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Microscopic Detection of Viable *Staphylococcus epidermidis* in Peri-Implant Tissue in Experimental Biomaterial-Associated Infection, Identified by Bromodeoxyuridine Incorporation▽

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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 bromodeoxyuridine (BrdU), a nucleotide analogue that is incorporated into bacterial DNA and can be detected with antibodies. Infected human tissue was obtained postmortem from patients with intravascular devices, and mouse biopsy specimens were obtained from mice with experimental biomaterial infection. *In vitro* experiments showed that *Staphylococcus epidermidis* incorporated BrdU, as judged from staining of the bacteria with anti-BrdU antibodies. After incubation of bacteria with BrdU and subsequent staining of microscopic sections with anti-BrdU antibodies, bacteria could be clearly visualized in the tissue surrounding intravascular devices of 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 pericatheter 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 immunolabeling with BrdU and BrdU antibodies.

Biomaterial-associated infection (BAI) is 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 (11, 25). Bacteria causing these infections can be present on the implanted material and form a biofilm (17, 23, 43), but they are also found in the surrounding tissue (3, 7–9). 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 (3–5, 7, 8). In the tissue the bacteria are associated with host inflammatory cells and are found in large numbers within macrophages (3), which suggests that these macrophages allow intracellular survival and possibly replication of the bacteria. In mice, tissue-residing *Staphylococcus epidermidis* bacteria persist despite rifampin/vancomycin treatment (7), whereas the antibiotics do clear the bacteria from the implanted biomaterial itself. We also detected viable bacteria in samples of tissue surrounding catheters retrieved from deceased patients (9). The tissue surrounding foreign bodies, therefore, must be considered a niche for bacteria that cause BAI.

The aim of our study was to investigate the spatio-temporal characteristics of the development of the infection at the microscopic level by labeling viable bacteria *in situ*. Available methods to detect and/or localize bacteria include plate and liquid broth culture (13, 36) and various molecular diagnostic techniques (PCR and reverse transcription-PCR [RT-PCR]) (1, 19, 34), whereas to visualize bacteria, methods such as Gram staining, green fluorescent protein (GFP) expression (15, 28), use of bioluminescent markers (12, 20, 42), *in situ* hybridization with probes for RNA or DNA (21, 24), live/dead fluorescent staining (16), bioluminescence imaging using *in vivo* imaging systems (IVIS) (14), and immunohistochemistry analysis (3) have been used. None of these methods, however, allows a timed start of labeling of viable cells combined with the possibility of microscopic detection of viable cells *in situ*. A suitable method to achieve this may be labeling with bromodeoxyuridine (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 (12, 30, 31, 37, 40) and also for detection of replicating bacteria in marine (27, 38) and soil samples (2, 6, 44). It is readily incorporated by *Escherichia coli* in broth culture (18, 35). In the present study we applied BrdU labeling to investigate the presence of viable bacteria in pericatheter tissue of deceased patients and the viability of *S. epidermidis* on implants and in surrounding tissue in a mouse BAI model. We used dexamethasone for immune suppression to assess whether infection in the mice could be resuscitated at 2 weeks after a low-dose challenge with *S. epidermidis*.

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MATERIALS AND METHODS

***S. epidermidis* strains and inoculum preparation.** *S. epidermidis* strain RP62a (ATCC 35984) was used in the mouse experimental biomaterial-associated in-

fection model. Strain RP62a harbors the biofilm-associated *icaA*, *atlE*, *aap*, and *sarA* genes and produces polysaccharide intercellular adhesin (PIA) and δ-toxin (8). MICs according to a standard Etest of strain RP62a were the following (μg/ml): rifampin, <0.016; teicoplanin, 3; gentamicin, 8; and vancomycin, 4. Bacteria retrieved after challenge of mice were always checked for susceptibility to these antibiotics. *S. epidermidis* inocula were prepared by inoculating tryptic soy broth (TSB) with 25 μl of thawed suspension from frozen stocks and incubation for 4 to 5 h at 37°C. After centrifugation and washing of the bacterial pellet with saline, suspensions containing approximately 4×10^8 CFU per ml of saline were prepared based on the optical density at 620 nm (OD_{620}) (4). Suspensions of *E. coli* ML35 (22), used as positive control strain for BrdU incubation in *in vitro* experiments, were prepared in the same way (18, 35).

To assess BrdU incorporation *in vitro*, 150 μl of the bacterial suspension was added to 3 ml of TSB containing 10 μM BrdU and 33 nM thymidine and incubated for 2 h at 37°C. Thymidine was added to suppress thymidilate synthase (18). Ten microliters of this suspension was added to an epoxy-coated glass slide and treated for light-microscopic detection of BrdU as described below. Concentrations of up to 100 μM BrdU did not influence the growth of *S. epidermidis* in TSB.

