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# Virulence of *Staphylococcus epidermidis* in a mouse model: significance of extracellular slime

M. A. DEIGHTON[1], R. BORLAND[2] AND J. A. CAPSTICK[1]

[1] *Department of Applied Biology and Biotechnology*
[2] *Faculty of Biomedical and Health Sciences, Royal Melbourne Institute of Technology, 124 LaTrobe St, Melbourne 3000, Australia*

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## SUMMARY

The ability to produce large quantities of biofilm on solid surfaces *in vitro* is believed to distinguish potentially pathogenic strains of *Staphylococcus epidermidis* from commensals. Biofilm consists of staphylococcal cells encased in a matrix of extracellular polysaccharide (also referred to as slime), firmly adherent to each other and to the underlying surface structure. The association of slime with colonization of catheter surfaces *in vivo* has been examined extensively. Less attention has been paid to the contribution of slime to infections that occur in the absence of an inserted device. In a mouse model of subcutaneous infection without an implanted device 10 *S. epidermidis* strains (5 slime-positive, 5 slime-negative) produced abscesses; thus a foreign body is not essential for the expression of virulence by *S. epidermidis*. Biofilm-positive strains produced significantly more abscesses, that persisted longer than biofilm-negative strains. In these chronic infections, large numbers of staphylococci were associated with macrophages and viable staphylococci were cultured from specimens of pus collected at autopsy. Thus slime or components of slime appear to delay the clearance of *S. epidermidis* from host tissues, possibly by interfering with intracellular killing mechanisms. However, differences in the capacity to produce abscesses, within both the slime-positive and slime-negative groups, indicate that other factors also contribute to the virulence of *S. epidermidis*.

## INTRODUCTION

Coagulase-negative staphylococci (CoNS), mainly *Staphylococcus epidermidis*, are the most common causes of nosocomial bloodstream infections [1]. The most important predisposing factor for these infections is the presence of an indwelling medical device such as a prosthesis or an intravascular catheter. Immunosuppressed patients and premature newborns are also at risk of CoNS infection [2, 3]. In these groups, infection can occur in the absence of a medical device [2].

The ability to produce large quantities of biofilm on solid surfaces *in vitro* is believed to distinguish potentially pathogenic strains of *S. epidermidis* from commensals [4–6]. Biofilm formation on device surfaces *in vivo* characterizes biomaterial-related infections [5]. Biofilm consists of staphylococcal cells encased in a matrix of extracellular polysaccharide (also referred to as slime), firmly adherent to each other and to the underlying surface structure [5]. Biofilm production is a complex process comprising a number of steps and involving several surface components [7]. Extracellular polysaccharide appears to be involved in the late accumulation stage of biofilm formation [8, 9]. Thus biofilm formation and slime are not terms that are interchangeable. Moreover, both characteristics are modulated by environmental conditions [10, 11].

Extracellular polysaccharide may assist staphylo-

cocci to evade host defences by providing a non-specific physical barrier [12]. Experiments to determine the specific effect of extracellular polysaccharide on the uptake of staphylococci by polymorphonuclear neutrophils have been inconclusive [13–17]. There is some evidence that extracellular extracts of *S. epidermidis* interfere with intracellular killing of staphylococci by neutrophils [14] and alveolar macrophages [18] and promote tissue damage by stimulating monocytes to produce inflammatory mediators [19]. It remains to be determined, however, whether these effects are due to polysaccharide *per se* or to contaminating material present in crude extracts.

If the antiphagocytic effects of *S. epidermidis* exopolysaccharide are important, biofilm-positive strains would be expected to be more virulent than biofilm-negative strains, regardless of the presence of a catheter. Studies comparing the virulence of biofilm-positive and biofilm-negative strains of CoNS in animal models of catheter-associated infection have produced conflicting results [20–24]. One study found no evidence of infection in mice inoculated subcutaneously with $10^9$ colony forming units (CFUs) of strongly slime-positive strains of *S. epidermidis* in the absence of a surgical incision or catheter [23].

The aims of this study were to (i) develop an animal model to study *S. epidermidis* subcutaneous infection in mice in the absence of surgery and of an implanted device and (ii) compare biofilm-positive and -negative strains for their virulence in the animal model.

## MATERIALS AND METHODS

### *Staphylococcal* strains

In an earlier investigation, 40 clinical isolates of *S. epidermidis* were examined for biofilm production throughout the growth cycle in tryptic soy broth (TSB) and in TSB made iron-limited by the addition of ethylenediamine-di-*o*-hydroxyphenol acetic acid (EDDA) at levels of 12·5, 25, 50 and 100 $\mu$g/ml [11]. On the basis of the amount of biofilm produced in 25 different combinations of medium and incubation period, strains were classified as strong (group A), moderate (group B) or weak or negative (group C) biofilm producers [11]. Four group A strains (A-73, A-263, A-285, RP62A [ATCC 35984] [25] and four group C strains (A-62, A-211, A-250, A-315) were selected for the current study to represent the strongest and weakest biofilm producers respectively. Strain A-73 is of particular interest as it produces heavy biofilm in all iron-limited media but not in standard TSB or in TSB containing equimolar amounts of EDDA and ferrous sulphate. In addition, we included strains A-204 (biofilm-negative) and A-205 (biofilm-positive), a pair of phenotypic variants isolated from valvular tissue of a patient with native valve endocarditis. These variants differ in the expression of several other phenotypic characteristics [26, 27]. The sources of strains used in the study are listed in Table 1. All strains had been identified previously as *S. epidermidis* by standard microbiological methods [28, 29].

### Examination of *S. epidermidis* strains for other possible virulence determinants

The ability to produce $\alpha$-toxin was determined by examining colonies on 6·0 % rabbit blood in Columbia agar base (Oxoid) for zones of haemolysis around single colonies [30] after incubation at 35 °C for 24 h. Delta lysin production was assessed by the synergistic haemolysis method [30]. Tests for lipolytic activity and DNAase were performed by spot inoculating $5 \times 10^5$ cells (suspended in saline) in the logarithmic phase of growth onto appropriate media. Sierra's medium [31] containing 1 % Tween 20, Tween 40, Tween 60 or Tween 80 was used to test for lipolytic activity, defined as precipitation within the medium, around the inoculum after incubation at 35 °C for up to 7 days. Baird Parker plates (Oxoid, containing 1 % egg yolk) were prepared without tellurite to test for 'egg yolk activity', defined as a zone of opacity surrounding the inoculum after incubation at 35 °C for 5 days. Nuclease activity was tested on DNase agar (Oxoid) and assessed according to the manufacturer's instructions. Proteolytic activity was assessed in brain heart infusion broth containing 4 % gelatin after incubation for up to 3 weeks [31] and on calcium caseinate agar [32]. Strains were examined for cell surface hydrophobicity by the xylene adherence assay [26].

