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Staphylococcus epidermidis originating from titanium implants infects surrounding tissue and immune cells

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ABSTRACT

Infection is a major cause of failure of inserted or implanted biomedical devices (biomaterials). During surgery, bacteria may adhere to the implant, initiating biofilm formation. Bacteria are also observed in and recultured from the tissue surrounding implants, and may even reside inside host cells. Whether these bacteria originate from biofilms is not known. Therefore, we investigated the fate of *Staphylococcus epidermidis* inoculated on the surface of implants as adherent planktonic cells or as a biofilm in mouse experimental biomaterial-associated infection. In order to discriminate the challenge strain from potential contaminating mouse microflora, we constructed a fully virulent green fluorescent *S. epidermidis* strain. *S. epidermidis* injected along subcutaneous titanium implants, pre-seeded on the implants or pre-grown as biofilm, were retrieved from the implants as well as the surrounding tissue in all cases after 4 days, and in histology bacteria were observed in the tissue co-localizing with macrophages. Thus, bacteria adherent to or in a biofilm on the implant are a potential source of infection of the surrounding tissue, and antimicrobial strategies should prevent both biofilm formation and tissue colonization.

1. Introduction

Medical devices such as prosthetic joints, dental implants, vascular grafts and catheters, collectively referred to as "biomaterials", are increasingly used in modern medicine. Unfortunately, the presence of a biomaterial in host tissue strongly enhances the susceptibility to biomaterial-associated infection (BAI) [1–4]. BAI is mostly caused by staphylococci, in particular by *Staphylococcus epidermidis* and *S. aureus*, and by streptococci, Gram-negative bacilli, enterococci and anaerobes like *Propionibacterium acnes* [5]. Infection of implants or the surrounding tissue may arise due to contamination during the implantation or insertion procedure by bacteria from the skin of the patients [6–8] and from the surgical environment (Fig. 1) [9–11]. Adherence of bacteria to the biomaterial surface and subsequent formation of biofilms are considered major factors in the pathogenesis of BAI [12–16]. Moreover, the presence of the biomaterial, particularly in the presence of bacteria, will cause local derangement of inflammatory responses with ensuing ineffectiveness of neutrophils to clear bacteria [4], and survival of phagocytosed bacteria within host cells [17–22].

Strategies to reduce the risk of contamination include hygiene measures prior to and during surgery [23,24] and single dose systemic peri-operative antimicrobial prophylaxis [25]. When BAI occurs despite these preventive measures, intervention with antibiotics often is ineffective. Reduced susceptibility of sessile bacteria present in biofilms and poor penetration of antibiotics through the biofilm matrix are considered the predominant causes of the limited efficacy of antibiotics against BAI. Treatment of BAI therefore often requires prolonged antibiotic therapy, and in many cases ultimately needs to be combined with adequate surgical intervention, as antibiotics alone are usually only able to suppress, but not eliminate, these infections [5,26].

Another important phenomenon in the pathogenesis of BAI is the survival of bacteria in the tissue surrounding implants, as observed both in experimental BAI [20,27–30] and in patients [8,21,31,32]. Despite the presence of macrophages and granulocytes around an implant, the microorganisms cannot be cleared,

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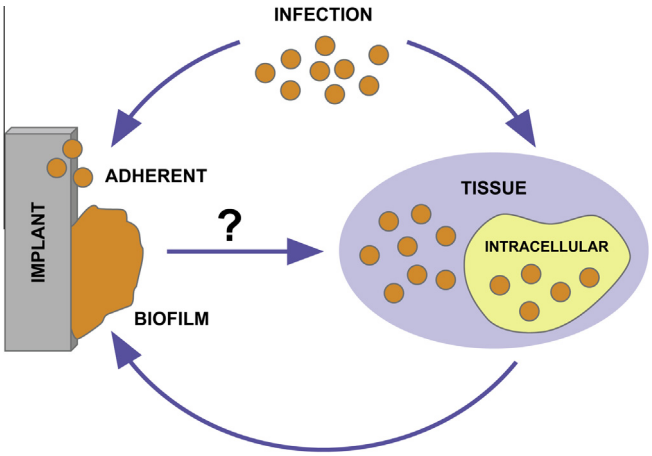

Fig. 1. A schematic representation of the pathogenesis of biomaterial-associated infection. Bacteria contaminating the surgical site enter the tissue surrounding the implant and may become internalized by host cells [4,20], or adhere to the implant and eventually develop a biofilm [12]. Infection of the implant and/or the surrounding tissue arising during surgery is modeled by injection of the *S. epidermidis* bacteria along the biomaterial implant. The development and localization of *S. epidermidis* infection in tissue surrounding the implant starting from bacteria present on a surface is studied by applying bacteria to the implant surface from a suspension, as a model for adherent bacteria, or bacteria pre-grown on the implant surface as a biofilm. Bacteria in tissue can form a reservoir for recurrence of infection after removal of an infected biomaterial and possibly even when present in host cells.

due to the frustrated phagocytosis caused by the implantation of a biomaterial. In revision surgery of prosthetic hips and knees, the presence of bacteria in peri-implant tissue indeed is a risk factor for reinfection [17,18]. Bacteria may even persist in large numbers within host cells, including in macrophages [20,33,34].