Catheters and surrounding tissue from deceased patients. Tissue samples of deceased patients were obtained in a study described previously (9). 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. When corpses are refrigerated shortly after death, their microbiological status is expected not to change significantly (10), and viable bacteria present in the tissue may replicate when the tissue is placed at 37°C.

Catheters with surrounding tissue were explanted from the corpses as described previously (9). In brief, with a carefully controlled procedure a biopsy specimen was excised (approximately 2 to 3 cm in diameter and 4 to 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 biopsy specimen was sliced and the catheter segments were removed for separate analysis (9). The tissue was homogenized briefly to allow BrdU penetration while preserving most of the tissue cell structures. To 100 μl of this homogenate 50 μl of TSB was added with BrdU and thymidine at final concentrations of 10 μM and 33 nM, respectively, and the mixture was incubated with shaking overnight at 37°C. The remainder of the homogenate was processed further for quantitative culture (9). After overnight incubation the samples from two patients with positive cultures were used to prepare slides for BrdU detection. Ten microliters of sample was transferred to epoxy-coated slides, fixed with 2% paraformaldehyde, and stained for immunodetection of BrdU by light microscopy, 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 (3–5, 7, 8). In a pilot experiment, four mice were anesthetized with FFM mix (1 ml of fentanylcitrate, 1 ml of midazalam, and 2 ml of distilled water), and a 1-cm-long segment of polyvinylpyrrolidone-grafted silicon elastomer (SEpvp) catheter (diameter, 2.5 mm; wall thickness, 0.6 mm [Medtronic PS Medical, Goleta, CA]) was implanted subcutaneously on each side of the back. The incisions were closed with a single stitch (Vicryl 6/0), and the bacterial inoculum (10^7 CFU in 25 μl of saline) was injected subcutaneously along the implanted segments. On day 2, along each implanted segment two mice were injected subcutaneously with 50 μl of 10 μM BrdU–33 nM thymidine, and two mice were injected with 50 μl of 0.9% NaCl. One BrdU-injected mouse and one control mouse were sacrificed after 4 h, whereas the other two mice were sacrificed after 24 h, as described below.

Subsequently, three groups of nine mice anesthetized with FFM mix received SEpvp catheter segments, and a bacterial inoculum of 10^6 *S. epidermidis* CFU in 25 μl of saline was injected alongside each implanted segment. Previous studies showed that after infection with this size of inoculum, only small numbers of bacteria are cultured from the mice after 14 days (4). 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 nine 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 injections of 50 μl of BrdU-thymidine at the implantation sites every 2 days between day 14 and day 21 under mild anesthesia (isoflurane/oxygen). One of these groups received daily intraperitoneal injections of dexamethasone (3 mg/kg of body weight), and the other (control) group received saline injections.

At day 21 the mice were sacrificed under full anesthesia. Prior to cardiac puncture a standardized biopsy specimen of 12 mm in diameter comprising the implant with surrounding tissue was taken from each implantation site. The right-side biopsy specimen was used for quantitative culture; the left-side biopsy specimen was cut into halves; one half was used for quantitative culture and the other half used for histology. The implants were rinsed and then sonicated for 30 s in 500 μl of saline. The sonicate was cultured quantitatively by plating six 10-μl aliquots of undiluted sonicate (detection limit of 5 CFU) and of 10-fold serial dilutions of the sonicate. The implant itself was placed in 80 ml of liquid Brewer Tween broth ([BT] 3% [wt/vol] thioglycolate broth containing 0.03% [wt/vol] polyanetholesulfonic acid and 0.5% Tween 80, adjusted to pH 7.6 with 1 M 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 12-mm-diameter tissue samples were homogenized and cultured quantitatively. In addition, a 1/10 volume of the homogenate was cultured in 80 ml of BT broth for up to 14 days at 37°C. If this BT broth culture was positive and the plate cultures were negative, the total homogenate was considered to have contained 10 CFU of bacteria since 1/10 of the homogenate had been cultured. Positive BT broth cultures of any of the implants or tissue samples were streaked on blood agar plates that were incubated overnight at 37°C. The culture results were used to calculate the total numbers of CFU present on the entire implant and in the entire tissue biopsy specimen. Twenty-five microliters of blood was cultured in 80 ml of BT broth for up to 14 days at 37°C. Blood cultures were negative for all samples.

For histological confirmation of the microbiological findings, mouse tissue biopsy specimens were fixed in formaldehyde and embedded in plastic (methylmethacrylate/buthylmethacrylate [MMA/BMA]; Merck Schuchart, Hohenbrunn, Germany). Sections of 5 μm were cut and transferred to microscopic slides. The plastic was removed, and sections were fixed in paraformaldehyde and rinsed with Tris-HCl buffer (50 mM, pH 7.4) for 15 min.