### Development of mouse model

Bacterial suspensions for inoculation of mice were prepared immediately before use in each series of experiments. After retrieval from storage at −70 °C, strains were inoculated into TSB and incubated for 6 h at 37 °C with shaking, centrifuged and resuspended in saline to give a suspension containing approximately $2 \times 10^9$ CFU/ml. The decision to prepare fresh inocula for each series of experiments rather than cells that had been stored at −70 °C was made on the basis of *in vitro* studies showing that cells stored at −70 °C performed poorly when used as direct inocula for

Table 1. *Characteristics of* S. epidermidis *strains used to inoculate mice*

| Strain | Source | Biofilm phenotype* | α-lysin† | δ-lysin‡ | Hydrolysis of Tween 80§ | 'Egg yolk reaction'‖ | Hydrophobic surface¶ |
|---|---|---|---|---|---|---|---|
| A-73[11] | Blood | A | − | − | − | + | − |
| A-263 | Peritoneal dialysis fluid | A | − | − | ++ | ++ | − |
| A-285 | Peritoneal dialysis fluid | A | − | − | + | + | + |
| RP62A[25] | Catheter sepsis | A | − | − | + | +++ | + |
| A-62 | Peritoneal dialysis fluid | C | − | − | + | ++ | − |
| A-211 | Peritoneal dialysis fluid | C | − | + | ++ | ++ | − |
| A-250 | Peritoneal dialysis fluid | C | − | − | + | ++ | − |
| A-315 | Peritoneal dialysis fluid | C | − | − | + | ++ | − |
| A-205[26,27] | Valvular tissue | B | − | + | − | variable | + |
| A-204[26,27] | Valvular tissue | C | − | − | + | − | + |
| *S. aureus* | RMIT 344/2-4 | not tested | +++ | − | +++ | +++ | not tested |

* Strains were classified as strong (group A), moderate (group B), weak or negative (group C) biofilm producers depending on the amount of biofilm produced in TSB and in TSB supplemented with EDDA at concentrations of 12·5, 25, 50 and 100 μg/ml for 14, 18, 24, 48 and 72 h [11].

† Haemolysis on 6% rabbit blood agar: −, no haemolysis; +++, a zone of complete haemolysis (5 mm annular radius).

‡ Synergistic haemolysis in the presence of *S. aureus* β-toxin. +, zone of complete haemolysis; −, no haemolysis or a zone of incomplete haemolysis.

§ Annular radius of precipitation within the medium (Sierra's medium containing 1% Tween 80) after incubation at 35 °C for 7 days: −, no zone; +, < 4 mm; ++, 4–6 mm; +++, > 6 mm.

‖ Annular radius of zone of opacity after incubation on Baird Parker medium, without tellurite, at 35 °C for 5 days: −, no zone; + < 3 mm; ++, 3–5 mm; +++, > 5 mm.

¶ Hydrophobicity index: +, ⩾ 80; −, < 80.

Not listed in Table 1: No strain produced DNAase or hydrolysed casein. All strains liquefied gelatin, but at a very slow rate. All hydrolysed Tween 20 and most hydrolysed Tween 60.

quantitative biofilm assays [11]. Longer incubation periods were also rejected because cells of heavy biofilm-producing strains grown for 18 h or more frequently autoagglutinated when centrifuged and were therefore unsuitable for preparation of even suspensions.

Male Swiss mice (average weight, 25–30 g) were inoculated subcutaneously over the rib-cage on the left of the dorsal midline with $2 \times 10^8$ CFU of the test strain in a volume of 0·1 ml of sterile saline. The same volume of sterile saline was inoculated to the right of the dorsal midline. Immediately after each series of experiments, the purity of all inocula was confirmed by subculture.

At autopsy, mice were examined for evidence of inflammation and abscess formation at the inoculation sites and scored on a five point scale as follows: − no evidence of inflammation; +/− no evidence of inflammation at autopsy but some roughness of formalized tissue; + small collection of purulent material; ++ moderate abscess (short diameter, < 2 mm); +++ large abscess (short diameter, 2–10 mm, mean about 5 mm). In practice, most assessments fell into categories − or +++. Samples for microbiological examination were taken with sterile tooth picks, from internal skin surfaces (right and left) and any inflammatory foci or abscess cavities. Immediately after collection, the samples were cultured on horse blood agar and used to prepare smears for Gram staining. Skin samples (approximately 1·5 cm by 2 cm in diameter) from the injection sites on the right and left sides of the chest were removed and fixed in neutral buffered formalin, embedded in paraffin, sectioned, stained with haematoxylin and eosin and examined for microscopic evidence of inflammation. The degree of inflammation observed in histological specimens was assessed according to the extent and stage of resolution of the lesion and the density and type of inflammatory cells. This assessment was made without knowledge of the identity of

samples. The following guidelines were used to score tissue reactions from 0 to 5: grade 0, no evidence of inflammation; grade 1, a small focal collection of inflammatory cells (approximate area 0·04–0·1 $mm^2$) consisting of macrophages alone or macrophages and polymorphs; grade 2, a small focal lesion (approximate area 0·1–0·5 $mm^2$) consisting of sparsely packed cells (macrophages, degraded polymorphs or both), macrophages predominating among incoming cells; grade 3, a larger focal lesion (approximate area 0·5–1·0 $mm^2$) consisting of densely packed polymorphs, tissue debris in some samples, macrophages comprising 50–100 % of incoming cells. Larger lesions (approximate area 1·0–2·5 $mm^2$) showing signs of resolution were also classified as grade 3; grade 4, large focal lesion (approximate area 1·0–2·5 $mm^2$) consisting of densely packed cells (predominantly polymorphs) and staphylococci. More advanced lesions with varying amounts of fibrin deposition were also classified as grade 4. In these advanced lesions, incoming cells were predominantly macrophages, but included approximately 20 % polymorphs; grade 5, this category was reserved for very large focal lesions ($> 2·5$ $mm^2$) associated with extensive tissue damage. These lesions contained densely packed polymorphs and staphylococci, they often contained areas of fibrin deposition, but they were still essentially active with polymorphs predominating among the incoming cells.

