Opsonic requirements of *Staphylococcus epidermidis*

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Summary. The opsonic requirements of 18 strains of *Staphylococcus epidermidis* were compared in pooled normal human serum and in peritoneal dialysate from patients undergoing continuous ambulatory peritoneal dialysis (CAPD). A serum concentration of 2.5% gave optimal opsonisation. The opsonisation of all strains was antibody- and complement-dependent, and there were no significant differences in the pattern of their opsonic requirements. Peritoneal-dialysis effluent from uninfected patients was a poor source of opsonins because of the low levels of immunoglobulin G and of the C3 component of complement it contained. Growth of *S. epidermidis* in peritoneal-dialysis effluent rather than in broth did not alter its opsonic requirements. Strains from patients undergoing CAPD and suffering from peritonitis were not more resistant to opsonisation and phagocytic killing than those from other sources.

Introduction

Peritonitis is one of the major complications of continuous ambulatory peritoneal dialysis (CAPD). Most infecting organisms come from the skin of patient or nurse, and *Staphylococcus epidermidis* is the most common cause of CAPD peritonitis. The main antibacterial defence of the peritoneal cavity is the opsonisation, phagocytosis and killing of invading bacteria, first by resident peritoneal macrophages and later by neutrophils that enter the peritoneal cavity as part of the early inflammatory response to infection. The large volumes of fluid used in dialysis tend to dilute available opsonins and reduce the concentration of peritoneal phagocytes, limiting the efficacy of this defence system (Verbrugh *et al.*, 1983). An association between the level of opsonic activity and the incidence of peritonitis in CAPD patients has been shown by Keane *et al.* (1984). Duwe *et al.* (1981) have shown that the low pH and high osmolality of dialysis fluids may inhibit phagocytosis.

The opsonic requirements of bacteria of the same species or even the same serotype within a species may vary quite markedly (Hastings and Easmon, 1981); and resistance to opsonisation might be a determinant of virulence in the peritoneal cavity (Easmon and Glynn, 1976). We therefore investigated the opsonic requirements of *S. epidermidis* strains isolated from cases of CAPD peritonitis and endocarditis, and from the normal skin, with both

serum and peritoneal-dialysis effluent as a source of opsonins. We also examined the effect on opsonic requirements of growing *S. epidermidis* in dialysis effluent rather than in broth.

Materials and methods

Bacteria

Coagulase-negative staphylococci isolated from cases of CAPD peritonitis and from other sources were kindly supplied by Dr R. R. Marples, Division of Hospital Infection, Central Public Health Laboratory, Colindale, London. The strains used (listed in table I) were characterised at the Staphylococcal Reference Laboratory, also at Colindale, by the method of Baird-Parker (1963) as modified by Marples *et al.* (1978).

Bacteria were stored in liquid nitrogen and grown overnight at 37°C in either Brain Heart Infusion Broth (Lab M Ltd) or in peritoneal-dialysis effluent from uninfected patients that had been passed through an 0.45- μ m membrane filter (Millipore Corp).

Before use the bacteria were washed twice in saline 0.9% w/v and resuspended to a concentration of $2-3 \times 10^8$ cfu/ml in Hanks's Balanced Salt Solution without phenol red (HBSS; Gibco Ltd).

Opsonins

Pooled normal human serum (NHS) was obtained from six healthy adult volunteers. Portions of this (0.5 ml in volume) were stored at -70°C. Complement was inactivated by heating the serum at 56°C for 30 min (HHS). The classical pathway of complement was inacti-

Table 1. Serial numbers (and biotypes*) of strains of *S. epidermidis* used in this study

peritoneal cavity of CAPD† patients	Strains from the	
	blood of endocarditis patients	normal skin
1026 (SVI)	607 (SII)	17370 (SII)
1040 (SII)	1110 (SII)	17381 (SVI)
1047 (SII)	1167 (SII)	17436 (SII)
1051 (SII)	1222 (SIV)	17418 (SVI)
1058 (SII)		
1075 (SII)		
1078 (SVI)		
1082 (SII)		
1093 (SII)		
1101 (SII)		

* See *Materials and methods*.

