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Production of extra-cellular slime by Staphylococcus epidermidis during stationary phase of growth: its association with adherence to implantable devices

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Abstract

A method of optimising slime production produced by *Staphylococcus epidermis* and its quantitative assay was developed, which gave a preliminary indication of its identity and an assessment of the correlation between slime production and adherence of the organism to implants. After inducing vigorous growth in brain heart infusion broth to stationary phase, all nutrients were removed by washing and the organisms resuspended in sterile deionised water with added magnesium. After further incubation the culture was centrifuged and the supernatant reacted with alcian blue in 50 mM magnesium chloride/ sodium acetate solution, and the amount of bound dye was measured spectrophotometrically at 620 nm after its resolubilisation using sodium dodecyl sulphate. Large quantities of slime were produced by some, but not all, strains. Preliminary electrophoresis of the slime showed mobility and staining similar to that of the glycosaminoglycans. Adherence was tested by growing strains in wells of tissue culture plates and aspirating the supernatant after incubation. After fixation and staining of adherent growth the amount of bound stain was determined spectrophotometrically after its elution with ethanol.

In this series of organisms there was no correlation between the result of tests for adherence or production of extra-cellular slime, and no correlation between either of these and the clinical source of the organisms.

Since the introduction of surgically implantable devices *Staphylococcus epidermidis* has been increasingly recognised as a major cause of their colonisation. Attempts at eradicating the organisms without removal of the implant have met with only limited success, and such infections often have a profound effect on patient management.

Eighteen years ago the importance of adherence of staphylococci in hydrocephalus shunt colonisation was recognised[1] and the organisms were found to produce a mucoid substance, now known as extra-cellular slime. This was later described in colonised intravenous catheters[2 3] and has since been noted in most other colonised devices. Initially, extra-cellular slime was thought to be the means by which the cocci adhered to the silicone elastomer, but adherence has since been shown to be mediated, at least in part, by other factors.[4] The importance of extra-cellular slime now seems to lie in its association with the failure of antimicrobial drugs to eradicate the colonising organisms,[5] though there is some evidence to suggest additional effects on host defences.[6 7] Several studies have shown that production of extra-cellular slime is more common in strains isolated from cases of implant colonisation confirmed by culture than in environmental strains or contaminants, and this has stimulated interest in laboratory tests for extra-cellular slime production as a means of assessing the clinical importance of isolates. Christensen *et al* have described a method of detecting extra-cellular slime production[8] which has since been modified to make it quantitative.[9] These tests detect adherence, though a correlation with extra-cellular slime production is implied.

Attempts have been made to characterise extra-cellular slime, and several fractions containing a variety of saccharides and amines have been examined.[10 11] Pure preparations of extra-cellular slime, however, have been difficult to obtain. Various media have been used to stimulate extra-cellular slime production,[8 11] but samples of extra-cellular slime are easily contaminated by media constituents. Despite the use of various enriched or defined culture media, allusions have been made from time to time to the probable poor nutritional state of coagulase negative staphylococci colonising implants in vivo.[12]

In 1953 Duguid and Wilkinson found that in cultures of *Aeromonas (Klebsiella) aerogenes* most of the extra-cellular slime was produced after growth had stopped because of nutrient limitation.[13] Similarly, Wilkinson and Stark noted the production of extra-cellular slime by washed stationary phase cells of *K aerogenes*,[14] and Bernheimer found similar results with pneumococcus.[15] We therefore decided to investigate the possible production of extra-cellular slime by stationary phase

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Table 1 Sources of organisms

Clinical group I		*Clinical groups III, IV*	
Number	*Origin*	*Number*	*Origin*
241	CSF Shunt infection	242	Blood culture contaminant
297	CSF Shunt infection	246	CSF shunt contaminant
311	CAPD peritonitis	274	CSF shunt contaminant
313	CAPD peritonitis	295	Skin pre-op shunt
314	CAPD peritonitis	298	CSF shunt contaminant
315	CAPD peritonitis	317	Nose, CAPD monitoring
332	CSF shunt infection	333	Skin pre-op shunt
336	CAPD peritonitis	366	Scalp pre-cleaning
363	CSF shunt infection	374	CSF Shunt contaminant
371	CSF shunt infection	407	Groin, CAPD monitoring
422	CSF shunt infection	409	Hand, CAPD monitoring
429	Thoracovenous shunt infection	411	Nose, CAPD monitoring
441	CAPD peritonitis	412	Nose, CAPD monitoring
442	CAPD peritonitis	413	Nose, CAPD monitoring
445	CAPD peritonitis	414	Nose, CAPD monitoring
476	CSF shunt infection	415	Umbilicus, CAPD monitoring
544	CSF shunt infection	416	Umbilicus, CAPD monitoring
563	CSF shunt infection	423	CSF shunt contaminant
616	CSF shunt infection	443	Axilla, CAPD monitoring
656	CSF shunt infection	444	Axilla, CAPD monitoring
708	CSF shunt infection		
8444	THR infection		
Controls			
F10	Christensen's control RP12		
F11	Internal control, CSF shunt infection		
F19	Christensen's control SP2		
F20	Christensen's control RP2A		