### Virulence of strains RP62A (biofilm-positive) and A-315 (biofilm-negative) in the mouse model

In the first series of 4 experiments, 40 mice were inoculated with strain RP62A (biofilm-positive) and 40 with strain A-315 (biofilm-negative). Ten mice from each group were sacrificed on days 1, 3, 7 and 14 days after inoculation and histological and microbiological examinations were made as described in the previous section.

To confirm the stability of the biofilm phenotype after storage and inoculum preparation and to determine whether any change in phenotype had occurred as a result of animal passage, staphylococci recovered from mice were examined concurrently with the respective inocula for colonial morphology on Congo red agar, biotype, biofilm phenotype and cell surface hydrophobicity. Congo red agar was prepared as previously described [26] by incorporating Congo red (0·003 %) into blood agar base no. 2 (Oxoid). Colonial phenotype was assessed after incubation for 48 h at 35 °C followed by another 48 h at room temperature [26]. Isolates were biotyped using a commercial identification kit (ID 32 STAPH, API Systems, La Balme Les Grottes, France) and examined for biofilm production by the tube adherence assay [4]. Biofilm production was also assessed spectroscopically, after growth in standard TSB and TSB containing 25 $\mu$g/ml EDDA. Cell surface hydrophobicity was assessed by the xylene adherence method as previously described [26]. To minimize possible changes in phenotype, these tests were performed on freshly isolated strains without subculture and from purity plates prepared from saline suspensions used to inoculate the mice.

### Comparison of other biofilm-positive and biofilm-negative strains for virulence in the mouse model

After demonstrating a statistically significant difference in the virulence of strains RP62A and A-315 in the mouse model, a second series of experiments was conducted using the other 8 *S. epidermidis* strains (4 biofilm-positive and 4 biofilm-negative) and including RP62A and A-315 as controls. Each strain was inoculated into the subcutaneous tissue of 12 mice in a series of 3 identical experiments, each with 40 mice (total 120). Of 12 mice, 3 were autopsied on days 1, 3, 7 and 14 respectively and examined macroscopically, microbiologically and histologically for evidence of infection as in the first set of experiments. After completion of the experiment, the biofilm phenotype of the suspension used for inoculation and of all isolates recovered from mice was confirmed by the microtitre adherence assay [26].

### Statistical analysis

The $\chi^2$ test of the independence of categorical samples was used to compare the host response in mice to the subcutaneous inoculation with (i) strain RP62A (biofilm-positive) and strain A-315 (biofilm-negative) and (ii) all biofilm-positive with all biofilm-negative strains. The following comparisons were made: presence and size of subcutaneous abscesses (5 levels), number of polymorphs and staphylococci in Gram-stained smears (5 levels), number of colonies of *S. epidermidis* recovered in culture (5 levels), histological evidence of local tissue inflammation (6 levels).

## RESULTS

### Characteristics of strains

The sources and possible virulence determinants of the ten strains used in the study are presented in Table 1. No strain produced DNAase or hydrolysed casein.

Table 2. *Microbiological and histological findings in mice inoculated with* S. epidermidis *strain RP62A (biofilm-positive) or A-315 (biofilm-negative)*

| Days after inoculation | Macroscopic abscess at autopsy* | | Polymorphs in Gram stain† | | Gram-positive cocci Gram stain† | | *S. epidermidis* grown in culture‡ | | Inflammatory response§ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RP62A | A-315 | RP62A | A-315 | RP62A | A-315 | RP62A | A-315 | RP62A | A-315 |
| 1 | +++ | +/− | +++ | − | +++ | − | +++ | +++ | 5 | 4 |
| 1 | +++ | + | +++ | +/− | +++ | +++ | +++ | +++ | 5 | 4 |
| 1 | +++ | +/− | +++ | − | +++ | + | ++ | ++ | 5 | 4 |
| 1 | ++ | + | +++ | + | +++ | ++ | ++ | ++ | 4 | 4 |
| 1 | +++ | +/− | +++ | − | +++ | − | +++ | + | 3 | 4 |
| 1 | +++ | +/− | +++ | − | +++ | +/− | +++ | ++ | 3 | 3 |
| 1 | +++ | + | +++ | − | +++ | + | ++ | + | 1 | 3 |
| 1 | − | + | − | + | − | ++ | − | ++ | 0 | 2 |
| 1 | − | + | − | − | − | + | +/− | + | 0 | 2 |
| 1 | − | − | − | − | − | − | +/− | + | 0 | 0 |
| 3 | +++ | + | ++ | − | +++ | − | +++ | + | 5 | 5 |
| 3 | +++ | + | ++ | − | ++ | + | +++ | + | 5 | 4 |
| 3 | +++ | − | +++ | − | +++ | − | ++ | − | 5 | 3 |
| 3 | +++ | +/− | +++ | − | ++ | + | ++ | +/− | 5 | 1 |
| 3 | +++ | +/− | +++ | − | ++ | +/− | ++ | +/− | 5 | 1 |
| 3 | +++ | − | +/− | − | ++ | − | ++ | − | 5 | 0 |
| 3 | +++ | − | ++ | − | ++ | + | ++ | − | 5 | 0 |
| 3 | +++ | − | ++ | − | ++ | − | + | − | 5 | 0 |
| 3 | +++ | − | +++ | − | +++ | + | +++ | − | 3 | 0 |
| 3 | +++ | − | +++ | − | +++ | + | ++ | − | 3 | 0 |
| 7 | +++ | + | − | − | − | + | − | +/− | 5 | 4 |
| 7 | +++ | − | +++ | − | ++ | − | ++ | − | 5 | 2 |
| 7 | +++ | − | +++ | − | +++ | ++ | ++ | − | 5 | 1 |
| 7 | +++ | − | +++ | − | +++ | + | + | − | 5 | 1 |
| 7 | +++ | − | +++ | − | +++ | − | ++ | − | 4 | 1 |
| 7 | +++ | − | +/− | − | + | − | + | − | 4 | 0 |
| 7 | +++ | − | ++ | − | +++ | − | +++ | − | 3 | 0 |
| 7 | − | − | − | − | + | − | − | − | 0 | 0 |
| 7 | +/− | − | − | − | − | − | − | − | 0 | 0 |
| 7 | − | +/− | − | − | − | ++ | − | − | 0 | 0 |
| 14 | +++ | − | +++ | − | +++ | − | +/− | − | 5 | 1 |
| 14 | +++ | +/− | +++ | − | +++ | − | ++ | − | 5 | 1 |
| 14 | +++ | − | +++ | − | +++ | − | + | − | 4 | 1 |
| 14 | +/− | − | − | − | − | − | − | − | 3 | 1 |
| 14 | +++ | − | − | − | ++ | − | +/− | − | 3 | 0 |
| 14 | − | − | − | − | − | − | − | − | 1 | 0 |
| 14 | − | − | − | − | − | − | − | − | 1 | 0 |
| 14 | − | − | − | − | − | − | − | − | 0 | 0 |
| 14 | − | − | − | − | − | − | − | − | 0 | 0 |
| 14 | − | − | − | − | − | − | − | − | 0 | 0 |

