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

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CHAPTER

Tissue surrounding implants is a niche for *Staphylococcus epidermidis*, identified by their ability to incorporate BrdU.
Tissue surrounding implants is a niche for *Staphylococcus epidermidis*, identified by their ability to incorporate BrdU

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**ABSTRACT**

Infection of biomedical devices is characterized by biofilm formation and colonization of surrounding tissue by the causative pathogens. To investigate whether bacteria detected microscopically in tissue surrounding infected devices were viable, we used BrdU, a nucleotide analogue that is incorporated into bacterial DNA and can be detected with antibodies. Human infected tissue was obtained from patients with intravascular devices, after their death, and from mice with experimental biomaterial infection. Preliminary experiments showed that *Staphylococcus epidermidis* stained with anti-BrdU antibodies could be discriminated from eukaryotic cells. By BrdU and antibody staining bacteria could be clearly visualized in the tissue surrounding intravascular devices in deceased patients. With this staining technique, relapse of infection could be visualized in mice challenged with a low dose of *S. epidermidis*, and treated with dexamethasone between 14 and 21 days after challenge to suppress immunity. This confirms and extends our previous findings that peri-catheter tissue is a reservoir for bacteria in biomaterial-associated infection. The pathogenesis of the infection and temporo-spatial distribution of viable, dividing bacteria can now be studied at the microscopic level by immunolabelling with BrdU and BrdU antibodies.

**INTRODUCTION**

Biomaterial-associated infections (BAI) are a common and serious problem in modern medicine. Infection necessitates extensive antimicrobial treatment and may eventually lead to removal or replacement of the device. This causes morbidity and mortality, and increases hospital costs considerably. Bacteria causing these infections can be present on the implanted material and form a biofilm, but are also found in the tissue surrounding it. Previously we have shown that in a mouse model of BAI bacteria are present in higher numbers in the tissue surrounding the implanted material than on the biomaterial itself. In the tissue the bacteria are associated with host inflammatory cells and are found in large numbers within macrophages, which suggests that these macrophages allow intracellular survival and possibly replication of the bacteria. In mice, tissue-residing *Staphylococcus epidermidis* persist despite rifampicin / vancomycin treatment, whereas the
antibiotics do clear the bacteria from the implanted biomaterials. We also detected viable bacteria in samples of tissue surrounding catheters retrieved from deceased patients. The tissue surrounding foreign bodies must be considered a novel niche for bacteria causing BAI.

The aim of our study was to investigate the spatiotemporal characteristics of the development of the infection at the microscopic level, by labelling viable bacteria in the tissues. Available methods to detect and localize bacteria include plate and liquid broth culture, various molecular diagnostic techniques (PCR, RT-PCR) and gram-staining, whereas to visualize bacteria, methods such as green fluorescent protein (GFP) expression or bioluminescent markers, in situ hybridization with probes for RNA or DNA, live/dead fluorescent staining and immunohistochemistry have been used. None of these methods, however, allows a timed start of labelling of viable cells combined with microscopic detection. A suitable method may be labelling with 5-bromo-2-deoxyuridine (BrdU). BrdU is a synthetic thymidine analogue which is incorporated into newly synthesized DNA during DNA replication and repair. BrdU has been used extensively to study eukaryotic cell proliferation and other cellular functions, and also for detection of replicating bacteria in marine and soil samples. It is readily incorporated by Escherichia coli in broth culture. In the present study we applied BrdU labelling to investigate the viability of S. epidermidis in peri-catheter tissue of deceased patients, and on implants and surrounding tissue in a BAI mouse model.