BrdU staining for light microscopy. A 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 of 3% H_2O_2 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 streptavidin-horseradish peroxidase (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 *E. coli* strain ML35 and with UV-killed *S. epidermidis* strain RP62a as positive (18, 35) and negative controls, respectively. Sections were either counterstained with hematoxylin-eosin (HE) or subjected to Gram staining.

Staining for fluorescence microscopy. BrdU detection was performed as described above, with anti-BrdU–Alexa Fluor 488. *S. epidermidis* bacteria cultured in the absence of BrdU were routinely included as controls in experiments with the anti-BrdU–Alexa Fluor 488 and were always negative (data not shown). Sections were also stained with anti-lipoteichoic acid (LTA; QED, Bioscience Ltd. San Diego, CA) (7) to identify *S. epidermidis* and to show that anti-BrdU-positive particles would, indeed, be replicated in *S. epidermidis.* 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 Fluor 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. Anti-LTA specifically binds to Gram-positive bacteria containing LTA in their cell walls. Gram-negative bacteria or uninfected mammalian tissue did not show any signal (data not shown).

For combined staining, slides were incubated for 1 h with anti-LTA–Alexa Fluor 568, rinsed, and incubated for 10 min with RetrieveAgen solution in a 89°C water bath. Slides were allowed to cool to room temperature (in approximately 20 min), rinsed, and then incubated for 1 h with anti-BrdU–Alexa Fluor 488 in a humid chamber. All slides were mounted with 15 μl of Vectashield hard-set mounting medium containing 4′,6′-diamidino-2-phenylindole ([DAPI] H1500; Brunswig Chemie) to visualize DNA and inspected using a Leica SP2 confocal microscope (Leica, Rijswijk, The Netherlands).

TABLE 1. Labeling of *S. epidermidis* with BrdU[a]

Bacterium	Treatment		Result	
	BrdU	Anti-BrdU	Anti-BrdU staining	Gram staining
S. epidermidis	+	+	+	+
S. epidermidis	−	+	−	+
S. epidermidis	+	−	−	+
S. epidermidis	−	−	−	+
Heat-killed *S. epidermidis*	+	+	−	+
E. coli	+	+	+	+

[a] Bacteria were incubated with or without BrdU for 4 h, and slides were treated with or without anti-BrdU–biotin and 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 the presence of bacteria on the slides.

RESULTS

BrdU incorporation and detection in *S. epidermidis* RP62a *in vitro*. To determine whether BrdU incorporation takes place in *S. epidermidis* RP62a, bacteria were incubated with BrdU and thymidine for 2 h, 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 when anti-BrdU was used in the staining procedure (Table 1). No staining was observed when heat-killed *S. epidermidis* cells were used. *E. coli*, the positive control, also was stained as a result of BrdU incorporation.