* −, no evidence of inflammation; +/−, no evidence of inflammation at autopsy but some roughness of formalized tissue; +, small collection of purulent material; ++, moderate abscess (short diameter, < 2 mm); +++, large abscess (short diameter 2–10 mm, mean about 5 mm).

† The numbers of polymorphs and staphylococci per oil immersion field were assessed as follows: +++, > 20; ++, 5–20; +, up to 5; +/−, < 1, −, none present.

‡ Cultures were assessed according to the amount of growth in the primary inoculum. +++, confluent growth; ++, > 50 colonies; +, 10–50 colonies; +/−, < 10 colonies; −, no growth.

§ The degree of inflammation was ranked on a 5-point scale from 0 (no evidence of inflammation) to 5 (large defined abscess), as described in the text.

There was a statistically significant difference between strains RP62A and A-315 for all parameters of infection ($P < 0{\cdot}005$, $< 0{\cdot}005$, $< 0{\cdot}005$, $< 0{\cdot}01$, $< 0{\cdot}005$ respectively).

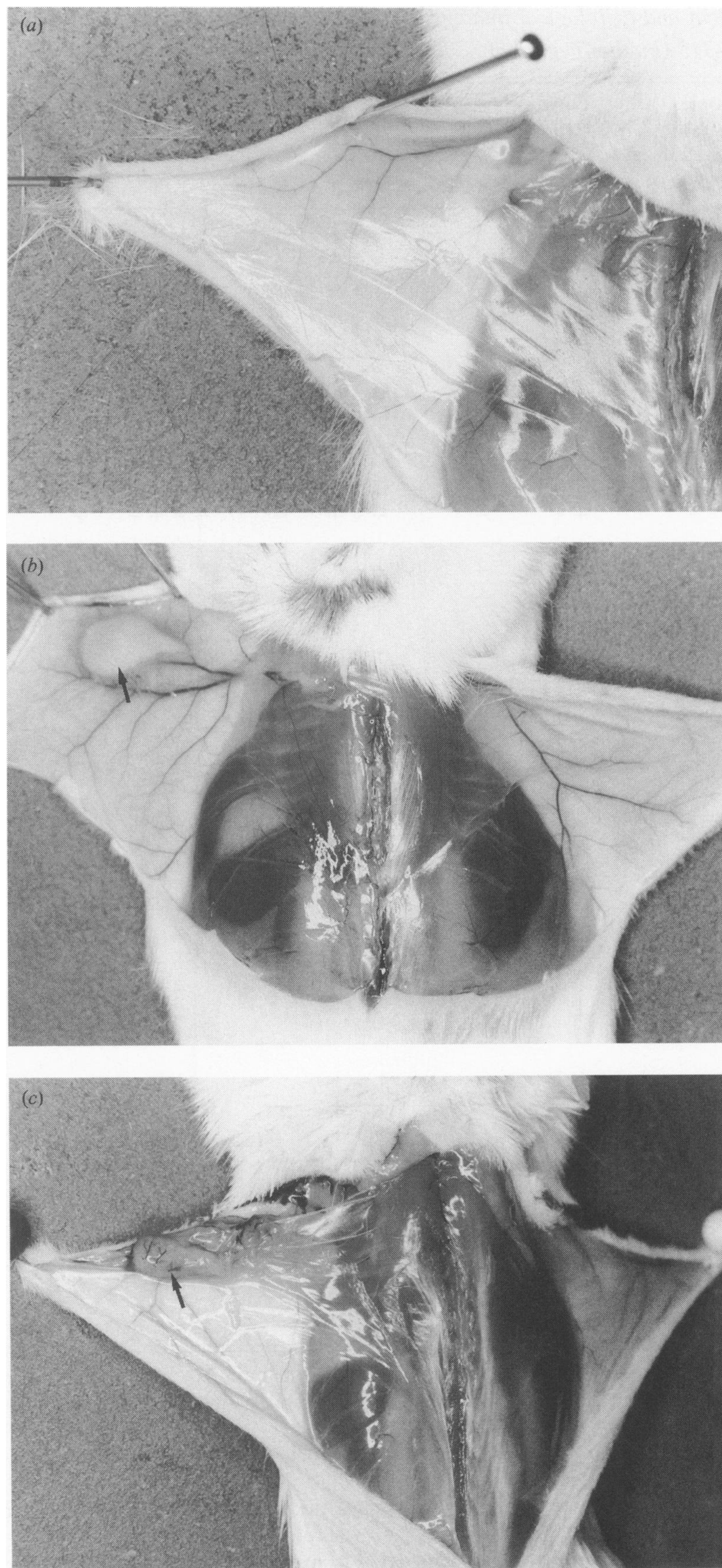


**Fig. 1.** Macroscopic findings in mice inoculated subcutaneously with *S. epidermidis* strains RP62A (biofilm-positive) and A-315 (biofilm-negative). Mice were inoculated subcutaneously over the rib-cage on the left of the dorsal midline with $2 \times 10^8$ CFU of the test strain, suspended in sterile saline, as described in the text. The same volume of sterile saline was inoculated

All strains were negative or very weakly positive for α-toxin. All strains liquefied gelatin, but at a very slow rate. All hydrolysed Tween 20 and most hydrolysed Tween 60. Strains differed in the production of δ-lysin, hydrolysis of Tween 80 and Tween 40 and cell surface hydrophobicity. Since Tween 80 and Tween 40 generated similar results, only Tween 80 hydrolysis is recorded in Table 1.