CSF: Cerebrospinal fluid.
CAPD: Continuous ambulatory peritoneal dialysis.
THR: Total hip replacement.

cells of *S epidermidis* in the absence of extraneous nutrients.

As we had originally used alcian blue to detect extra-cellular slime in hydrocephalus shunts[1] we decided to use an assay method requiring this dye.[16] The results were compared with those obtained in a modified conventional test[9] and the extra-cellular slime was also preliminarily examined by electrophoresis.

Methods

Strains of *S epidermidis*, identified by API Staph (Biomérieux UK) and from known clinical sources (table 1) were stored at −70°C. The organisms were classified for other purposes into clinical groups. Groups II and V were not relevant to this study. Group I comprised those organisms which, on clear clinical and microbiological grounds, were causing an infection involving an implant. In this group there were 12 hydrocephalus shunt infections, eight cases of peritonitis in continuous ambulatory peritoneal dialysis (CAPD), one infected thoracovenous shunt for chylothorax, and one infected total hip replacement, a total of 22 strains. Group III comprised strains isolated as contaminants from removed implants where the clinical findings, serological findings if available, and the circumstances of testing and isolation clearly contraindicated implant colonisation. Group IV contained isolates from skin or mucous membranes obtained during projects involving monitoring of skin flora in patients undergoing hydrocephalus shunt surgery or CAPD. There were six strains in group III and 14 in group IV, and the two groups were combined. Controls consisted of three strains kindly donated by Dr Gordon Christensen (Memphis, Tennessee) (F10 and F20, both of which were "positive", and F19, a "negative" isolate), and an internal control, F11, which had been isolated from a hydrocephalus shunt infection and whose characteristics were well known. F10, F19, and F20 were ATCC 35983, ATCC 39582, and ATCC 35984, respectively.

ADHERENCE TEST

This was based on Christensen's spectrophotometric method with some modifications.[9] Briefly, the organisms were inoculated into tryptone soya broth (TSB, Oxoid) and incubated at 37°C for 18 hours. Aliquots of 20 μl were then added to each of three wells of a sterile enzyme linked immunosorbent assay (ELISA) plate (Sterilin UK) and 200 μl of fresh sterile TSB were added. The plate was covered and incubated at 37°C for a further 18 hours, after which the TSB was aspirated by gentle suction. Each well was washed three times in phosphate buffered saline, and buffered glutaraldehyde was added as a fixative. After 20 minutes at room temperature this was aspirated and 200 μl of crystal violet (GURR) as used in Gram's stain were added. After five minutes this was shaken out and the tray washed under running tap water to remove free stain. The tray was then dried for two hours at 37°C. To each well were then added 200 μl of industrial methylated spirit, and after 10 minutes the contents were mixed, 100 μl transferred to a cuvette, diluted with 1 ml of water, and the $A^{1\,cm}{}_{570}$ recorded.

PRODUCTION OF EXTRA-CELLULAR SLIME

Two to three colonies of each organism were inoculated into 15 ml of brain heart infusion (Oxoid) and vortexed, after which they were incubated at 37°C on an orbital shaker for 18–20 hours. They were then centrifuged and

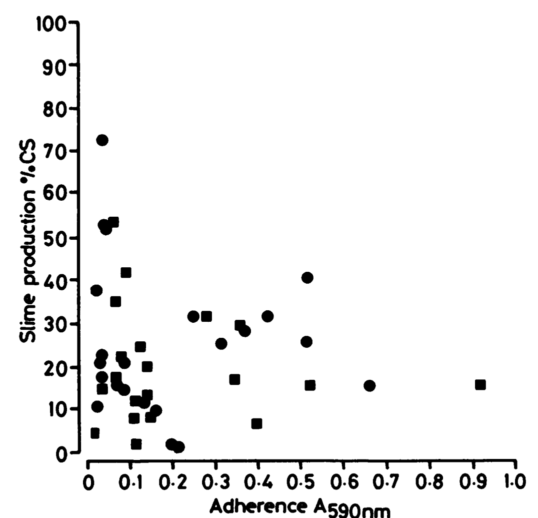

Figure 1 Scattergram of extra-cellular slime (slime) production, expressed as percentage of the reading given by the standard 25 mg/l chondroitin sulphate; and adherence, expressed as absorbance at a wavelength of 590 nm. ● = clinical group I; ■ = clinical groups III and IV.
Kendall's rank correlation test showed that there was no correlation between extra-cellular slime production and adherence (r = 0·0074).