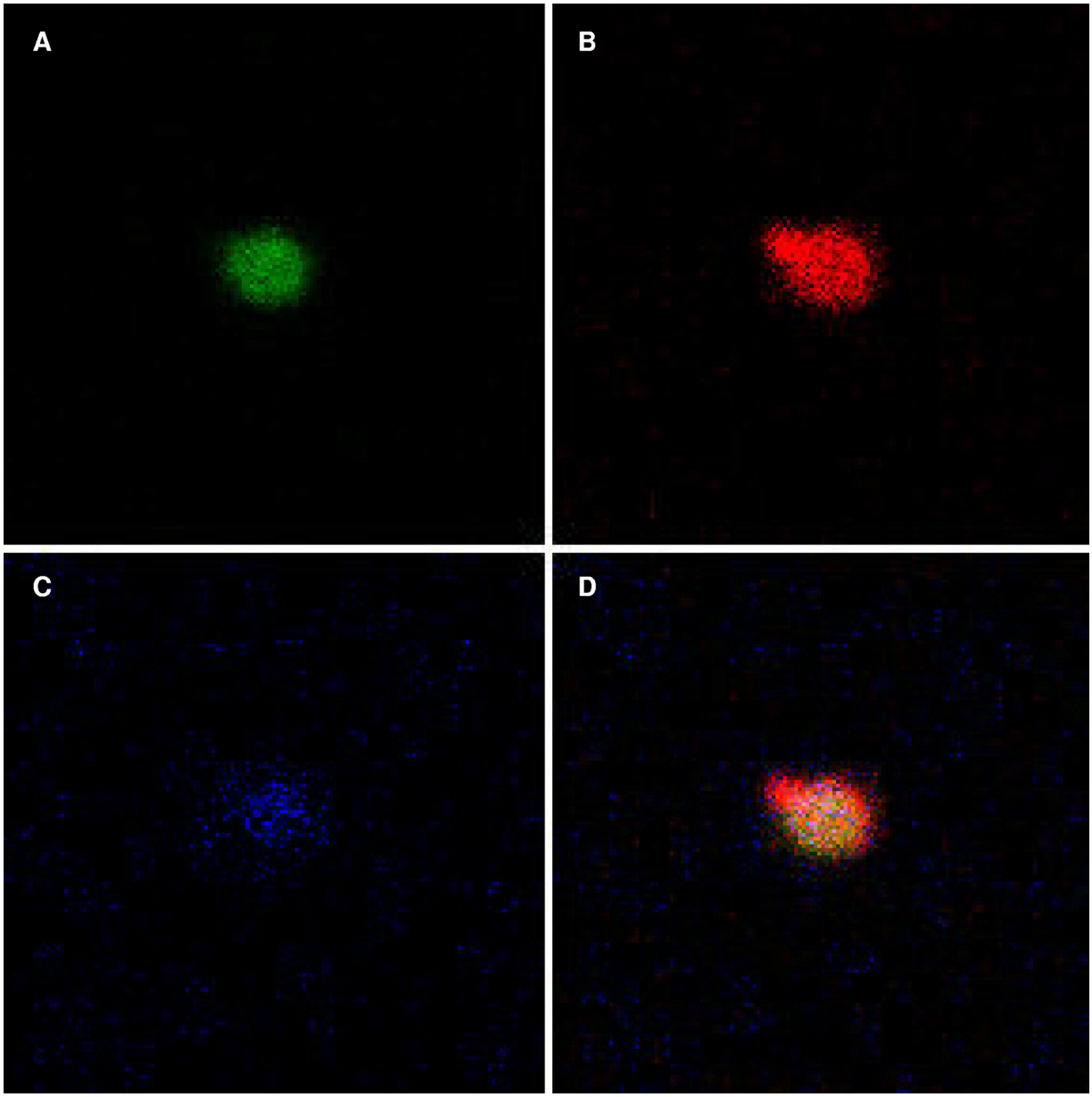

FIG. 1. Confocal image of *S. epidermidis* RP62A grown with BrdU, stained with anti-BrdU–Alexa Fluor 488 (green signal; A) and anti-LTA–Zenon Alexa Fluor 594 (red signal; B). DNA is stained blue by the DAPI included in the Vectashield mounting medium (C). An overlay of all the fluorescence channels (D) clearly shows full overlap of all signals.

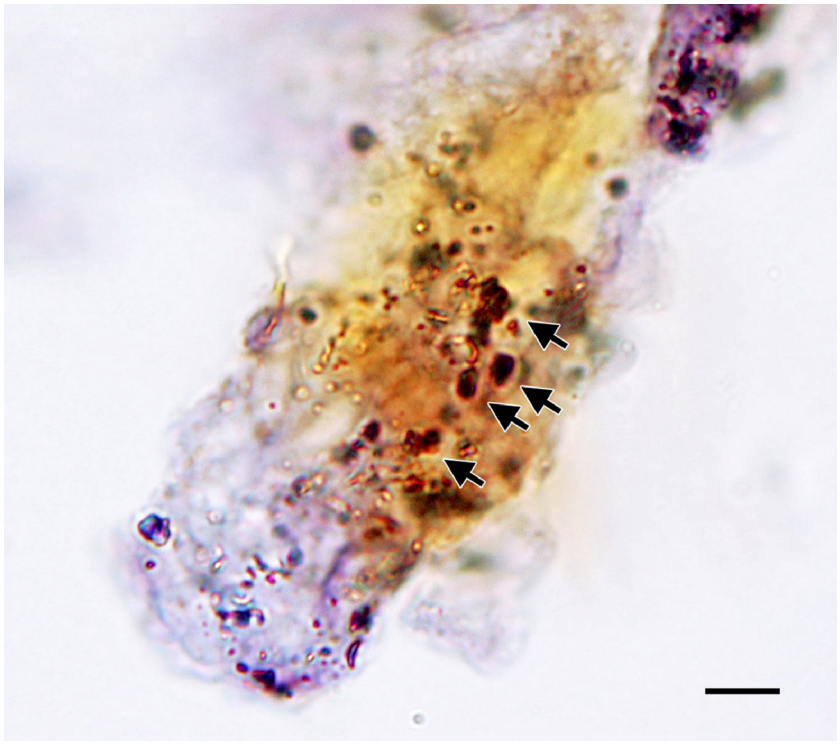

FIG. 2. Microscopic analysis of homogenate of the pericatheter tissue of a deceased patient. Homogenates were incubated overnight with BrdU, and BrdU incorporation was investigated by immunomicroscopy. Brown staining indicates the presence of bacteria that have incorporated BrdU (arrowheads). Bar, 10 μm.