MATERIALS AND METHODS

S. epidermidis strains and inoculum preparation. Staphylococcus epidermidis RP62a (ATCC 35984) was used in the experimental mouse model. RP62a harbours the biofilm-associated icaA, atLE, aap and sarA genes, and produces polysaccharide Intercellular Adhesin (PIA) and δ-toxin. MIC values (µg/ml) according to standard E-test of strain RP62a were: rifampicin < 0.016, teicoplanin 3, gentamicin 8, and vancomycin 4. Bacteria retrieved after challenge of mice were always checked for susceptibility to these antibiotics. The bacterial inocula were prepared by inoculating Tryptic Soy Broth (TSB) with 25 µl of thawed S. epidermidis RP62a or Escherichia coli ML35 suspension from frozen stocks, and incubation for 4-5 h at 37°C. After centrifugation and washing of the bacterial pellet with saline, suspensions containing
S. epidermidis identified by their ability to incorporate BrdU

approximately $4 \times 10^8$ colony forming units (cfu) per ml saline were prepared based on the OD$_{620}$

To assess BrdU incorporation in vitro, 150 µl of the bacterial suspension was added to 3 ml TSB containing 10 µM BrdU and 33 nM thymidine, and incubated for 2 h at 37°C. Thymidine was added to suppress thymidilate synthase. Ten µl of this suspension was added to an epoxy-coated glass slide and treated for light microscopic detection of BrdU as described below.

Opsonophagocytosis of S. epidermidis RP62a by HL-60 cells. HL60 cells (a human granulocyte cell-line; ATCC CCL-240) were grown in complete RPMI medium without phenol red with 20% FBS (both from Gibco Invitrogen). Cells were centrifuged at 160xg for 7 min, resuspended in RPMI with N,N-dimethylformamide (100 mM) and incubated for 6 days to allow differentiation into polymorphonuclear leucocytes (PMN’s). At day 4 BrdU and thymidine were added to the culture to final concentrations of 10 µM and 33 nM respectively, to allow the cells to incorporate BrdU during the last 2 days of incubation. S. epidermidis was cultured in TSB with 10 µM BrdU and 33 nM thymidine for 4 h. Twenty µl of this bacterial suspension was opsonized with 10 µl 0.05% inactivated pooled human serum for 30 min at 37°C. Ten µl of rabbit complement (Harlan, Horst, The Netherlands) and 40 µl of differentiated HL60 cells were added to the bacterial suspension, and the mixture was incubated shaking for 25 min at 37°C. Phagocytosis was stopped by placing the mixtures on ice.

The assay was performed with HL60 cells in the presence and absence of S. epidermidis (1x10$^7$ cfu) and with and without the addition of BrdU. Fourty µl of the samples were transferred to epoxy-coated slides, fixed with 2% paraformaldehyde (Merck, Schuchart, Hohenbrunn, Germany), and stained for detection of BrdU as described below. Sections were counterstained with haematoxylin and inspected by light microscopy.

Catheters and surrounding tissue from deceased patients. Tissue samples of deceased patients were obtained in a study described previously. The need for review by the Institutional Review Board of studies involving deceased patients was waived by the Dutch Central Committee involving Human Subjects (CCMO), provided that informed consent for autopsy and removal and preserving of tissues
for scientific research was given by the next of kin, in accordance with Dutch law.

Catheters with surrounding tissue were explanted from the corpses as described. In brief, with a carefully controlled procedure a biopsy was excised (approximately 2-3 cm in diameter and 4-8 cm in length), which contained a catheter segment with its subcutaneous and deeper surrounding tissue. Sample processing was performed in a laminar flow cabinet, where the tissue was briefly homogenized. To 100 µl of the homogenate 50 µl TSB was added with BrdU and thymidine at final concentrations of 10 µM and 33 nM, respectively, and the mixture was incubated shaking o/n at 37°C. The remainder of the homogenate was processed further for quantitative culture. After overnight incubation the samples from patients with positive cultures were used to prepare slides for BrdU detection. Ten µl of sample was transferred to epoxy-coated slides, fixed with 2% paraformaldehyde and stained for light detection of BrdU as described below.

