Biomaterial-associated infection : peri-implant tissue is an important niche for Staphylococcus epidermidis survival
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ABSTRACT

Biomaterial-associated infections (BAI), which are predominantly caused by Staphylococcus epidermidis, are a significant problem in modern medicine. Biofilm formation is considered the pivotal element in the pathogenesis, but in previous mouse studies we retrieved S. epidermidis from peri-implant tissue. To assess the kinetics and generality of tissue colonization, we investigated BAI using two S. epidermidis strains, two biomaterials, and two mouse strains. With small inocula all implants were culture negative, whereas surrounding tissues were positive. When higher doses were used, tissues were culture positive more often than implants, with higher numbers of cfu. This was true for the different biomaterials tested, for both S. epidermidis strains, at different times, and for both mouse strains. S. epidermidis co-localized with host cells at a distance that was >10 cell layers from the biomaterial-tissue interface. We concluded that in mouse experimental BAI S. epidermidis peri-implant tissue colonization is more important than biofilm formation.

INTRODUCTION

Infections of indwelling medical devices, mainly caused by Staphylococcus epidermidis and other coagulase-negative staphylococci, are a major problem in modern medicine. The formation of a biofilm consisting of bacteria, bacterial products, and host proteins on the biomaterial surface is considered a pivotal element in the pathogenesis of these biomaterial-associated infections (BAI). Biofilm formation involves direct adherence of S. epidermidis to the biomaterial surface through autolysin AtlE and to deposited host proteins by fibronectin-binding and fibrinogen-binding proteins. Subsequent accumulation is attributed to polysaccharide intercellular adhesin, elaborated by the products of the icaADBC genes, and to accumulation-associated protein. Mutation of either S. epidermidis atlE or icaABC results in a biofilm-negative phenotype in vitro and strongly reduces the numbers of cfu retrieved from implants in animal models. However, several studies have failed to demonstrate a requirement for the ica biofilm genes for virulence in experimental BAI, and...
between 40 and 75% of clinical *S. epidermidis* isolates lack one or more of the biofilm genes. This suggests that biofilm formation is not the only mechanism underlying BAI.

As the presence of a biomaterial reduces the efficacy of the local host immune response, bacteria may not be cleared from peri-implant tissue. Therefore, we studied the kinetics of colonization of implants, as well as peri-implant tissue, with two *S. epidermidis* strains, two biomaterials, and two mouse strains, C57BL/6 and BALB/c. Our data show that colonization of tissues surrounding implants by *S. epidermidis* is a general phenomenon, which may be an important element in the pathogenesis of BAI that has been overlooked previously.

**MATERIALS AND METHODS**

**Animals.** Animal experiments were approved by the Animal Ethical Committee of the University of Amsterdam. Specific-pathogen-free C57BL/6 and BALB/c female mice that were 6 to 8 weeks old and weighed 15 to 20 g were used (Harlan, Horst, The Netherlands). These mouse strains were chosen because they favor the development of Th1- and Th2-type inflammatory responses, respectively. Mice were housed in a pathogen-free environment and were provided with sterile food and water. After surgery mice were housed singly in individually ventilated cages.

**Biomaterials.** Catheters of silicon elastomer grafted with poly(vinylpyrrolidone) hydrogel (SEpvp) (Medtronic PS Medical, Goleta, CA) (used for hydrocephalus shunts) and of polyamide grafted with poly(vinylpyrrolidone) (PApvp) (experimental biomaterial) with an external diameter of 2.5 mm and a wall thickness of 0.6 mm were used. SEpvp and PApvp were chosen as model biomaterials because they induce different grades of inflammation in C57BL/6 mice infected with *S. epidermidis* RP62a; SEpvp provokes a strong proinflammatory response, and PApvp provokes only a mild proinflammatory response.