Subsequently, *S. epidermidis* RP62a cells grown in the presence of BrdU were stained with both anti-LTA–Zenon Alexa Fluor 594 and anti-BrdU–Alexa Fluor 488. Slides were inspected by confocal microscopy. The confocal image showed fluorescently labeled bacteria due to anti-BrdU (Fig. 1A, green) and anti-LTA (Fig. 1B, red) binding, with DNA stained blue due to the presence of DAPI in the mounting medium (Fig. 1C). In the overlay (Fig. 1D) all colors merged, showing that the bacteria had incorporated BrdU. Bacteria incubated without BrdU showed only red and blue fluorescence (data not shown). As a result of 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 Fluor 594 complex, anti-LTA covalently coupled to Alexa Fluor 568 was used in further experiments.

Bacterial presence and growth in homogenates of tissue surrounding catheters from deceased patients. Slides of pericatheter tissue from biopsy specimens obtained from deceased patients incubated overnight with BrdU-thymidine showed positively stained cocci in immunomicroscopy with anti-BrdU (Fig. 2, arrowheads; the result is representative for both patients), proving that BrdU had been incorporated and that bacteria had replicated *in situ*. The tissue was not stained, showing that no nonspecific binding had taken place and that viable bacteria could clearly be discriminated in these ex vivo tissue samples.

BrdU incorporation by *S. epidermidis* in experimental BAI. Sections were prepared of biopsy specimens of mice that had received either BrdU or saline at 3 days after challenge and were sacrificed 1 day later. Alternating slides were stained by Gram and fluorescent staining. Gram-positive bacteria were seen both at the biomaterial-tissue interface and within the tissue surrounding the implants, often in association with cells (Fig. 3A to C). In confocal microscopy, slides treated with anti-BrdU–Alexa Fluor 488 and anti-LTA–Alexa Fluor 568 showed particles the size of bacteria which were stained green and red, respectively (Fig. 3D and E). In the overlay, these particles were yellow (Fig. 3F), thus confirming that they were *S. epidermidis* cells which had replicated within the host tissue *in vivo*.

Incorporation of BrdU by *S. epidermidis in vivo* after immune suppression of mice with dexamethasone. Next, we assessed whether *S. epidermidis* bacteria were still viable and able to replicate between 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 nine mice were sacrificed to determine the level of colonization prior to the start of dexamethasone administration. Nine of the 18 tissue biopsy specimens yielded growth, whereas only 1 of the 18 implants was culture positive (Fig. 4). Between 14 and 21 days the remaining 18 mice received BrdU injections combined with either dexamethasone or saline injections. In the group of nine mice that had received BrdU as well as dexamethasone injections, 10 of 18 tissue biopsy specimens and 2 of 18 implants yielded bacterial growth after 21 days. In the control group,