BrdU incorporation in *S. epidermidis* bacteria in a mouse BAI model. Mouse BAI studies were approved by the Animal Ethical Committee of the University of Amsterdam and were performed as described earlier. In a pilot experiment, 4 mice were anesthetized with FFM-mix (1 ml fentanylcitrate, 1 ml midazalam, and 2 ml distilled water) and a 1 cm long polyvinylpyrrolidone-grafted Silicon Elastomer (SEpvp, 2.5 mm diameter, wall thickness 0.6 mm: Medtronic PS Medical, Goleta, CA, USA) catheter segment was implanted subcutaneously on each side of the back. The incisions were closed with a single stitch (vicryl 6/0) and the bacterial inoculum (1x10^7 cfu in 25 µl saline) was injected subcutaneously along the implanted segments with a repetitive injector (Stepper, model 4001-025, Tridak division, Brookfield, CT). On day 2, two mice were injected with 50 µl 10 µM BrdU / 33 nM thymidine, and 2 mice with 50 µl 0.9% NaCl injection subcutaneously along each implanted segment. One BrdU-injected and one control mouse was sacrificed after 4 h, whereas the other two were sacrificed after 24 h as described below.

Subsequently, three groups of 9 mice anesthetized with FFM-mix received SEpvp catheter segments as described above and a bacterial inoculum of 1x10^6 cfu in 25 µl saline was injected alongside each implanted segment. Previous studies showed that after infection with this inoculum size only small numbers of bacteria are cultured from the mice after 14 days. When such bacteria are dividing in situ, they should incorporate BrdU, and should be detectable after immunostaining for BrdU.
We hypothesized that treatment of mice with the immunosuppressive agent dexamethasone starting 14 days after challenge might result in reactivation or relapse of the infection, and that multiplying bacteria would incorporate BrdU. Therefore, one group of 9 mice was sacrificed after 14 days, to determine the number of cfu present at this time point of the experiment. The other two groups of mice received subcutaneous 50 µl BrdU / thymidine injections at the implantation sites every 2 days between day 14 and day 21. One of these groups received daily intraperitoneal injections of dexamethasone (3 mg/kg) and the control group daily saline injections.

At day 21 mice were sacrificed under full anesthesia. Prior to cardiac punction a standardized biopsy of 12 mm in diameter was taken from each implantation site, comprising the implant with surrounding tissue. The right side biopsy was used for quantitative culture; the left side biopsy was cut in 2 halves, 1 for quantitative culture and 1 for histology. The implants were rinsed and then sonicated for 30 s in 500 µl saline. The sonicate was cultured quantitatively by plating 6 aliquots of 10 µl of the undiluted sonicate (detection limit of 5 cfu) and of ten-fold serial dilutions of the sonicate. The implant itself was placed in 80 ml liquid Brewer Tween broth (BT; 3% (w/v) thioglycolate broth containing 0.03% (w/v) polyanetholesulfonic acid, and 0.5% Tween 80, adjusted to pH 7.6 with 1M NaOH). Cultures were incubated for up to 14 days at 37°C. When plate cultures were negative while the corresponding BT culture was positive, we considered the implants to have been colonized with 5 cfu of bacteria. The tissue samples were homogenized and cultured quantitatively. In addition, 1/10 volume of the homogenate was cultured in 80 ml BT for up to 14 days at 37°C. If this BT culture was positive and the plate cultures were negative, the total homogenate was considered to have contained 10 cfu of bacteria, since one tenth of the homogenate had been cultured. Positive BT cultures of any of the implants or tissue samples were streaked on blood agar plates which were incubated overnight at 37°C. 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.

For histological confirmation of the microbiological findings, mouse tissue biopsies were fixed in formaldehyde and embedded in plastic (methylmethacrylate/ butylmethacrylate (MMA/BMA); Merck Schuchart, Hohenbrunn, Germany). Sections of 5 µm were cut with a microtome, transferred to microscopic slides, deplastified, fixed in paraformaldehyde and rinsed with Tris / HCl buffer [50 mM, pH 7.4] for 15 min.
BrdU staining for light microscopy. The BrdU detection kit (BD Biosciences) was used according to the manufacturer’s protocol. Briefly, slides were incubated in fixation and diluent buffer (BrdU detection kit, BD Biosciences), for membrane permeabilization, followed by an incubation in 10 µl 3% H₂O₂ and RetrievAgen A working solution (BrdU detection kit, BD Biosciences) at 89°C. Slides were cooled to room temperature, rinsed and incubated with anti-BrdU-biotin for 1 h, and subsequently with streptavidine-HRP for 30 min. Bound antibodies were visualized with 3,3’ Diaminobenzidine (DAB) substrate and slides were inspected by light microscopy for characteristic dark brown staining. This procedure was also performed with *Escherichia coli* strain ML35, and with UV-killed *S. epidermidis* strain RP62a, as positive and negative controls, respectively. Sections were counter-stained with hematoxylin-eosin (HE) or gram stained.