**S. epidermidis strains.** *S. epidermidis* RP62a (= ATCC 35984), a well-studied BAI strain, and *S. epidermidis* clinical isolate AMC5 were used. Both strains were capable of producing slime, as determined by the method of Christensen et al., and δ-toxin, as determined by the method of Hebert and Hancock, and were
Peri-implant tissue is an important niche for Staphylococcus epidermidis positive for the *icaA*, *atlE*, *aap*, and *sarA* genes as determined by PCR. The primers used for *icaA*, *atlE*, and *aap* were the primers described by Vandecasteele et al. 32. *SarA* was detected using primers sarA_d (AGCAAATGCTACATTGCTAATT) and sarA_r (ATTGCTTCTGTGATACGGTT). The MICs of antibiotics for strains RP62a and AMC5 (as determined by E tests) were as follows: rifampicin, < 0.016 and < 0.016 µg/ml, respectively; teicoplanin, 3 and 1.5 µg/ml, respectively; gentamicin, 8 and 0.125 µg/ml, respectively; and vancomycin, 4 and 4 µg/ml, respectively. The following antibiotic tablets were used for antibiogram analysis of challenge bacteria and cfu recovered from the model: spectinomycin (200 µg), penicillin low (5 µg), chloramphenicol (60 µg), vancomycin (5 µg), erythromycin (78 µg), rifampicin (30 µg), minocycline (80 µg), and gentamicin (40 µg). RP62a is resistant to spectinomycin and erythromycin. The AMC5 strain is susceptible to all antibiotics tested.

**Inoculum preparation.** Thirty milliliters of tryptic soy broth inoculated with the *S. epidermidis* test strain was incubated for 4 h at 37°C on a rotary shaker at 120 rpm. The culture was centrifuged at 2,200xg for 10 min, and the pelleted bacteria were washed twice with 30 ml of pyrogen-free 0.9% NaCl (saline) (Fresenius Kabi, Sévres, France). Suspensions containing 4x10^6 to 4x10^9 cfu per ml were prepared based on the optical density at 620 nm.

**Mouse biomaterial-associated infection model.** Mouse BAI studies were performed as described previously 25,28. In short, mice anesthetized with a mixture of 1 ml fentanyl-citrate, 1 ml midazalam, and 2 ml distilled water received 1-cm-long subcutaneous implants in a laminar flow cabinet. The incisions were closed with a single stitch. Inocula (25 µl) were injected along the implants with a repetitive injector (Stepper model 4001-025; Tridak Division, Brookfield, CT).

Mice were anesthetized with FFM mixture between 5 and 21 days after challenge, and standardized biopsies (diameter, 12 mm) of biomaterial implants with subcutaneous tissue and skin were taken. The right side biopsy was used for quantitative culture. The left side biopsy was cut into two halves; one half was used for quantitative culture, and the other half was used for histology. Cardiac puncture was performed to collect blood and terminate the mice. The time intervals between challenge and termination of mice in the different experiments were matched but were not always identical due to variation in the availability of operating rooms in the
animal facility. The catheter segments were rinsed twice to remove bacteria loosely associated with the implant and placed in sterile tubes containing 500 µl of saline. The tubes were sonicated for 30 s in a water bath sonicator (Bransonic B-2200 E4) to dislodge adherent bacteria for quantitative culture. The segment itself was placed in 80 ml of Brewer Tween (BT) thioglycolate broth at 37°C for 2 to 14 days to detect low numbers of bacteria or bacteria released from host cells lysed by the Tween. If the BT culture was positive and plate cultures were negative, the biomaterial segment was considered to have had 5 adherent cfu.

The tissue samples were weighed, a volume of saline corresponding to four times the weight of each sample (range, 50 to 300 mg) was added, the samples were homogenized on ice (model 985-370 Tissue Tearor; Biospec Products, Bartlesville, OK), and quantitative cultures were grown. In addition, 0.1 volume of the homogenate was cultured in 80 ml BT for 2 to 14 days at 37°C. If the BT culture was positive and plate cultures were negative, the total homogenate was considered to have contained 10 cfu. Positive BT cultures were streaked on blood agar plates and incubated at 37°C. Cultured bacteria were analyzed by the Gram stain and antibiogram methods. In all cases reisolated bacteria had the same antibiograms as the challenge strain.

**Blood culture.** Twenty-five microliters of blood was cultured in 80 ml of BT for up to 14 days at 37°C. Blood cultures were negative for all samples.