In order to design strategies to combat BAI, it is vital to understand the precise mechanism of this pathogenic process of tissue colonization. The aim of the present study therefore was to investigate whether bacteria present on the implant surface – either adherent or as a biofilm – can be the source for colonization of the surrounding tissue. In the mouse experimental BAI model, intraoperative infection of tissue and of the implant itself were modeled by injecting *S. epidermidis* bacteria along subcutaneous titanium implants and by directly applying planktonic bacteria to the implant surface, respectively. Biofilm infection was modeled by the use of implants with a pre-grown biofilm. In order to directly visualize the challenge strain by microscopy and to discriminate it from potential contaminating mouse flora in culture we constructed a GFP-expressing *S. epidermidis* strain for these studies.

2. Materials and methods

2.1. Bacteria and plasmid

The bacteria used in this study were *Escherichia coli* DH5α (Invitrogen), *S. aureus* RN4220 (ATCC 35556) and *S. epidermidis* O-47 [35]. Bacteria were made fluorescent using plasmid WVW189, which is the *E. coli* – staphylococcal shuttle plasmid pALC1484 [36] carrying a constitutive *S. aureus* promoter in front of the gfp_{uvr} gene. The plasmid contains ampicillin and chloramphenicol resistance genes for selection in *E. coli* and staphylococci, respectively [37].

2.2. Transformation

A 50 ml volume of B2 medium [38] was inoculated with 500 µl of an *S. epidermidis* O-47 preculture, and the bacteria were cultured to an optical density at 620 nm (OD_{620}) of 0.5–0.6 at 37 °C (OD_{620} of 0.3 corresponds to 1×10^8 colony forming units (CFU) ml^{-1}), shaking. Bacteria were pelleted by centrifugation at 3500×*g* for 10 min at 4 °C, the pellet was washed four times, with 1, 0.5, 0.25 and 0.1 volumes of 10% glycerol, respectively, and stored at −80 °C in aliquots of 50 µl. For electroporation, one aliquot was thawed on ice, ~2 µg of plasmid WVW189, isolated from *S. aureus* RN4220 WVW189 [39], was added and the mixture was incubated for 30 min at room temperature (RT). Electroporation was performed in 1 mm cuvettes (VWR) in a pulse electroporator (Biorad Gene Pulser II) at 25 µF, 100 Ω and 2.0–2.2 kV. After electroporation bacteria were immediately transferred to fresh recovery medium (combination of B2 and SMMP medium of Lee [40], final composition: B2 medium with 0.5 M sucrose, 0.02 M maleic acid, 0.02 M $MgCl_2$ and 1% BSA) and incubated for 2 h at 37 °C, shaking. After recovery the suspensions were plated on tryptic soy agar (TSA) with 10 µg ml^{-1} chloramphenicol (CAM) for the selection of WVW189-harboring transformants, and incubated overnight at 37 °C. A transformant colony exhibiting strong green fluorescence when exposed to UV light was selected and designated *S. epidermidis* O-47 GFP. The presence of the full length plasmid was confirmed by plasmid isolation and restriction analysis. For all further experiments, *S. epidermidis* O-47 GFP was distinguished from *S. epidermidis* O-47 by inspection under exposure to UV light.

2.3. Plasmid stability

Bacteria were cultured overnight on TSA plates with 10 µg ml^{-1} CAM at 37 °C and recultured on fresh plates for three consecutive passages. Bacteria of each of the four culture plates were collected using cotton swabs and suspended in phosphate buffered saline (PBS), and analyzed for expression of the GFP protein by flow cytometry (FACS Caliber, BD).

2.4. Inoculum preparation

S. epidermidis O-47 and *S. epidermidis* O-47 GFP were cultured in tryptic soy broth (TSB; BD Difco) and TSB with 10 µg ml^{-1} CAM, respectively, to the logarithmic growth phase at 37 °C, shaking. The bacteria were pelleted by centrifugation at 14,000×*g*, washed twice with 1 volume of 0.9% NaCl (saline) and resuspended in saline to desired concentrations, based on the OD_{620}. For selected experiments, mixtures of equal numbers of CFUs of *S. epidermidis* O-47 and *S. epidermidis* O-47 GFP were prepared.

2.5. Growth curves

To assess the possible influence of plasmid WVW189 on the growth of *S. epidermidis* O-47, the bacteria were cultured in media optimally supporting their growth (brain heart infusion broth (BHI) and TSB) and media not designed for staphylococci (lysogeny broth (LB) [41] and Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal calf serum (FCS)), to model optimal and less favorable conditions, respectively. *S. epidermidis* O-47 and *S. epidermidis* O-47 GFP were pre-cultured overnight in 5 ml of the respective media at 37 °C, shaking. Subsequently, these overnight cultures were diluted to an OD_{620} of 0.1 in the respective media, and the OD_{620} was recorded every hour during 8 h of incubation at 37 °C, shaking.

2.6. Mouse experimental biomaterial-associated infection model

2.6.1. Animals

All animal experiments were approved by the Animal Ethical Committee of the Academic Medical Center at the University of Amsterdam. Specific pathogen-free C57BL/6 OlaHsd immune competent female mice (Harlan, Horst, The Netherlands), aged 7 to

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