### Macroscopic findings, strains RP62A and A-315

Of mice inoculated with the biofilm-positive strain (RP62A), abscesses were found in 24 of 30 (80%) autopsied within a week of inoculation and 4 of 10 (40%) autopsied on day 14 (Table 2, Fig. 1). In contrast, mice inoculated with the biofilm-negative strain (A-315) often showed collections of purulent material on day 1, but these rarely persisted for longer than 3 days (Table 2, Fig. 1).

### Microbiological evidence of infection – strains RP62A and A-315

Gram-stained smears prepared from mice inoculated with strain RP62A suggested acute infection, which failed to clear and progressed to chronic abscess formation (Table 2). Typically, specimens collected at autopsies performed on day 1 showed large numbers of polymorphs and staphylococci. From day 3 onwards, macrophages associated with numerous staphylococci which appeared to be intracellular, became evident and were a predominant feature of smears collected on day 14 (Fig. 2*b*, Fig. 2*c*). Cultures of the 14-day specimens yielded fewer bacteria than would be expected from examination of the Gram-stained smears, suggesting that at least some of the bacteria associated with macrophages were dead, but that others had escaped killing by the macrophages. In contrast, strain A-315 evoked a weaker polymorph response and staphylococci were usually cleared from the site of inoculation by day 3 (Table 2, Fig. 2*a*).

The inoculated strain was isolated from 29 of 40 (73%) mice infected with strain RP62A compared with 15 of 40 (38%) mice infected with strain A-315. Strain RP62A persisted in mouse tissues for longer than A-315. Strain RP62A was recovered from 25 of 30 (83%) mice autopsied on days 1, 3 or 7 and from 4 of 10 (40%) mice autopsied on day 14 (Table 2). In contrast, strain A-315 was rarely recovered beyond day 3 and then only in small numbers (Table 2).

Isolates of strain RP62A recovered from mice were indistinguishable from the inoculated culture. All isolates produced distinctive red transparent colonies on Congo red agar, were phosphatase-negative and generated the same API 32 STAPH profile number (3660 3021, *S. epidermidis* 97·4%). The mean hydrophobicity index was not significantly different from that of the inoculum. All isolates produced heavy biofilm under all test conditions. Although strain RP62A is recognized as highly variable [9], there was no evidence that phenotypic switching occurred during animal passage.

Isolates from mice infected with strain A-315 were also indistinguishable from the inoculated culture. On Congo red agar, all produced distinctive red colonies that were difficult to dislodge from the agar. All isolates were biofilm-negative after incubation periods of up to 72 h and in TSB containing EDDA at concentrations ranging from 0–100 μg/ml. All generated the same API 32 STAPH profile number (3660 3221, *S. epidermidis* 98·1%) and were strongly hydrophilic. There was no evidence to suggest that the predominant colonial morphotype changed or that a minor phenotype was enriched by animal passage.

These findings are similar to those of Christensen and colleagues [21] who found that strain RP62A and a non-adherent variant (RP62A-NA) were stable through animal passage. However, given the random and unpredictable nature of phenotypic variation of *S. epidermidis in vitro* it is possible that we would have observed phenotypic variation if a larger number of animals had been studied.

### Histological evidence of infection, strains RP62A and A315

The degree of inflammation, assessed by the examination of stained sections of subcutaneous tissue, differed significantly between the strains ($P < 0{\cdot}005$), as did the kinetics of abscess development and tissue repair. Mice infected with strain RP62A typically developed large focal subcutaneous abscesses within 3 days of inoculation. The centres of these abscesses contained numerous polymorphs and many staphylococci. Polymorphs predominated among the incoming inflammatory cells. Between days 3 and 7, samples

to the right of the dorsal midline. (a) no abscess in a mouse autopsied 3 days after inoculation with strain A-315, (b) large subcutaneous abscess (arrow) in a mouse autopsied 3 days after inoculation with strain RP62A, (c) chronic granuloma (arrow) in a mouse autopsied 7 days after inoculation with strain RP62A.