**Histological examination.** Biopsies were fixed in formaldehyde, embedded in plastic (methylmethacrylate / buthylmethacrylate; Merck Schuchart, Hohenbrunn, Germany), and 3- to 5-µm sections were cut at different levels. These sections were stained with hematoxylin and eosin or with Gram stain and examined by light microscopy.

**Statistics.** The Fisher's exact test was used to test the significance of differences in the frequencies of positive cultures of biopsies and biomaterial implants from different groups of mice. Differences in numbers of cfu were analyzed with the Mann-Whitney test. For both tests, a P value of < 0.05 was considered significant.
RESULTS AND DISCUSSION

Experimental BAI due to \textit{S. epidermidis} RP62a in C57BL/6 mice with SEpvp implants. Our experimental BAI model was based on the model of Christensen \textit{et al.} \textsuperscript{33} and required relatively high doses of \textit{S. epidermidis} \textsuperscript{28,33-35} to achieve infection. In the original model only the explanted biomaterials were cultured. We cultured both the biomaterial implants and peri-implant tissue samples \textsuperscript{25,26}. After 5 days there was a dose-dependent increase in the frequency of positive cultures for implant samples from mice challenged with \num{5e6} cfu or more, but even at the highest inoculum dose \textsuperscript{(1e8 cfu)} not all implanted biomaterial segments were positive (Figure 1A). As observed for the implants, the frequency of culture-positive tissue samples increased with the inoculum dose (Figure 1A). However, cultures of tissue samples were culture positive more often than the corresponding implants for all inoculum sizes between \num{1e6} and \num{5e7} cfu \textsuperscript{(P < 0.05)} and contained higher numbers of cfu \textsuperscript{(P < 0.05)} (Figure 1B). Biopsies from mice challenged with \num{1e8} cfu were all culture positive, and the numbers of cfu were much higher than the numbers of cfu obtained for the corresponding implants \textsuperscript{(P = 0.0002)} (Figure 1B).

Persistence of \textit{S. epidermidis} RP62a around SEpvp in experimental BAI. At 5 or 14 days after challenge cultures of SEpvp implants from mice challenged with \num{2e6} cfu were all negative. In contrast, at 5 days all tissue biopsies were culture positive. At 14 days a liquid culture for one tissue sample was positive. Implants from mice challenged with \num{1e7} cfu and sacrificed after 5 days were all culture negative, but after 14 days 3/12 implants were culture positive (difference not significant). At 5 days as well as 14 days, more tissue samples than corresponding implants \textsuperscript{(P < 0.003)} were culture positive, with higher numbers of cfu at 14 days \textsuperscript{(P = 0.0046)} (Figure 1C). Thus, \textit{S. epidermidis} RP62a persisted in peri-implant tissue rather than on the implant itself.

Experimental BAI due to \textit{S. epidermidis} AMC5 in C57BL/6 mice with SEpvp or PApvp implants. For mice carrying SEpvp implants, as well as for mice with PApvp implants, we chose conditions expected to yield approximately 50 to 90\% culture-positive tissue samples (Figure 1) \textsuperscript{26}. At the early time point (10 days) 67\% of the tissues, but none of the implants, of mice carrying SEpvp implants and challenged
with $3 \times 10^6$ cfu of *S. epidermidis* AMC5 were culture positive ($P = 0.0013$) (Figure 2A). Even with an inoculum of $3 \times 10^7$ cfu not more than 25% of the implants yielded growth, whereas all tissue biopsies were culture positive ($P = 0.0003$). The tissue biopsies yielded significantly higher numbers of cfu than the implants yielded for both inocula tested (Figure 2A). In mice carrying PApvp implants challenged with $3 \times 10^6$ cfu, 92% of the tissues and 25% of the implants yielded positive cultures after 8 days ($P = 0.003$), and the numbers of cfu cultured from the tissues were significantly higher (Figure 2B). After challenge with $3 \times 10^7$ cfu, 42% of the implants and 67% of the tissues were culture positive after 8 days (difference not significant), and the numbers of cfu retrieved were similar.