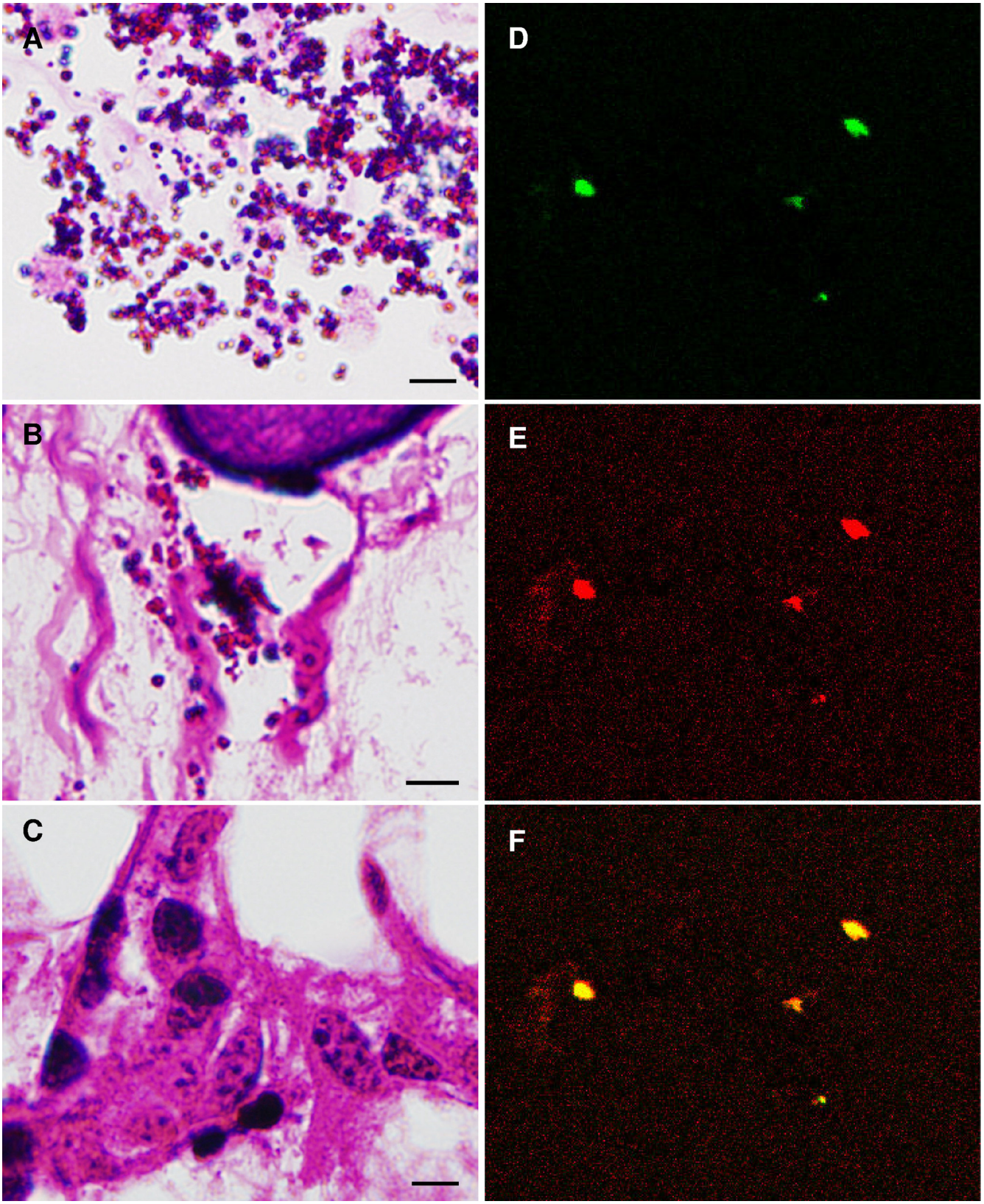

FIG. 3. Microscopic analysis of biopsy specimens of mice with experimental biomaterial-associated infection injected with BrdU. (A to C) Gram staining of representative sections of biopsy specimens from mice sacrificed at 4 and 24 h after bacterial challenge. (D and E) Confocal microscopy image of a mouse biopsy specimen slide stained with anti-BrdU (D) and anti-LTA (E). (F) Overlay of the images shown in panels D and E. Bar, 10 μm.

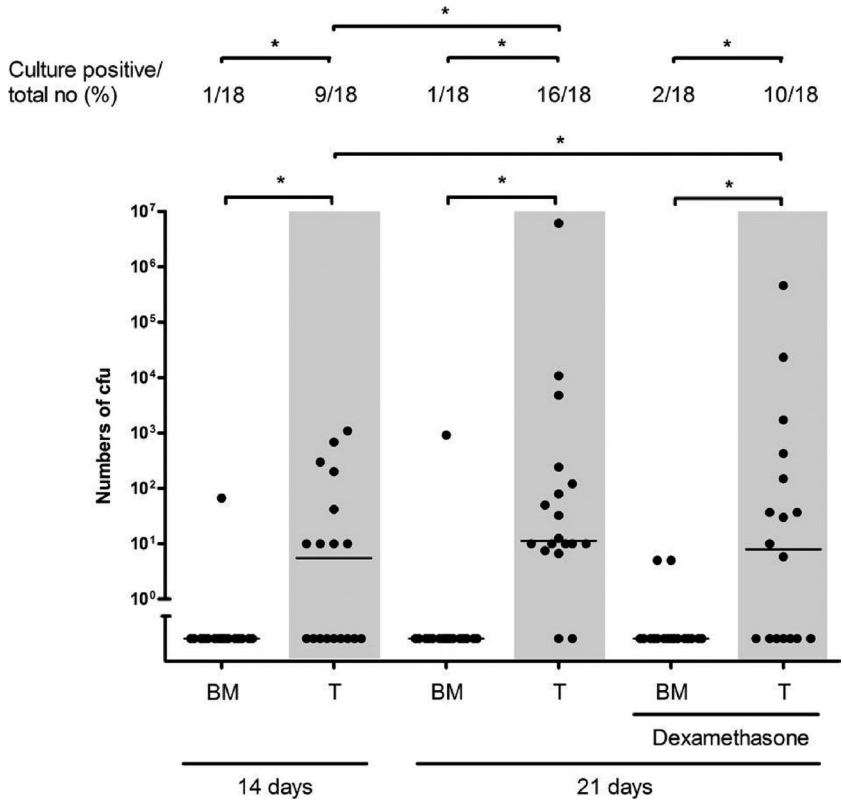

FIG. 4. Effect of dexamethasone on biomaterial and tissue colonization by *S. epidermidis* RP62a in the mouse model. Frequencies of positive cultures and total numbers of CFU cultured from the 12-mm-diameter tissue biopsy specimens (T) and from the biomaterial implants (BM) of C57BL/6 mice at 14 and 21 days after challenge with 1×10^7 CFU of *S. epidermidis* RP62a are indicated. Frequencies of positive cultures are given above the lanes. *, $P < 0.05$.

which had received BrdU and saline, 16 of 18 tissue biopsy specimens and 1 of 18 implants yielded growth.