† CAPD=continuous ambulatory peritoneal dialysis.

vated by treating NHS (800 µl) with 200 µl of a solution containing 100 mM magnesium chloride and 100 mM ethylene glycol tetra-acetate (Mg EGTA; Sigma Co.). *S. epidermidis* antibody was removed from NHS by absorbing three volumes of NHS with one volume of packed *S. epidermidis* (strain 1101) for 1 h at 4°C. Complement levels in absorbed serum were checked by testing on C3c Radial Immunodiffusion Plates (Behring Co.) and by luminol-dependent chemiluminescence with zymosan as the opsonised particle (see below). Centrifuged, filtered, peritoneal-dialysis effluent from uninfected patients undergoing CAPD, and an immunoglobulin preparation (Sandoglobulin; Sandoz Co., Basle) made from pooled normal human plasma, were also used. Antibody-free baby-rabbit serum kindly provided by Dr D. Moreno, Wellcome Research Laboratories, Beckenham, was used as a source of complement.

Polymorphonuclear leukocytes (PMN)

PMN were separated from heparinised venous blood by incubation with dextran 6% w/v in saline 0.9% w/v for 45 min. The leukocyte-rich plasma layer was removed and residual red cells were lysed by treatment for 10 min with 10 ml of Tris-buffered ammonium chloride 0.83% w/v. Cells were washed twice and resuspended to a concentration of 1.5×10^6 /ml in HBSS supplemented with fetal calf serum 1% v/v.

Opsonisation

Zymosan (500 µl; 20 mg/ml) was opsonised with 10% NHS. Similar volumes of bacteria were opsonised with serum or dialysis effluent. The reaction mixture was made up to 1 ml with HBSS and was incubated at 37°C for 5 and 15 min. The reaction was stopped by the addition of 1 ml

of HBSS at 4°C. Bacteria or zymosan were washed and resuspended in HBSS at the original concentration.

Phagocytosis assays

Microscopy. Opsonised bacteria were diluted 1 in 100 in HBSS, and 200 µl of this suspension were incubated with an equal volume of PMN at 37°C for 15 min, the tube being placed on a roller to ensure thorough mixing. Smears were prepared in a cytospin centrifuge (Shandon Co.), fixed with methanol and stained by Giemsa's method. Results were expressed as the percentage of PMN with ingested bacteria.

Luminol-dependent chemiluminescence. Suspensions of opsonised bacteria or zymosan (200 µl) were added to 500 µl of PMN (10^6 /ml) and 900 µl 10^{-5} M luminol (Sigma Co.) dissolved in dimethyl sulphoxide and then diluted in HBSS. The mixture was placed in the reaction chamber of a 1250 luminometer (LKB Co.) and the light generated recorded in mV at 10-s intervals. The level of chemiluminescence after 15 min was recorded in each case.

Complement and IgG levels

These were measured on normal and low-range radial immunodiffusion plates for C3 and IgG (Behring Co.). The low-range plates were used for peritoneal-dialysis effluents.

Results

Preliminary work showed that phagocytosis of *S. epidermidis* was not increased by opsonisation with NHS at concentrations exceeding 2.5%. At serum concentrations below this, the total amount of phagocytosis of all of the strains tested was considerably decreased; the results obtained with three of the strains, as measured by luminol-dependent luminescence and by microscopy, are illustrated in fig. 1.

Fig. 2 shows the uptake of nine strains, of various biotypes and derived from several different body-sites, after opsonisation with 2.5% serum; similar results were obtained with the other nine strains tested. *S. epidermidis* was well opsonised by MgEGTA-chelated NHS but poorly opsonised by HHS. Increasing the serum concentration to 10% or even 20% did not improve the opsonic capacity of HHS (results not shown). These experiments demonstrated the importance of complement for the opsonisation of *S. epidermidis*.

We investigated the importance of antibody using serum absorbed with *S. epidermidis* cells and IgG prepared from pooled normal human plasma. Although, as expected, there was little difference between the uptake of bacteria opsonised for 15 min with 2.5% and 10% NHS, absorption with *S.*

Fig. 1. Effect of serum concentration on total uptake of *S. epidermidis* strains, measured by: (a) luminol-dependent chemiluminescence and (b) microscopy.

epidermidis strain 1101 reduced the opsonic capacity of 2.5% NHS to a greater extent than that of 10% NHS (fig. 3). Even with 10% NHS the effect of absorption was to slow the rate of opsonisation (fig. 4); thus, after opsonisation for 5 min, the test strain of *S. epidermidis* (no. 1101) was poorly taken up. Only after opsonisation for 15 min did the uptake approach that after opsonisation with unabsorbed 10% NHS.

