean CL responses were compared by the unpaired *t*-test.

Results

Effect of slime production on CL response

Strain-to-strain variation in CL induction was observed, especially with opsonised bacteria (fig. 1). Mean CL induction by unopsonised slime-producing strains was significantly greater than mean CL generated by unopsonised non-slime-producers (68 vs 39, $t=4.3$, $df=41$, $p<0.005$; $SD=25.1$ vs 19.4). The difference between the mean CL values of the opsonised strains was not statistically significant (183 vs 190, $t=0.2$, $df=41$, $p>0.1$; $SD=106$ vs 104). When the CL values of opsonised slime-producers grown in NB were compared with the CL values of the same strains

grown in TSB there was no significant difference (results not shown).

Opsonic requirements

CL induction by opsonised CNS increased stepwise up to a serum concentration of 10% (fig. 2). Both slime-producing and non-producing CNS induced only low CL values when complement-depleted (i.e., heated) serum was used as an opsonin (fig. 3). Absorption of serum with either slime-producing or non slime-producing CNS strains also reduced opsonisation but to a much smaller extent (fig. 3).

Killing assays

Killing assays were performed on nine slime-producing and eight non-slime-producing *S. epidermidis* strains (fig. 4). With all strains there was a reduction in viable counts after 45 min. There was considerable variation between isolates but this did not appear to be related to slime production—mean reduction 60.0% (slime producers) vs 60.5% (non-producers).

Four slime-producing *S. epidermidis* strains grown in TSB and NB were compared in PMNL killing assays (fig. 5). Although these strains showed increased slime-production in TSB, results in the killing assay were not significantly different.

Discussion

Opsonisation is considered to be a pre-requisite for efficient phagocytosis and intracellular killing of bacteria. Resistance to opsonisation is a virulence factor for some organisms (Quie *et al.*, 1981). All 43 CNS strains in our study depended on complement for efficient opsonisation. Clark and Easmon (1986) investigated 18 *S. epidermidis* isolates and found a similar pattern. However, other authors have reported strains of *S. epidermidis* that are well opsonised by complement-deficient serum (Fleer *et al.*, 1985). Direct comparisons are difficult because the latter workers used a phagocytosis assay based on radio-labelled organisms, whereas we relied on luminol-dependent CL and bacterial killing assays. Other variations in experimental conditions may also account for different findings; studies of the kinetics of opsonisation have shown that serum concentration and incubation time are important variables when studying opsonic requirements (Tofte *et al.*, 1980; Williams *et al.*, 1980). The CNS-specific antibody concentration in the serum pools may also have been different. Despite these reservations, variations in susceptibility to serum opsonisation may be a feature of *S. epidermidis*. Indeed, we found considerable variation between CNS strains in susceptibility to opsonisation by the same pool of serum (fig. 1). These observations emphasise the need to assess a range of strains when studying pathogenic mechanisms.

The presence of extracellular slime had no influence either on the opsonic requirement or on the efficiency of opsonisation at any of the serum concentrations employed. Bacterial killing assays with selected strains again failed to demonstrate a role for slime in mediating resistance of CNS to phagocytic killing. The bacterial glycocalyx, whether loosely bound (slime) or firmly bound

Fig. 1. CL induced by non-slime-producing (N-SL) and slime-producing (SL-P) *S. epidermidis* strains opsonised with whole serum 20%. Bars represent mean values.

Fig. 2. CL induced by *S. epidermidis* strains opsonised with different concentrations of whole serum. Histograms represent mean values $\pm$ SEM (bars); $\square$ Non-slime producers; ▨ slime-producers.

Fig. 3. CL (mV) induced by 20 *S. epidermidis* strains. Histograms represent mean values $\pm$ SEM (bars); UN=unopsonised; WS=whole serum; HS=heated serum; AS=serum absorbed by a slime-producing strain; AN=serum absorbed by a non-slime-producing strain.

Fig. 4. Phagocytic killing of non-slime-producing (N-SL) and slime-producing (SL-P) *S. epidermidis* strains. The dotted line represents the mean reduction in viable counts.

(capsule), may inhibit opsonisation by virtue of its ability to bind and neutralise immunoactive compounds. Our data suggest that the extracellular, loosely bound mucopolysaccharide that surrounds some CNS isolates has no such specific anti-opsonic property.

Bacterial killing assays and, to a lesser extent, CL studies, rely on the premise that one colony is equivalent to one organism, therefore our assay procedure included vortex mixing of bacterial suspensions to disperse clumps. It may be argued that, because of its water solubility, slime is washed off during this procedure. However, we believe that

enough slime remains on the surface to maintain the physicochemical properties of the cells. This premise is supported by the fact that, after vortex mixing, slime producers demonstrated the same stickiness or readiness to clump as before treatment. Furthermore, previous scanning electronmicroscopy studies had demonstrated some surface slime even after vortex mixing (K. G. Kristinsson, unpublished observation).

CL is a by-product of the PMNL respiratory burst and is a function of membrane stimulation. Light production is related to the efficiency of PMNL-bacterial surface contact. The increased

Fig. 5. Phagocytic killing of slime-producing *S. epidermidis* strains grown in TSB or NB. The dotted line represents the mean reduction in viable counts.

PMNL CL induced by non-opsonised slime-producing strains compared with that induced by non-slime-producing isolates may be related to changes in charge or hydrophobicity of the bacterial cell surface caused by the presence of an extracellular slime layer. These observations with PMNL CL were not reflected in the killing assays; neither slime-producing nor non-slime-producing CNS strains showed any reduction in viable counts in the serum-free experiments.

Electronmicroscopy has shown that slime production on plastic surfaces allows microcolonies of CNS to flourish beneath a protective coating of

slime (Peters *et al.*, 1981, 1982). Under these circumstances, effective opsonisation and phagocytosis may be significantly hampered by the physical presence of slime. Support for this suggestion has been given by a recent report in which reduced phagocytosis of slime-producing CNS was demonstrated by radio-labelled bacteria adherent to plastic wells (Johnson *et al.*, 1986).

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