In all groups significantly more tissue biopsy specimens than corresponding implants were culture positive. In mice treated with BrdU and dexamethasone as well as in mice that had received BrdU and saline, the numbers of CFU in the tissue at 21 days were significantly higher than at day 14 ($P = 0.007$) (Fig. 4) when dexamethasone and BrdU administration had been started. In mice treated with BrdU and saline, the number of culture-positive tissue biopsy specimens had increased between 14 and 21 days ($P = 0.03$).

In microscope slides of biopsy specimens of the mice sacrificed after 14 days, Gram-positive bacteria were observed in the tissue (data not shown). Positive staining for BrdU was observed in samples of both groups that had received BrdU injections (Fig. 5). This showed that BrdU allows detection of replicating *S. epidermidis in situ* in the mouse model. Moreover, the results show that *S. epidermidis* bacteria were not just surviving within the tissue surrounding implants but that they had actually 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 (3–5, 7, 8) as well as around catheters in deceased patients (9). In the present study, we used BrdU to be able to microscopically detect replicated bacteria *in situ*. Indeed, replication of bacteria was shown to occur within tissue surrounding catheters from deceased intensive care unit (ICU) patients. In our mouse BAI model we subsequently showed, both by CFU counts and BrdU incorporation, that *S. epidermidis* replicated within peri-implant tissue between 14 and 21 days after challenge. These data provide evidence that the bacteria were not just persisting but were actually able to multiply while residing within the tissue surrounding the biomaterial implants.

Studies with BrdU have mainly been performed on eukaryotic cells (12, 30, 31, 37, 40) and marine (27, 38) and soil bacteria (2, 6, 44). To the best of our knowledge, this is the first study where BrdU incorporation has been used to detect replicating *S. epidermidis*. Previously, *S. epidermidis* bacteria have been detected by fluorescence microscopy (29, 32), green fluorescent protein expressed in the bacteria (15, 28), bacterial luminescence (20, 42), PCR (1, 19, 34), and culture techniques (13, 36). None of the above described methods, however, allows the possibility of microscopic detection *in situ* by labeling viable cells at specific time points. A new method used to study *Staphylococcus aureus* biomaterial-associated infection is bioluminescence imaging using the IVIS imaging system. This is a powerful method that allows infection to be monitored longitudinally and nondestructively in the same animal throughout the duration of an experiment but does not offer the possibility