Neither HHS nor the IgG preparation showed any opsonic activity against *S. epidermidis* on their own. Even IgG at 10 mg/ml had no effect. However,

when complement was added the opsonic effect of both heated serum and purified IgG was enhanced (table II). For a given IgG concentration, HHS was a more effective opsonin than purified IgG when combined with complement.

Peritoneal-dialysis effluents from 18 uninfected patients were examined; in all samples the content of complement (C3c) and IgG was low, and none showed any significant opsonic activity (table III). *S. epidermidis* grew poorly in peritoneal-dialysis fluid but very much better in dialysis effluent; its opsonic requirements when grown in the latter were similar to those when grown in Brain Heart Infusion Broth (fig. 5).

Discussion

The 18 *S. epidermidis* strains investigated belonged to several different biotypes and had been isolated from a variety of sites in the human body. We found no difference in their basic opsonic requirements, all requiring both complement and antibody. Blocking the classical pathway of complement activation had little effect on opsonisation. Serum concentrations as low as 1% provided near optimal opsonisation of *S. epidermidis*, but below this opsonic activity dropped sharply.

Verhoef *et al.* (1977) examined the opsonic requirements of three *S. epidermidis* strains using 10% pooled NHS. Two of the three showed the same pattern that we found, while the third was well opsonised by HHS. Keane and Peterson (1984) examined four strains and found no differences in opsonic requirements. A serum concentration of 1% was the lowest that gave optimal opsonisation: HHS at a concentration of 20% was as good an opsonin as NHS, suggesting an antibody-mediated effect independent of complement. Other publications by workers of this group (Keane *et al.*, 1984; Verbrugh *et al.*, 1984), who used 10% HHS, also suggest antibody-mediated complement-independent opsonisation, but it is not clear whether the same *S. epidermidis* strain was used on each occasion. Keane, Verbrugh and their colleagues measured the uptake of radiolabelled staphylococci while we used chemiluminescence and microscopy, but it is unlikely that this would account for our differing results with NHS and HHS. Although we examined more strains of *S. epidermidis* than they did, some degree of heterogeneity of properties is to be expected.

There is general agreement that a serum concentration of 0.5–1.0% is the lowest that will give good opsonisation of *S. epidermidis*. Other workers have also shown that the content of C3 and IgG in

Fig. 2. Uptake of *S. epidermidis* strains: U = unopsonised; P = opsonised with 2.5% normal human serum; M = opsonised with serum chelated with MgEGTA; H = opsonised with heat-inactivated serum; measured by (a) luminol-dependent chemiluminescence and (b) microscopy. Strains from: CAPD = peritoneal fluid of patients on continuous ambulatory peritoneal dialysis; E = blood of endocarditis patients; S = normal human skin.

Fig. 3. Effect of opsonisation with 2.5% and 10% normal human serum, and with these sera after absorption with *S. epidermidis* strain 1101, on the uptake of *S. epidermidis* strains, measured by (a) luminol-dependent chemiluminescence and (b) microscopy. ■ = unopsonised bacteria; ▨ = bacteria opsonised with normal human serum or absorbed serum; □ = bacteria opsonised with normal human serum only.

peritoneal-dialysis effluent from uninfected patients is very low (Verbrugh *et al.*, 1983; Keane *et al.*, 1984). Verbrugh *et al.* (1983) found that some peritoneal-dialysis effluents from uninfected CAPD patients did have opsonic activity for *S. epidermidis* and that there was a good correlation between this and IgG and C3 levels. The mean concentrations of IgG (108 mg/L) and C3 (28 mg/L) that they found in

Fig. 4. Effect of time of opsonisation with: ■ 2.5% NHS; □ 2.5% absorbed serum; ● 10% NHS; and ○ 10% absorbed serum on uptake of *S. epidermidis* strain 1101.