washed twice in sterile deionised water, following which they were resuspended in 10 ml of sterile deionised water containing 20 mg/l magnesium chloride. After vortexing, 200 μl were withdrawn and the $A^{1\,cm}{}_{490}$ recorded. The suspensions were then incubated statically at 37°C for 18–20 hours. They were then centrifuged at 2800 rpm at 4°C for 30 minutes and the supernatant retained.

EXTRA-CELLULAR SLIME ASSAY

The method is based on that of Whiteman.[16] All strains were tested in duplicate. Alcian blue (ICI Ltd) was dissolved in sodium acetate/magnesium chloride solution, pH 5·8, containing 50 mM of each, to give a dye concentration of 50 mg/100 ml.

One millilitre of supernatant, prepared as above, was mixed with 4 ml of alcian blue solution and left to stand at room temperature for one hour. After centrifugation at 2800 rpm at +4°C for 20 minutes the supernatant was gently aspirated by suction and replaced by 4 ml of absolute ethanol. After gentle mixing this was again centrifuged as above and the supernatant gently aspirated and discarded, being replaced by 4 ml of sodium dodecyl sulphate (100 g/l in 50 mM sodium accetate, pH 5·8). The mixture was vortexed to dissolve the precipitate and the $A^{1\,cm}{}_{620}$ was recorded. A background control in which 1 ml of deionised water was substituted for the 1 ml supernatant, and a standard consisting of 1 ml of 25 mg/l chondroitin sulphate (Sigma Chemical Co UK) was assayed, were included.

To calculate the result the background $A^{1\,cm}{}_{620}$ was subtracted from all other readings, which were then divided by the $A^{1\,mc}{}_{490}$ reading for each strain to standardise extra-cellular slime production for bacterial cell density. The resulting figure was then expressed, as a percentage of the $A^{1\,cm}{}_{620}$ of the chondroitin sulphate standard.

Table 2 Results

Clinical group I			*Clinical groups III and IV*		
Organism	*Slime*	*Adherence*	*Organism*	*Slime*	*Adherence*
241	26	0·305	242	9	0·147
297	26	0·502	246	8	0·105
311	38	0·029	274	54	0·043
313	11	0·029	295	5	0·007
314	15	0·079	298	32	0·270
315	16	0·062	317	15	0·033
332	32	0·246	333	16	0·511
336	23	0·024	366	17	0·336
363	32	0·412	374	30	0·354
371	12	0·126	407	2	0·109
422	73	0·028	409	18	0·063
429	16	0·657	411	20	0·130
441	52	0·031	412	25	0·114
442	22	0·020	413	7	0·392
445	10	0·155	414	12	0·119
476	2	0·189	415	42	0·083
544	0·9	0·203	416	35	0·062
563	18	0·026	423	16	0·914
616	29	0·361	443	22	0·073
656	53	0·030	444	13	0·130
708	21	0·087			
8444	41	0·507			
Controls	*Slime*	*Adherence*			
F10	41	0·237			
F11	35	1·397			
F19	1·8	0·020			
F20	36	1·304			

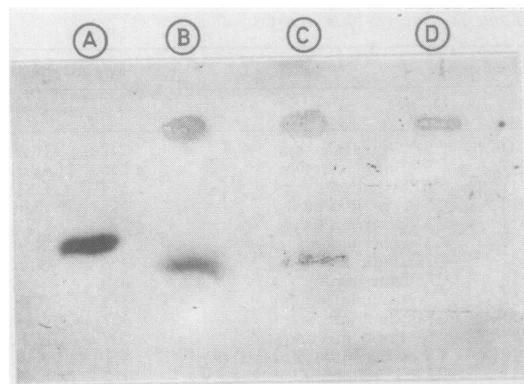

Figure 2 Sample results of electrophoresis. The results of extra-cellular slime assay and adherence of the strains are shown in Table 2. A chondroitin sulphate standard; B F11; C F422; D F19.

ELECTROPHORESIS

The method used was that described by Wessler,[17] using glucose acetate and staining with alcian blue.

Results

The results of both tests are shown in table 2. In each case they are the means of three readings. The readings for the quantitative slime assay in group I ranged from 0·9% to 73% of the standard, which contained 25 mg/l of chondroitin sulphate. In groups III and IV the readings ranged from 2% to 54%. The three "positive" controls gave readings of 35% to 41% and the "negative" control (F19) gave a reading of 1·8%.