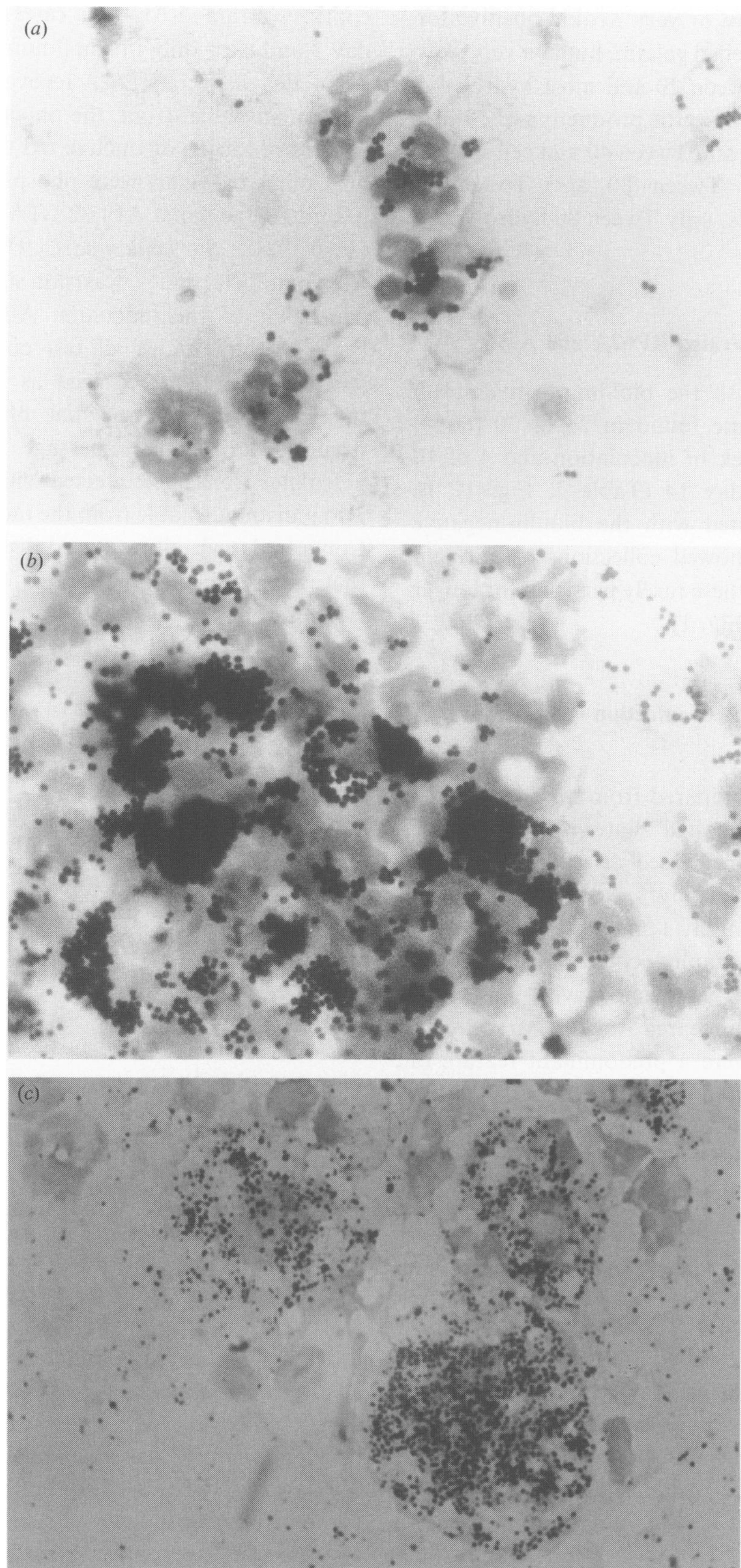


**Fig. 2.** Microbiological findings in mice inoculated subcutaneously with *S. epidermidis* strains RP62A (biofilm-positive) and A-315 (biofilm-negative). Gram-stained smears from subcutaneous abscesses of mice autopsied (a) 1 day after inoculation with strain A-315, (b) 1 day after inoculation with strain RP62A, (c) 14 days after inoculation with strain RP62A. Note the numerous Gram-positive cocci associated with macrophages. Culture of material from this lesion yielded *S. epidermidis* RP62A. Photographs were taken under oil immersion microscopy.

from some mice showed a change to a predominantly macrophage response but others showed few signs of resolution. Samples from lesions still present on day 14 usually showed chronic abscesses that contained collections of macrophages associated with bacteria that appeared to be located intracellularly (Table 2, Fig. 3).

Mice inoculated with strain A-315 usually showed evidence of acute inflammation when autopsied 24 h after inoculation. By the third day, however, there was either complete resolution of the infection or the presence of a resolving lesion with an influx of macrophages (Table 2, Fig. 3).

Skin inoculated with a saline suspension showed no evidence of infection either by histological or microbiological examination.

### Virulence of other biofilm-positive and biofilm-negative *S. epidermidis* strains

For 3 of the 5 indicators of infection examined by the $\chi^2$ test (size of subcutaneous abscesses, number of polymorphs and staphylococci in Gram-stained smears) there was a statistically significant difference between the biofilm-positive group and the biofilm-negative group ($P < 0{\cdot}01$, 0·01, 0·05 respectively). Biofilm-positive strains of *S. epidermidis* were recovered more frequently in culture and produced more inflammatory lesions than biofilm-negative strains but the differences did not reach statistical significance ($P < 0{\cdot}25$ and $< 0{\cdot}10$ respectively).

A summary of the microbiological and histological findings obtained in the second series of experiments is presented in Table 3. For the purpose of this comparison, an active infection was defined as the presence of a macroscopic abscess at autopsy together with microbiological or histological evidence of inflammation. A low grade infection was defined as a lesion associated with a mild inflammatory response (grade 1 on histological examination or 1–4 polymorphs/5 oil immersion fields in Gram-stained smears). In a small number of cases staphylococci were identified in Gram-stained smears or cultures in the absence of inflammation. These samples were recorded as negative. Using these criteria 35 (73%) mice inoculated with the biofilm-positive strains developed infections compared with 17 (35%) inoculated with the biofilm-negative strains. If the same criteria are applied to the initial comparison of RP62A and A-315 and to control mice used in the second series of experiments the figures are 28 (70%), 40 (40%) and 9 (75%), 6 (50%) respectively.

Low grade inflammatory responses appeared to be associated with inability to clear the infection (Tables 2, 3). It is interesting to note that almost three times as many infections occurred with A-205 compared with its biofilm-negative variant A-204. The difference was due to a greater number of low grade chronic infections occurring in mice infected with A-205.

Thus, there were considerable differences in the virulence of the 10 *S. epidermidis* strains examined. In general, the biofilm-positive strains were more virulent than the biofilm-negative strains. However, the most virulent of the biofilm-negative strains (strain A-250) produced almost as many abscesses as the least virulent of the biofilm-positive strains. None of the potential virulence determinants listed in Table 1 correlated with the capacity to produce abscesses in our mouse model.

### Variability between animals

Daily palpation of the inoculation site, together with data on the presence and appearance of microscopic abscesses at autopsy, revealed two major types of response to infection; progression to chronic abscess formation and rapid resolution, exemplified by strains RP62A and A-315 respectively. In the former group, early abscesses consisting of diffuse collections of polymorphs and staphylococci, were subsequently converted into large focal lesions that persisted for 10 days or longer. The second group showed no palpable lesions at any time during the study period but autopsy sometimes revealed small collections or purulent material early in the infection. The outcome for a given animal could be predicted by palpation of the inoculation site for the first 3 days, suggesting that progression to chronic lesion formation was determined early in the course of the infection. While the first and second patterns of infection were observed most frequently with biofilm-positive and -negative strains respectively, there were also differences between mice, both between and within experiments. This occurred despite strict adherence to protocols for preparation of inocula and inoculation of mice.

There are a number of possible reasons for the variations that were observed between animals. The capacity of *S. epidermidis* for phenotypic variation with respect to slime and other virulence factors [17, 21, 26, 27] could have resulted in differences between the inocula used in different experiments. Alternatively, host factors, such as the ability to mount an early polymorph response, could affect the outcome.