Fig. 5. Effect of growth of *S. epidermidis* strain 1101 in Brain Heart Infusion Broth (closed symbols) or peritoneal-dialysis effluent (open symbols) on opsonisation by 2.5% NHS (squares) and 2.5% MgEGTA-chelated serum (circles).

effluents with good opsonic activity were higher than the mean levels (45 mg/L and 3.7 mg/L respectively) that we found in our 18 patients. In fact our levels were similar to those that Verbrugh

Table II. Opsonic activity of heated human serum (HHS) and of IgG for *S. epidermidis* strain 1101 in the presence and absence of complement

Source of immunoglobulin	Concentration of IgG (mg/ml)	Complement*	Chemiluminescence (mV)	Percentage of phagocytosis
...	0	—	8	10
...	0	+	12	10
HHS	0.3	—	11	12
HHS	1.2	—	13	13
HHS	2.4	—	15	12
IgG (Sandoglobulin)	1.0	—	10	12
HHS	0.3	+	22	19
HHS	1.2	+	48	30
HHS	2.4	+	60	38
IgG (Sandoglobulin)	0.3	+	10	11
IgG (Sandoglobulin)	1.0	+	16	24

* Baby rabbit serum (10%); + = added; — = not added.

Table III. IgG and C3c content and opsonic activity of peritoneal-dialysis fluid from 18 uninfected patients, and of pooled normal human serum (NHS)

Description	Opsonin		Phagocytic activity		
	IgG (mg/L): mean (range)	C3c (mg/L): mean (range)	Number of tests	Chemiluminescence (mV): mean (range)	Percentage phagocytosis: mean (range)
Dialysis effluent*	45 (5–115)	3.7 (1.1–8.2)	54	15 (3–49)	11 (4–18)
2.5% NHS	300	14	54	92 (37–237)	49 (28–76)
None	...	...	54	13 (5–24)	9 (3–15)

* Undiluted.

found in effluents with no opsonic activity. This at least supports the view that differences in the techniques employed were not responsible for the lack of conformity between their results and ours.

Work to date has concentrated on the opsonic activity of dialysis effluents from uninfected patients and the use of this as an indicator of future infection (Keane *et al.*, 1984). What is less clear is how rapidly IgG, C3 and PMN enter the peritoneal cavity during the earliest phases of infection. Differences at this stage rather than before infection may be crucial in deciding whether infection is controlled early or allowed to develop. Verbrugh *et al.* (1984) have shown that at least 10^6 phagocytic cells/ml are needed to control bacterial growth. We are now studying the early phase of peritonitis before antibiotics are used to see if cells, IgG and complement enter at different rates in different individuals.

The major target for opsonic antibody in *S. aureus* is peptidoglycan. Cell-wall ribitol teichoic

acid does not appear to be concerned in this (Wheat *et al.*, 1974; Peterson *et al.*, 1978). Strains of *S. aureus* containing protein A are well opsonised in IgG-deficient sera (Verhoef *et al.*, 1977). *S. epidermidis* does not contain protein A and its cell-wall teichoic acid contains glycerol but not ribitol. The peptidoglycan of *S. epidermidis* differs from that of *S. aureus* only in the amino-acid content of the interpeptide cross links between the glycopeptide chains. In *S. epidermidis*, some of the glycine residues are replaced by L-serine, but the position of the serine substitution is not constant (Schliefer, 1983). The structural basis for heterogeneity of opsonic requirements in normal human serum is not known. Absorption studies with purified cell-wall components might clarify the point. Keane and Peterson (1984) reported that absorption with *S. epidermidis* peptidoglycan did remove the opsonic activity from serum, but provided no data.

In addition to cell-wall components, some strains

of *S. epidermidis* produce extracellular slime. This may play an important role in adherence to catheters and prostheses (Bayston and Penny, 1972; Peters *et al.*, 1982). Extracellular material of this type might interfere with opsonisation and phagocytosis, thus acting as a virulence factor for *S. epidermidis* in the peritoneal cavity. We have started to examine the effects of slime on opsonisation and phagocytosis. There are, however, technical problems. The slime does not form a well defined extracellular layer and is easily lost from the surface of the staphylococci as they are being prepared for phagocytosis studies. We have not yet attempted to purify the slime from *S. epidermidis*.

Although we could find no differences in the

opsonic requirements of *S. epidermidis* from various sites, the literature suggests that at least some strains do not require complement for opsonisation. As far as infection is concerned the main point is whether such variation could be linked to virulence. With the exception of slime production, which merits further investigation, there seems no evidence to suggest that certain strains of *S. epidermidis* are more likely to cause peritonitis in CAPD by virtue of their greater resistance to opsonisation and phagocytic killing.

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