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...
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 Hank’s Buffered Salts Solution (HBSS) to a final concentration of (0.5-1) × 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-μl volume of PMNL suspension (10^6 cells/ml), prepared as described above, was mixed with 200 μl of bacteria (10^9 cfu/ml) and 200 μl of serum. A 100-μl sample was immediately transferred to 10 ml of ice-cold sterile water, and a viable count performed (t0) 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-μl sample was then transferred to 10 ml of ice-cold sterile water and a second viable count (t45) 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).

![Graph showing 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.]

**Killing assays**

Killing assays were performed on nine slime-
producing and eight non-slime-producing S. epider-
midis 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 reser-
vations, variations in susceptibility to serum opson-
isation 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. 2. CL induced by *S. epidermidis* strains opsonised with different concentrations of whole serum. Histograms represent mean values ± SEM (bars); □ Non-slime producers; ■ slime-producers.

Fig. 3. CL (mV) induced by 20 *S. epidermidis* strains. Histograms represent mean values ± 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.
SLIME AND OPSONOPHAGOCYTOSIS OF S. EPIDERMIDIS

![Graph showing phagocytic killing of non-slime-producing (N-SL) and slime-producing (SL-P) S. epidermis strains. The dotted line represents the mean reduction in viable counts.]

Fig. 4. Phagocytic killing of non-slime-producing (N-SL) and slime-producing (SL-P) S. epidermis 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
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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