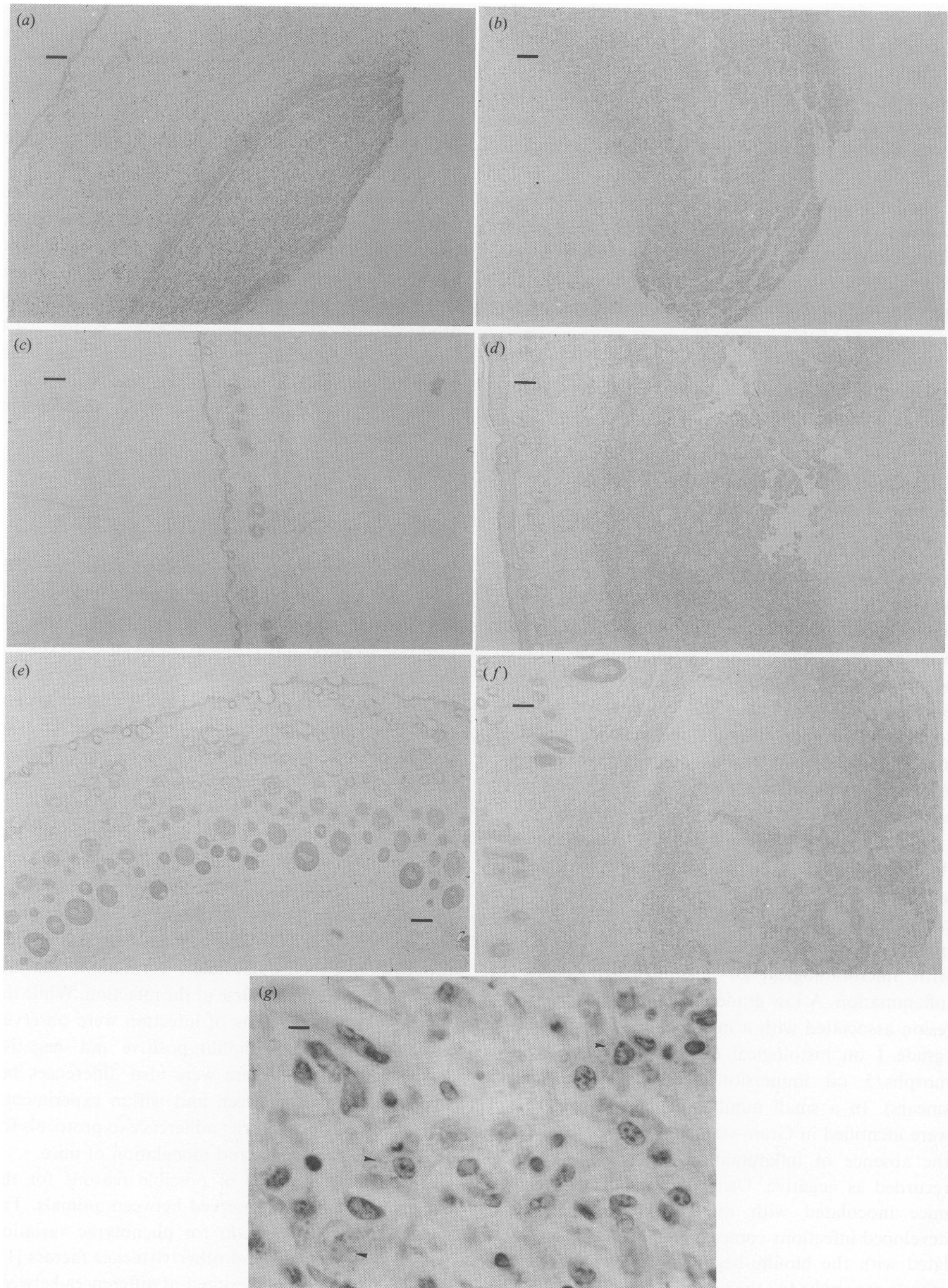


**Fig. 3.** Histological findings in mice inoculated subcutaneously with *S. epidermidis* strains RP62A (slime-positive) and A-315 (slime-negative). Haematoxylin and eosin-stained sections of tissue from mice autopsied after inoculation with strains A-315 and RP62A. (a) mouse epidermis, dermis and subcutis 1 day after inoculation with A-315, showing a circumscribed acute

Table 3. *Subcutaneous infections due to biofilm-positive and biofilm-negative* S. epidermidis *strains*

| | 1 day | | 3 days | | 7 days | | 14 days | | |
|---|---|---|---|---|---|---|---|---|---|
| Strains | Active | Low grade | Active | Low grade | Active | Low grade | Active | Low grade | All |
| Biofilm-positive | | | | | | | | | |
| A-73 | 2 | 1 | 2 | 0 | 1 | 1 | 2 | 0 | 9 |
| A-205 | 1 | 1 | 2 | 1 | 1 | 0 | 0 | 2 | 8 |
| A-263 | 2 | 1 | 3 | 0 | 2 | 0 | 2 | 0 | 10 |
| A-285 | 2 | 0 | 3 | 0 | 1 | 1 | 1 | 0 | 8 |
| All | 7 (44) | 3 (19) | 10 (63) | 1 (6) | 5 (31) | 2 (13) | 5 (31) | 2 (13) | 35 (73) |
| Biofilm-negative | | | | | | | | | |
| A-62 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 |
| A-204 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| A-211 | 3 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 5 |
| A-250 | 3 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 7 |
| All | 9 (56) | 0 (0) | 4 (25) | 1 (6) | 3 (19) | 0 (0) | 0 (0) | 0 (0) | 17 (35) |

Of 3 experiments, involving 40 mice and 10 *S. epidermidis* strains (5 biofilm-positive, 5 biofilm-negative), experiment 2 resulted in a higher rate of abscess formation than experiments 1 and 3. The total number of abscesses observed at autopsy for the experiments 1, 2 and 3 were 3, 9 and 3 respectively for biofilm-negative strains and 12, 14 and 10 for biofilm-positive strains. These differences between experiments were not statistically significant. It is our practice to have mice delivered to the laboratory by air-conditioned transport on the day before commencing experiments. Mice for experiment 2, although apparently normal on arrival, were delivered on an unusually hot day (39 °C), reported as the hottest December day in Melbourne for over 60 years. We speculated that the higher number of abscesses in this group was due to depression of immunity related to stress. While possible variations in the inoculum or in the cohort of animals do not explain variability within experimental groups, our observations suggest that both host and bacterial factors affect the outcome in a given animal.