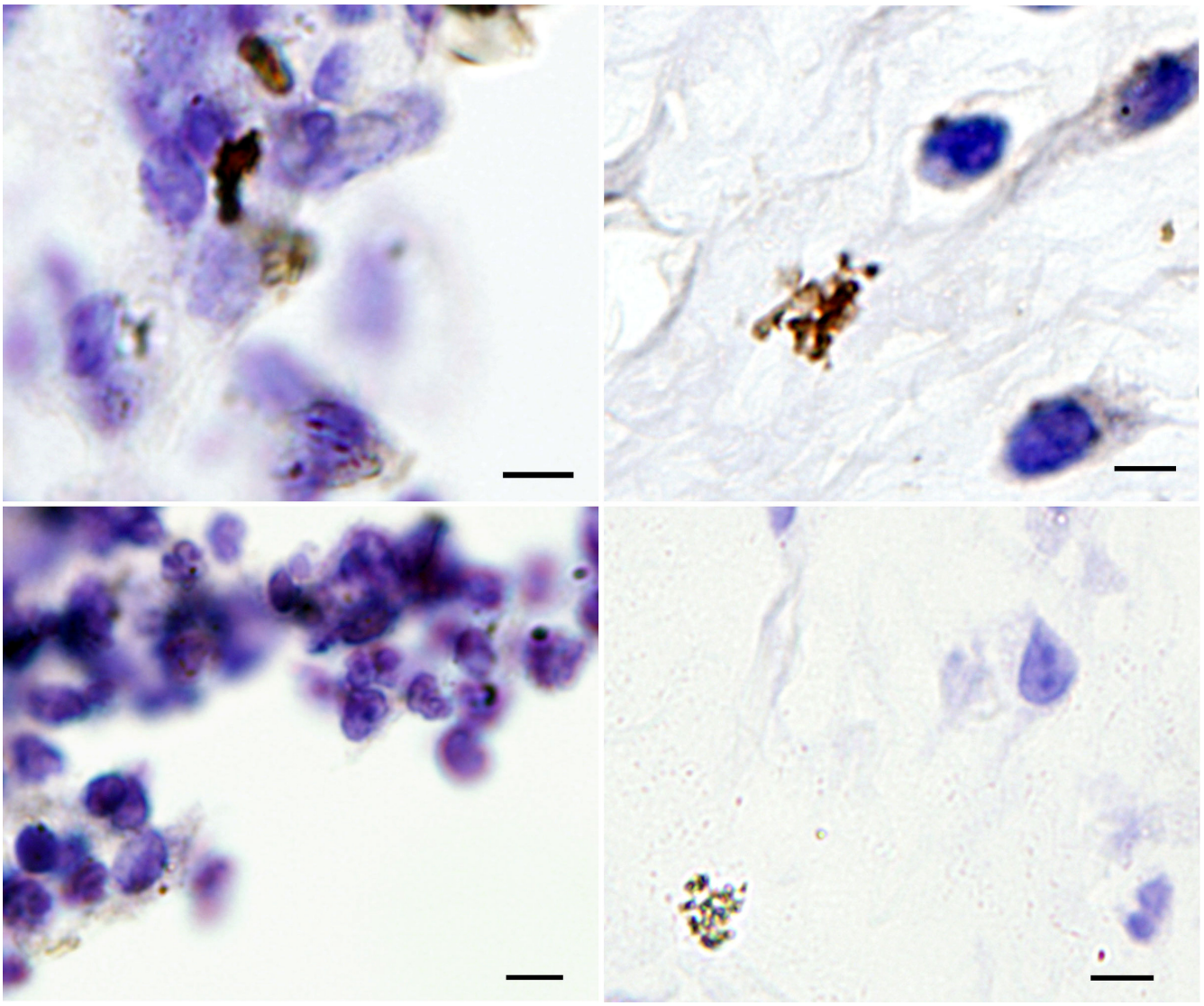

FIG. 5. Light microscopy of biopsy specimen slides of mice that received subcutaneous BrdU injections (top panels) and of one group of mice that additionally received intraperitoneal dexamethasone injections (bottom panels). BrdU incorporation is shown by the brown staining. Bar, 10 μm.

of microscopic detection of pathogens (14). BrdU incorporation thus complements the above methods. It allows studies of the spatio-temporal distribution of viable bacteria at the cellular level during the infectious process.

In order to assess whether BrdU-incorporating bacteria could also be identified in host cells and tissues, we investigated pericatheter tissue of deceased patients. We previously reported that this tissue is an additional niche for bacteria potentially causing catheter-associated infections. In 26% of the cases, the pericatheter tissue samples were positive in culture, whereas the corresponding catheter samples yielded lower numbers of bacteria or were culture negative (9). In the present study, we microscopically detected BrdU incorporation ex vivo in bacteria within tissue from deceased patients from the study described above. This shows that viable bacteria present in the tissue can be identified and localized by this approach. Furthermore, this is additional evidence that tissue surrounding a foreign body is, indeed, a niche for bacteria.

van Diepen et al. (39) 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 *S. epidermidis* infection. We challenged mice with a small inoculum of 10^6 CFU of *S. epidermidis* RP62a since after 14 days only a few *S. epidermidis* bacteria persist (4, 8). This, then, was considered the optimal starting point to suppress the immune system in order

to reactivate the infection. However, numbers of *S. epidermidis* CFU increased not only in the dexamethasone group but also in the controls receiving only BrdU with saline. Confocal microscopy of tissue biopsy specimens clearly showed double-labeled bacteria which were positive for both LTA and BrdU, indicating that the bacteria had replicated. The increase in numbers of bacteria may have been due to the fact that *S. epidermidis* can multiply in peri-implant tissue even without immune suppression (3). Alternatively, an immune-suppressive effect of BrdU might have contributed to survival although we are not aware of reports describing immune suppression by BrdU.

Evidence of the importance of bacterial colonization of tissue surrounding biomedical implants in biomaterial-associated infection is increasing. Recent studies using IVIS have indicated that *S. aureus* colonizes the tissue surrounding abdominal wall meshes implanted in a mouse infection model (14). Virden et al. investigated tissue surrounding breast implants and were able to culture bacteria from the tissue of a patient although the implant itself was culture negative (41). In cases of infected hip prostheses, bacteria were observed within fibroblasts in the bone tissue surrounding the metal prosthesis (26). As bacteria are present and able to replicate in the tissue and therefore are not entirely removed by removal of the infected device, the tissue may be a reservoir for reinfection. It is well known that BAI in total hip revisions are often recurrent, particularly if a new implant is inserted too soon and without proper antibiotic treatment. Prolonged antibiotic treatment is often required before a novel prosthesis can be placed with a relatively low risk of relapse of the infection (33). The role of tissue as a reservoir for infection is expected to apply also to other biomedical devices, and this possibility warrants detailed microscopic investigation of tissue around retrieved infected devices and in models of infection.

In conclusion, we developed a method to detect replicating *S. epidermidis* within tissue at the microscopic level by application of BrdU. Our study shows that replicating bacteria are present in pericatheter tissue of deceased ICU patients and that *S. epidermidis* is able to replicate in peri-implant tissue in mice between 14 and 21 days after infection with a small inoculum. This confirms and extends our previous findings that pericatheter tissue is a reservoir for bacteria in biomaterial-associated infection, which can now be studied at the microscopic level using BrdU.

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