The $A^{1\,cm}{}_{590}$ readings of the adherence test for group I strains ranged from 0·020 to 0·657, and for groups III and IV strains from 0·007 to 0·914. Readings for the three "positive" controls ranged from 0·237 to 1·397, and F19 gave a reading of 0·020. When analysed by Kendall's rank correlation test (fig 1) there was no correlation between results for the quantitative slime test and those for the adherence test (r = 0·0074). In group I and in groups III and IV, however, there were strains which showed high readings for slime production and adherence and a few which showed low readings for both tests. Controls gave consistently reproducible readings in both tests.

The results of electrophoresis of two strains giving a high extra-cellular slime test reading are shown as examples in fig 2, along with strain F19 and the chondroitin sulphate standard. In the two "positive" strains single bands were produced at a point near to that reached by chondroitin sulphate. In the "negative" strain no such band was produced. In all cases except the standard, alcian blue staining material also remained at the origin. Strain F422 produced a typical mobile band even though it was negative by adherence testing.

Discussion

The role of extra-cellular slime production as a virulence marker has been investigated previously. In most reported studies there are more "slime-positive" strains among those isolated from infections than among those from

skin or environment, though these differences vary greatly, do not always reach significance,[18–25] and are not found at all in some studies.[26–28]

In each case a version of Christensen's adherence test was used.[8 9] Some results were assessed by naked eye, while others were read using a spectrophotometer. The criteria used to determine infection also varied. There was also a tendency in some cases to assume that the terms "adherence" and "slime production" were interchangeable, though Younger *et al* suggested that some adherent organisms might not produce extra-cellular slime.[25]

Colonisation of implants seems to take place in two phases.[3 29] In the first phase the organisms adhere to the implant, and in the second phase, which follows a few hours later, they begin to multiply and produce microcolonies and, in some cases, extra-cellular slime. The production of extra-cellular slime at this stage seems to be important as several studies have shown an apparent association between this and the failure of antimicrobial drugs to eradicate the organisms.[5 19–22 30]

The clinical importance of strains which produce extra-cellular slime, but which are incapable of adhering, and vice versa, is unknown, however, and this aspect cannot be studied unless the two factors can be assayed separately. Our results suggest that this is now possible. Furthermore, the direct quantitative assay for extra-cellular slime production should serve as a reference test with which to evaluate more rapid, perhaps indirect, tests that are more suitable for use in the clinical laboratory.

We found no correlation between clinical grouping and extra-cellular slime production or adherence, or between the two factors themselves, but the strains did fall into four groups: those showing poor adherence and little or no extra-cellular slime production; those showing poor adherence and producing large amounts of extra-cellular slime; those showing strong adherence and poor extra-cellular slime production; and those able to adhere moderately well and to produce moderate amounts of extra-cellular slime. The clinical implications of these results are currently being studied, but it is possible that strains such as 423 or 544 that adhere well but are poor extra-cellular slime producers might cause implant colonisation which is amenable to antimicrobial treatment, while those such as 422 or 656, though producing large amounts of extra-cellular slime, might fail to adhere to implants, leaving those strains such as 241, 363, and 8444 as the most important clinically. The results of such tests might have a major impact on the management of implant colonisation, allowing the decision to attempt eradication without removing the device, or its converse, to be made more rationally.

As extra-cellular slime can be produced in a system which is not medium dependent and in which no other nutrients are needed, purification and characterisation of extra-cellular slime is facilitated. The crude substance was found to be mainly carbohydrate by Ludwicka *et al*,[10] who also showed the presence of uronic acids among the hydrolysis products. Our original demonstration of extra-cellular slime production in vivo was done with alcian blue,[1] and this dye has also been used by Christensen *et al.*[31] Alcian blue, a solubilised copper phthalocyanine, bears cationic groups which react with polyanions[32] that make it ideal for differential demonstration of glycosaminoglycans.[33] The extra-cellular slime produced in the system reported here seems to be similar to the glycosaminoglycans (GAG). Electrophoresis shows that there are probably two substances present which react to alcian blue, one of which is produced by all strains tested and which is immobile, and a second which has a mobility similar to, but not identical with, that of chondroitin sulphate. If the proposed identity of extra-cellular slime as a GAG is confirmed this may explain some of the biological behaviour of *S epidermidis*. A direct quantitative assay has also allowed us to proceed towards such confirmation, and to begin to investigate the factors governing the production of extra-cellular slime under various conditions.

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