## DISCUSSION

All 10 strains of *S. epidermidis* used in this study were capable of producing abscesses in subcutaneous tissues of mice not traversed by a foreign body. Most other animal models of CoNS infections have assessed virulence in the presence of a catheter inserted into the skin. Patrick and colleagues [23] found no abscesses in mice, 8 days after inoculation with any *S. epidermidis* strain, in the absence of a catheter or of a surgical incision. However, there was a high frequency of abscess formation in mice that underwent a sham operation (surgery without subcutaneous implantation of a catheter). Moreover, Ferguson and colleagues [33] demonstrated macroscopic abscesses in 11–88% of sham-operated mice inoculated with $2 \times 10^8$ *S. epidermidis* cells postoperatively. Both the present study and those of Ferguson and colleagues [33] and Patrick and colleagues [23] demonstrate that infection with *S. epidermidis* does not require the presence of a foreign body. In catheter-associated infections, the foreign body may simply provide easy access into the host. Supporting this hypothesis is the

inflammatory response in the subcutis largely consisting of polymorphonuclear leucocytes (PMNs) (grade 4). Bar 100 $\mu$. (b) mouse epidermis, 1 day after inoculation with RP62A, showing part of a very extensive inflammatory reaction in the subcutis consisting largely of PMNs (grade 4). Bar 100 $\mu$. (c) mouse epidermis, dermis and subcutis, 7 days after inoculation with A-315, showing normal tissue (grade 0). Bar 100 $\mu$. (d) mouse epidermis, dermis and subcutis, 7 days after inoculation with RP62A, showing an extensive acute granulomatous reaction undermining the dermis and subcutis and consisting largely of PMNs, macrophages and necrotic PNMs scattered throughout the lesion. Areas of focal necrosis are present towards the centre of the lesion (grade 5). Bar 100 $\mu$. (e) mouse epidermis, dermis and subcutis, 14 days after inoculation with A-315, showing normal tissue (grade 0). Bar 100 $\mu$. (f) mouse dermis and subcutis, 14 days after inoculation with RP62A, showing an extensive granulomatous lesion in the subcutis, consisting largely of monuclear cells, macrophages and multiple areas of focal necrosis throughout (grade 4). Bar 100 $\mu$. (g) subcutaneous tissue of mouse autopsied 7 days after inoculation with *S. epidermidis* RP62A. Note the stained material, which appears to be located within macrophages (arrowhead). Bar 5 $\mu$.

observation that CoNS inoculated onto entry sites of implanted catheters move very rapidly along external surfaces and give rise to acute inflammatory lesions within 2 days [34].

We have demonstrated significant differences between biofilm-positive and biofilm-negative strains of *S. epidermidis* in their capacity for subcutaneous abscess formation in a mouse model. Acute abscesses caused by biofilm-positive strains frequently progressed to chronic lesions that yielded viable staphylococci and contained numerous macrophages with associated staphylococci that appeared to be located intracellularly. In contrast, those caused by biofilm-negative strains usually resolved within a few days. These findings suggest that staphylococcal exopolysaccharide may protect the staphylococci from the intracellular killing mechanisms of macrophages and they support *in vitro* evidence that slime induces inflammation, possibly by stimulating monocytes to produce the inflammatory cytokines, prostaglandin $E_2$, interleukin-1 and tumour necrosis factor-$\alpha$ [19].

These results are in agreement with those of Christensen and colleagues [21] who demonstrated a threefold increase in the $ID_{50}$ of a biofilm-negative variant in a mouse model of catheter-related sepsis compared with that of the biofilm-positive parent. In Christensen's study, infection was defined as recovery of the inoculated strain from catheters 10 days after inoculation. We found, however, that abscess formation, assessment of Gram-stained smears and histological sections all provided better discrimination between biofilm-positive and biofilm-negative strains than the number of staphylococci recovered. In contrast to the findings of Christensen and colleagues [21], Patrick and colleagues [23] found no differences between biofilm-positive and biofilm-negative clinical isolates in their ability to produce visible abscesses in the subcutaneous tissues of mice in the presence of a catheter. However, the same group recently reported that biofilm-positive *S. epidermidis* strains caused abscesses in the presence of a catheter at significantly lower titres than biofilm-negative strains [24].

The reasons for these conflicting findings are unclear, however strain selection is likely to be important. The kinetics of biofilm expression differ between strains as do the optimal environmental conditions for optimal biofilm production [11]. It is possible that the strains used in some studies, although biofilm-negative when tested by conventional methods, might produce biofilm when subjected to iron-stress as in host tissues [11]. The strains used in the present study were chosen from a large collection as the strongest and weakest biofilm producers under a variety of conditions including growth for up to 4 days at 35 °C, under iron-limited and iron-replete conditions [11]. It is noteworthy that strain A-73, an isolate from blood, which produced the second largest number of abscesses, produces very heavy biofilm, in iron-limited TSB but not in standard TSB or in iron-restored TSB. It is possible that the expression of slime is enhanced under the iron-stressed conditions encountered *in vivo* and that such enhancement is strain dependent.

Our findings suggest that the ability to produce heavy biofilm *in vitro* is a marker which distinguishes strains of *S. epidermidis* with enhanced virulence. Extracellular polysaccharides are probably responsible for both attributes. It is clear, however, that other factors determine the virulence of *S. epidermidis*, since all biofilm-negative strains were capable of early abscess formation. In agreement with Lambe and colleagues [35], we found no evidence that haemolysins or extracellular enzymes contributed significantly to the virulence of *S. epidermidis*. Other factors that have been identified as possible virulence determinants include a protein adhesin [36] and surface structures mediating attachment to fibronectin, fibrinogen or other proteins present in damaged tissue [37].

Several investigators have identified surface structures of *S. epidermidis* that contribute to biofilm formation and are immunogenic in animal models. A capsular polysaccharide, believed to be involved in the early attachment phase of biofilm formation was identified by Tojo and colleagues [38]. Capsular polysaccharide adhesin interferes with neutrophil phagocytosis [39]. Moreover specific anti-capsular antibody was protective in an animal model of *S. epidermidis* endocarditis [39]. Two antigens associated with the late accumulation phase of biofilm formation have been described. Christensen and colleagues [40] isolated slime-associated antigen, which is immunologically distinct from capsular polysaccharide adhesin. More recently, Mack and colleagues [41] described a polysaccharide antigen associated with the biofilm-producing phenotype. It is not known whether these two antigens are related to each other [41]. Capsular polysaccharide adhesin and factor(s) responsible for cell aggregation are apparently closely linked, since transposon mutagenesis has yielded mutants deficient in both capsular polysaccharide and biofilm production, but it has not been possible to

isolate mutants deficient in only one of these properties [42].

The precise structures responsible for the evasion of host defence and tissue damage described in the present study are unknown. Use of isogenic mutants, such as those described by Muller and colleagues [42], in our mouse model would provide additional information on the contribution of the glycocalyx of *S. epidermidis* to virulence but would not separate the effects of capsular polysaccharide and factors responsible for cell aggregation. However, since virulence can seldom be attributed to a single factor [43], it is important that pathogenesis studies are performed on clinical isolates as well as mutant strains.

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