Staining for fluorescence microscopy. BrdU detection was performed as described above, with anti-BrdU-alexa-488. Sections were also stained with anti-Lipoteichoic Acid (anti-LTA; QED, Bioscience Ltd. San Diego, CA, USA) or by a combination of anti-BrdU and anti-LTA. Anti-LTA was labeled with a Zenon-alexa-fluor-488 mouse IgG1 labeling kit (Invitrogen-Molecular Probes, Breda, The Netherlands), or covalently linked with alexa-568 (Invitrogen-Molecular Probes), according to the manufacturer’s protocols. The labeled antibodies were applied to the slides at a concentration of 2.5 µg/ml and slides were incubated for 60 min at room temperature.

For combined staining slides were incubated for 1 h with anti-LTA-alexa-568, rinsed and incubated for 10 min with RetrieveAgen solution in a 89°C waterbath. Slides were allowed to cool to room temperature in approximately 20 min, rinsed and then incubated for 1 h with anti-BrdU-alexa-488 in a humid chamber. All slides were mounted with 15 µl vectashield hard set mounting medium containing DAPI (Brunswig Chemie, H1500) to visualize DNA, and inspected using a Leica SP2 confocal microscope (Leica, Rijswijk, the Netherlands).

RESULTS

BrdU incorporation and detection in *S. epidermidis* RP62a *in vitro* and after opsonophagocytosis by HL-60 cells. To determine whether BrdU incorporation
takes place in *S. epidermidis*, strain RP62a bacteria were incubated with BrdU for two hours, and incorporation of BrdU was investigated by immunomicroscopy with anti-BrdU-HRP. A positive signal (brown staining of the bacteria) was observed only when bacteria had been grown in the presence of BrdU, and anti-BrdU was used in the staining procedure (Table 1). No staining was observed when heat-killed *S. epidermidis* were used. *E. coli*, the positive control, also was stained due to BrdU incorporation. These results show that viable *S. epidermidis* were able to incorporate BrdU, while killed *S. epidermidis* did not, and that immunohistochemical detection was possible.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Treatment</th>
<th>Result</th>
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<tbody>
<tr>
<td></td>
<td>BrdU (+/-)</td>
<td>Anti-BrdU (+/-)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heat killed <em>S. epidermidis</em></td>
<td>+</td>
<td>-</td>
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<tr>
<td><em>E. coli</em></td>
<td>+</td>
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Table 1: Labelling of *S. epidermidis* with BrdU. Bacteria were incubated with or without BrdU for 4 h, and slides were treated with or without anti-BrdU-biotin, incubated with streptavidin-HRP and DAB-substrate. Brown stained bacteria were positive for incorporation of BrdU. As a control, slides were gram stained, to verify presence of bacteria on the slides.

Subsequently, *S. epidermidis* RP62a grown in the presence of BrdU was stained with both anti-LTA-Zenon-alexa-594 and anti-BrdU-alexa-488. Slides were inspected by confocal microscopy. The confocal image showed fluorescently labelled bacteria due to anti-BrdU (green, Figure 1A) and anti-LTA (red, Figure 1B) binding, with DNA stained blue due to the presence of DAPI in the mounting medium (Figure 1C). In the overlay (Figure 1D) all colours merged, showing that the bacteria had incorporated BrdU. Bacteria incubated without BrdU only showed red and blue fluorescence (not shown).