At the late time point, after 18 days, low numbers of SEpvp implants were culture positive, and the numbers of cfu were low (Figure 2A). The corresponding tissue samples yielded 50% positive cultures, which was a 50% decrease compared with the day 10 value ($P = 0.0137$). Tissue colonization around SEpvp and tissue colonization around PApvp at the late time point were similar, but higher numbers of cfu were retrieved from PApvp implants than from SEpvp implants ($P = 0.0253$) (Figure 2A and B). Thus, like strain RP62a, strain AMC5 was able to colonize and persist in peri-implant tissue around different biomaterials.

Clinical *S. epidermidis* isolates differ in the capacity to colonize implants in experimental BAI. *S. epidermidis* RP62a and AMC5 showed similar levels of implant colonization. Both strains colonized the tissue more avidly than they colonized the implants. The physicochemical characteristics of biomaterials may also influence the susceptibility to infection *in vivo* either due to differences in adherence of the bacteria or due to differences in the nature of the foreign body response or the immune response provoked by the presence of the biomaterial. Despite the differences in the inflammatory response known to be induced around SEpvp and PApvp during infection, tissue colonization around these materials was very similar.

*Figure 1 (p.37): Percentages of culture-positive samples (A) and numbers of cfu cultured from tissue biopsies (T) and biomaterial implants (BM) from C57BL/6 mice with SEpvp implants at 5 days after challenge with graded inocula (B) and at 5 and 14 days after challenge with $2 \times 10^6$ and $1 \times 10^7$ cfu (C) of *S. epidermidis* RP62a. The frequencies of positive cultures are indicated at the top. An asterisk indicates that the $P$ value is < 0.05. Samples that were positive as determined by liquid broth culture but negative as determined by plate culture are indicated by open circles.*
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Figure 2: Frequencies of positive samples and numbers of cfu cultured from tissue biopsies (T) and biomaterial implants (BM) from C57BL/6 mice with SEpvp implants sacrificed at 10 and 18 days (A) and from mice with PApv implants sacrificed at 8 and 15 days (B) after challenge with 3\times10^6 and 3\times10^7 cfu of S. epidermidis AMC5. The frequencies of positive cultures are indicated at the top. An asterisk indicates that the P value is < 0.05. Samples that were positive as determined by liquid broth culture but negative as determined by plate culture are indicated by open circles.
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**Experimental BAI due to *S. epidermidis* RP62a in BALB/c mice with SEpvp implants.** Since BALB/c mice are reported to be more susceptible to bacterial infection than C57BL/6 mice, we used small *S. epidermidis* RP62a inocula (1x10^4, 1x10^5, and 1x10^6 cfu). The mice were sacrificed 8 days after challenge. As observed for the C57BL/6 mice, the level of tissue colonization around the SEpvp implants was dependent on inoculum size and was higher than the level of colonization of the implants themselves after challenge with 1x10^5 and 1x10^6 cfu (Figure 3A and B).

To assess bacterial persistence, BALB/c mice were challenged with an inoculum containing 1x10^6 cfu of *S. epidermidis* RP62a and sacrificed 6, 14, or 21 days later. The numbers of culture-positive implants and surrounding tissues and the numbers of cfu retrieved from implants, as well as from tissues, decreased over time (Figure 3C and D). The peri-implant tissues were more often culture positive than the implants, but the difference was not significant. However, after 14 days RP62a was cultured from 44% of the tissue samples from BALB/c mice and from only 8% of the samples from C57BL/6 mice challenged with a similar inoculum (P < 0.05). Thus, BALB/c mice were more susceptible than C57BL/6 mice to persistent tissue colonization by *S. epidermidis*RP62a. This is in accordance with the higher susceptibility of BALB/c mice to infection by other bacteria, particularly pathogens residing in cells or tissues. This might be related to the Th2 predominance in these mice.

**Histology.** To investigate where the bacteria were located, we selected biopsies for histology which had yielded high numbers of cfu and were expected to contain sufficient bacteria for microscopic analysis. Sections were collected from different levels in the biopsies. The images in Figure 4 are representative of the biopsies examined from mice challenged with *S. epidermidis* AMC5 and RP62a.