Due to the heating step required to denature the DNA in order to make it accessible for anti-BrdU, the anti-LTA signal was rather weak and had to be amplified. As this most likely was due to dissociation of the anti-LTA-Zenon-alexa-594 complex, anti-LTA covalently coupled to alexa-568 was used in further experiments.
As eukaryotic cells also incorporate BrdU into their DNA, we examined whether replicating *S. epidermidis* could be identified when they were incubated with HL60 cells. HL60 cells were grown in presence and absence of bacteria, and of BrdU. Both BrdU-labeled HL60 cells and BrdU labeled bacteria were detected when incubated with anti-BrdU and streptavidine-HRP (Table 2) and bacteria could be well discriminated from the host cell nuclei (not shown).
S. epidermidis identified by their ability to incorporate BrdU

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BrdU-HRP</td>
<td>No anti-BrdU-HRP</td>
</tr>
<tr>
<td>HL60 cells with BrdU</td>
<td>S. epidermidis with BrdU</td>
</tr>
<tr>
<td>HL60 cells without BrdU</td>
<td>S. epidermidis with BrdU</td>
</tr>
</tbody>
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Table 2: Opsonophagocytosis results; HL60 cells and bacteria were incubated with or without BrdU and detection with anti-BrdU-biotin and streptavidine-HRP and visualized by DAB substrate.

Figure 2: Microscopic analysis of homogenate of the peri-catheter tissue of a deceased patient. Homogenates were incubated o/n with BrdU, and BrdU incorporation was investigated by immunomicroscopy. Brown staining indicates the presence of bacteria that have incorporated BrdU (arrowheads).
Bacterial presence and growth in homogenates of tissue surrounding catheters from deceased patients. Catheter and surrounding tissue removed under axenic conditions from deceased patients were separated. Tissue segments were homogenized briefly to allow BrdU penetration while preserving most of the tissue cell structures. Under light immunomicroscopy, slides prepared from homogenates after overnight incubation with BrdU showed positively stained cocci (Figure 2, arrowheads, representative for both patients), proving that BrdU had been incorporated and that bacteria had replicated in situ during the overnight incubation. The remainder of the tissue was not stained, showing that no aspecific binding had taken place and that bacteria could clearly be discriminated in these ex vivo tissue homogenates.

BrdU incorporation by *S. epidermidis* in experimental BAI. Sections were prepared of mouse biopsies which had received either BrdU or saline at 3 days after challenge, and which were sacrificed one day later. Alternating slides were stained by gram and fluorescent staining. Gram-positive stained bacteria were seen both at the biomaterial-tissue interface and within the tissue surrounding the implants, often in association with cells (Figure 3A-C). In confocal microscopy slides, stained with anti-BrdU-alexa-488 and anti-LTA-alexa-568, showed particles stained green and red, respectively (Figure 3D and E), which in overlay were yellow (Figure 3F), thus confirming the presence of bacteria which had replicated within the host tissue.

Incorporation of BrdU by *S. epidermidis* in vivo after immunosuppression of mice with dexamethasone. Next we assessed whether *S. epidermidis* were still viable and able to replicate 14 to 21 days after inoculation in the mouse BAI model, and whether replication would be enhanced if the mice were immunosuppressed by dexamethasone treatment. Twenty-seven mice carrying biomaterial implants were challenged with *S. epidermidis*. After 14 days 9 mice were sacrificed to determine the level of colonization prior to the start of dexamethasone administration. Nine of the 18 tissue biopsies yielded growth, whereas only 1 of the 18 implants was culture positive (Figure 4). Between 14 and 21 days the remaining 18 mice received BrdU injections and either dexamethasone or saline injections. In the group of 9 mice that had received BrdU as well as dexamethasone injections, 10 of 18 tissue biopsies and 2 of 18 biomaterial segments yielded bacterial growth after 21 days. In the
control group, which had received BrdU and saline, 16 of 18 tissue biopsies and 1 of 18 biomaterial segments yielded growth.