Figure 4 shows Gram-stained sections (Figure 4A to D) and hematoxylin- and eosin-stained sections (Figure 4E) of peri-implant tissue from C57BL/6 mice 8 days after implantation of SEpvp and challenge with 1x10^7 cfu of strain AMC5. Fibroblasts, lymphocytes, and granulocytes were present in the peri-implant tissue, indicating that there was severe inflammation (Figure 4E). Bacteria were co-localized with cells in the tissue with the morphology of macrophages at a distance that was 10 to 20 cell layers from the biomaterial-tissue interface (Figure 4C and D). No bacteria were visible at the biomaterial-tissue interface (Figure 4A and B), where a biofilm would have been localized.
Figure 3: Frequencies of positive samples (A and C) and numbers of cfu (B and D) cultured from tissue biopsies (T) and biomaterial implants (BM) from BALB/c mice with SEpvp implants at 8 days after challenge with $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ cfu S. epidermidis RP62a (A and B) and at 6, 14, and 21 days after challenge with $1 \times 10^6$ cfu S. epidermidis RP62a (C and D). An asterisk indicates that the P value is < 0.05. Samples that were positive as determined by liquid broth culture but negative as determined by plate culture are indicated by open circles.
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In the tissue of mice carrying PApvp implants and challenged with $3 \times 10^7$ cfu of S. epidermidis AMC5, signs of active inflammation were observed 8 days after challenge (Figure 4F to J). Fibroblasts, neutrophils, monocytes, and plasma cells were present (Figure 4J). At the tissue-implant interface no bacteria were observed (Figure 4F and G), but large numbers of gram-positive cocci co-localized with cells in peri-implant tissue that was > 30 cell layers from the implant (Figure 4H and I). Under conditions that compromise the intracellular microbicidal mechanisms, S. epidermidis survives intracellularly in various types of phagocytic cells. Our results imply that the presence of the implants indeed compromised the local immunity, providing S. epidermidis with a niche in the peri-implant tissue.

To assess whether our findings with the mouse model are relevant for the human situation, we recently collected central venous catheters and surrounding tissue from deceased patients. We cultured bacteria more often from the peri-catheter tissue than from the catheters and the numbers of bacteria were higher, and we also cultured bacteria from tissue not directly bordering the catheters. These findings support the hypothesis that peri-implant tissue is a niche for bacteria causing persistent biomaterial-associated infections in humans and imply that the mouse model is a proper model for studying these infections.

Our findings may have important implications for patients with an implanted biomedical device. If S. epidermidis is indeed able to survive and replicate in peri-implant tissue in humans and possibly intracellularly, antibiotic treatment protocols might need adjustment. S. epidermidis BAI is often treated with vancomycin, but vancomycin has relatively poor tissue penetration and is inefficient in reaching intracellular bacteria. When vancomycin rifampicin combinations are used for therapy, S. epidermidis in tissue or even inside host cells is reached and affected predominantly only by the rifampicin. As S. epidermidis readily develops resistance to rifampicin monotherapy, the use of a second antibiotic with good tissue and host cell penetration may be warranted.

Figure 4 (p.42): Gram-stained (A to D and F to I) and hematoxylin- and eosin-stained (E and J) sections of peri-implant tissue from C57BL/6 mice with SEpvp implants (A to E) challenged with $1 \times 10^7$ cfu of S. epidermidis AMC5 and with PApvp implants (F to J) challenged with $3 \times 10^7$ cfu of S. epidermidis AMC5 and sacrificed after 8 days. The arrows in panels A and F indicate the biomaterial-tissue interface, and the arrows in panels D and I indicate bacteria.
CONCLUSION

In our mouse BAI model, peri-implant tissue proved to be a major niche for *S. epidermidis*. After challenge with low doses, *S. epidermidis* was recovered only from the peri-implant tissue and not from the implants themselves. At higher challenge doses, bacteria were found in association with the biomaterial, but higher numbers of cfu were cultured from the surrounding tissue. Moreover, bacteria persisted longer in the tissue than on the biomaterial implants. This was observed for two strains of *S. epidermidis*, RP62a and AMC5, for two different biomaterials, SEpvp and PApvp, and in two different mouse genetic backgrounds, C57BL/6 and BALB/c. Thus, contrary to the general contention, the biomaterial implant itself was not the major site of colonization, but *S. epidermidis* resided predominantly in the peri-implant tissue.

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