Figure 3: Microscopic analysis of biopsies of mice with experimental biomaterial-associated infection injected with BrdU. A-C: Gram staining of representative sections of biopsies from mice sacrificed at 4 and 24 h after bacterial challenge. D-E: Confocal microscopy image of a mouse biopsy slide stained with anti-BrdU (3D) and anti-LTA (3E); 3F is an overlay of 3D and 3E.
Chapter 5

In all groups the tissue biopsies were significantly more often culture positive than the corresponding biomaterial segments. Dexamethasone did not significantly influence the frequency of culture positive tissue biopsies and biomaterial segments compared to controls receiving saline (Figure 4, 21 days), but the numbers of cfu in the tissue had increased significantly compared to the numbers at day 14 (p = 0.007), when dexamethasone and BrdU administration were started. The number of positive cultures of the mice treated with BrdU and saline had also increased compared to the number at 14 days (p = 0.03).

![Figure 4: Effect of Dexamethasone on biomaterial and tissue colonization by S. epidermidis RP62a in the mouse model. Frequencies of positive cultures and numbers of cfu recovered from tissue biopsies (T) and biomaterial implants (BM) of C57Bl/6 mice, 14 and 21 days after challenge with 1x10^7 cfu of S. epidermidis RP62a are indicated. Frequencies of positive cultures are given above the lanes. * indicates p < 0.05.](image)

In microscopic slides of biopsies of the mice sacrificed after 14 days, gram-positive bacteria were observed in the tissue (not shown). Positive staining for BrdU was observed in samples of both groups that had received BrdU injections (Figure 5).
This showed that BrdU allows detection of replicating *S. epidermidis* in our mouse model. Moreover, the results show that *S. epidermidis* present in tissue surrounding implants had replicated between 14 and 21 days after challenge.

**DISCUSSION**

We previously showed that *S. epidermidis* is present in high numbers in tissue surrounding implants in experimental biomaterial-associated infection in mice \(^7\text{-}^{11}\) as well as around catheters in deceased patients \(^1\). In the present study, we assessed whether *S. epidermidis* is able to replicate within the tissue surrounding an implant. The BrdU technique was used to label replicating bacteria *in vitro* as well as *in vivo*. 

![Figure 5: Light microscopy of biopsy slides of mice which received subcutaneous BrdU injections (top figures); with one group which additionally received intraperitoneal dexamethasone injections (bottom figures). BrdU incorporation is shown by the brown staining.](image)
These replicating bacteria were identified by different immunomicroscopic techniques. In mice, colonization of tissue surrounding implants by \textit{S. epidermidis} increased between 14 and 21 days after challenge when BrdU was given in combination with dexamethasone, but also when it was combined with saline. This indicates that the bacteria were able to replicate while residing within the tissue surrounding the biomaterial implants.

Studies with BrdU have mainly been performed on eukaryotic cells \textsuperscript{19,25-28} and marine \textsuperscript{29,30} and soil bacteria \textsuperscript{31-33}. To our knowledge this is the first study where BrdU incorporation is used to detect replicating \textit{S. epidermidis}. In line with our earlier studies, \textit{S. epidermidis} was mainly found in the tissue surrounding implants, more than on the implants themselves \textsuperscript{7-9}. Previously, \textit{S. epidermidis} has been detected by fluorescence microscopy \textsuperscript{37,38}, detection of green fluorescent protein expressed in the bacteria \textsuperscript{17,18}, bacterial luminescence \textsuperscript{20,21}, PCR \textsuperscript{14-16} and culture techniques \textsuperscript{12,13}. Using these methods either the presence or the location of bacteria can be determined, but not whether they are able to replicate at a certain place and time during infection. Using BrdU, the start of incorporation can be timed and bacteria can be detected at cellular levels by microscopy.

In order to assess whether BrdU-incorporating bacteria could also be identified in host cells and tissues, we first assessed that BrdU-labelled \textit{S. epidermidis} could be discriminated from BrdU-labelled HL60 cells. Subsequently, we investigated peri-catheter tissue of deceased patients. We previously investigated whether peri-catheter tissue is an additional niche for bacteria potentially causing catheter-associated infections \textsuperscript{1}. In that study, catheters and surrounding tissue were excised from deceased patients under axenic conditions, quantitatively cultured and examined by histology and immunohistochemistry. In 26\% of the cases, the peri-catheter tissue samples were positive in culture, whereas the corresponding catheter samples yielded lower numbers of bacteria or were culture negative. This indicates that the tissue surrounding these biomedical devices forms a niche for bacteria. In the present study, we detected BrdU incorporation in bacteria within tissue from deceased patients from the study described above. This showed that viable bacteria present in the tissue can be identified with this approach. Furthermore, this is additional evidence that the tissue surrounding implants is indeed an important niche for bacteria.

To assess viability of \textit{S. epidermidis} in the mouse BAI model after 14 days,
S. epidermidis identified by their ability to incorporate BrdU

and the potential infection-enhancing effects of local immune suppression when bacterial numbers are low, we challenged mice with a small inoculum of $1 \times 10^6$ cfu S. epidermidis RP62a. As shown earlier $^9,^{11}$, after 14 days only few bacteria persisted $^{11}$. This was considered the optimal starting point to suppress the immune system in order to reactivate the infection. In addition to BrdU, dexamethasone, a potent immunosuppresser $^39$ was therefore administered, while control mice received BrdU and saline. No significant differences in bacterial culture results were recorded in the mice treated with BrdU and saline compared to mice treated with BrdU and dexamethasone. There was however a significant increase in numbers of cfu in the BrdU with dexamethasone group, and in the frequency of culture positive samples in the BrdU with saline group, respectively, compared to the group sacrificed at 14 days. Confocal microscopy of tissue biopsies clearly showed double labelled bacteria which were positive for both LTA and BrdU, an indication that the bacteria had replicated.

Van Diepen et al. $^{40}$ used gamma irradiation to reactivate Salmonella enterica serovar Typhimurium infection in a mouse intestinal infection model, at a time point when bacteria were undetectable by culture. After total body irradiation the numbers of bacteria in liver and spleen increased to numbers similar to those in the primary infection, indicating that by $\gamma$-irradiation, assumed to cause immunosuppression, reactivation of a Salmonella infection can take place. In our study we aimed to cause immune suppression with dexamethasone in order to reactivate the infection. We expected that the infection would not relapse in the mice that were treated with BrdU and saline injections. To the best of our knowledge BrdU has not been reported to suppress immune function. However, in the mice receiving BrdU and dexamethasone as well as in the mice receiving BrdU and saline, increase in bacterial colonization occurred between day 14 to day 21. This may be due to the fact that numbers of S. epidermidis cfu in peri-implant tissue increase over time after 14 days, as we have observed previously $^7$. It can however not be ruled out that both dexamethasone and BrdU exerted an immune suppressive effect, which may have induced the relapse of the infection.

Although not many studies have focussed on colonization of tissue surrounding biomedical implants in patients, there is accumulating evidence supporting a role for the peri-implant tissue as a niche for bacteria. Virden et al. investigated tissue
surrounding breast implants and were able to culture bacteria from the tissue of a patient while the implant itself was culture negative. In cases of infected hip prostheses bacteria were observed within fibroblasts in the bone tissue surrounding the metal prosthesis. As bacteria are present and able to replicate in the tissue and therefore are not all removed by removal of the infected device, the tissue may be a reservoir for reinfection. After removal of infected hip prostheses, prolonged antibiotic treatment is often required before a novel prosthesis can be placed with relatively low risk of relapse of the infection. A role of tissue as a reservoir may apply for other biomedical devices as well, and warrants detailed investigation of tissue around retrieved infected devices.

In conclusion, we developed a method to detect replicating bacteria at microscopic level in animal models of BAI by application of BrdU. Our study shows that S. epidermidis is able to replicate in peri-implant tissue in mice at 14 to 21 days after infection with a small inoculum of S. epidermidis, and that bacteria present in peri-catheter tissue of deceased ICU patients can also replicate. This confirms and extends our previous findings that peri-catheter tissue is a reservoir for bacteria in biomaterial-associated infection.

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REFERENCES


38. Seaver M, Crookston JC, Roselle DC, Wagner SJ. First results using automated epifluorescence microscopy to detect Escherichia coli and Staphylococcus epidermidis in WBC-reduced platelet concentrates. Transfusion 2001; 41: 1351-5.


