Late hematogenous infection of subcutaneous implants in rats

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Late biomaterial-centered infection is a major complication associated with the use of biomaterial implants. In this study subcutaneously implanted biomaterials in rats were hematogenously challenged with bacteria 4 weeks after implantation. Bacteria were spread by either intravenous injection or stimulation of bacterial translocation. It was found that none of the biomaterials was infected by hematogenous spread, whereas 5% of the implants were infected by perioperative contamination. We conclude that late hematogenous infection of subcutaneous biomaterials does not occur in the rat. Also in man, there are growing doubts whether implants actually become infected through hematogenous routes or whether late infections are caused by delayed appearance of perioperatively introduced bacteria.
Introduction

A severe complication associated with the use of biomaterial implants is failure due to infection. About half of all biomaterial-centered infections occur months to even years after deep tissue implantation. Controversy exists concerning the origin of the infecting microorganisms in these late infections. Either bacteria spread hematogenously from endogenous foci are inserted during implantation and stay clinically unnoticed for a long time, designated as delayed infections [1].

Most hematogenous infections are believed to arise from infected skin lesions producing relapsing bacteremia [2]. This is supported by the fact that in most (56%) infections, where hematogenous spreading is suspected, staphylococci are involved, which are part of the normal skin flora. Also dental or other surgical interventions, bacteriuria, intestinal surgery or pneumonia have been proposed as possible causes of hematogenous spreading of bacteria. Streptococci are found in 15% of all late biomaterial-centered infections, while common intestinal bacteria, for example *Escherichia coli* and *Pseudomonas aeruginosa*, are responsible for 23% of the infections [1,2]. Another possible mechanism for hematogenous spreading from the intestinal tract is bacterial translocation [3], i.e. the escape of mainly Gram-negative rods through the intestinal wall [4].

Bacterial translocation (BT) can be promoted by nutritional factors, like total parenteral nutrition, fluid elemental nutrition, protein malnutrition [5] and vitamin A deficiency [6], hemorrhagic shock, extensive thermal injury and endotoxin [7]. Interestingly, also intraperitoneal implants promote BT [8,9].

In animal studies on biomaterial-centered infections, man-derived bacteria are frequently used. In man, however, biomaterial-centered infections are caused in most cases by their own commensal microflora toward which the immune system is more tolerant than to foreign flora [10,11]. As tolerated microorganisms probably survive longer in the circulatory system, it can be expected that their chance to cause biomaterial-centered infections is larger as compared with non-immunotolerated microorganisms.

The aim of this study was to determine whether hematogenous spreading of bacteria, after healing of the implantation wound, infects subcutaneous (s.c.) implants in rats. To this end, rats were intravenously (i.v.) injected with *Staphylococcus aureus*, *Staphylococcus epidermidis* or *P. aeruginosa* or their own total fecal flora, 4 weeks after implantation of a
biomaterial. To investigate the possibility of infection with translocating intestinal bacteria, BT was promoted either through special diets or through an intraperitoneal implant.

**Materials and methods**

**Rats**

Forty-eight male, 12 weeks old, specific-pathogen free Albino Oxford rats weighing 220-260 g were used. The animals were housed in a standard temperature-controlled environment (22°C), in macrolon cages and kept on a 12 h light/dark cycle. The rats were fed normal rat chow, unless otherwise stated, and had sterile tap water supplied *ad libitum*. Animals were allowed to acclimatize to our laboratory conditions for 2 weeks before the experiments. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” (NIH Publication No.85-23, revised 1985) and the Dutch Law on Experimental Animal Care.

**Bacteria**

Man-derived *S. aureus* ATCC 12600 and *S. epidermidis* HBH₂ 102 were cultured in tryptone soya broth (OXOID, Basingstoke, UK) in phosphate buffered saline (PBS) and man-derived *P. aeruginosa* AK1 in nutrient broth (OXOID) in PBS. First, a strain was streaked and grown overnight at 37°C from a frozen stock on a blood agar plate. A colony was used to inoculate 5 ml of growth medium, which was incubated at 37°C in ambient air for 24 h and used to inoculate a second culture (150 ml) that was grown for 18 h. The bacteria from the second culture were harvested by centrifugation (5 min, 5000 g for staphylococci and 10,000 g for *P. aeruginosa*) and washed twice with sterile Millipore-Q water. Subsequently, the bacteria were resuspended in sterile 0.9% NaCl solution, and *S. epidermidis* was sonicated on ice to disrupt aggregates.

Gut bacteria were harvested from fresh feces of the rats. The feces (2-3) were suspended in 10 ml anaerobic 0.9% NaCl solution. The suspension was centrifuged for 2 min at 250 g to remove larger particles. Supernatants were centrifuged at 10,000 g for 20 min to spin down the bacteria. Finally, the pellets were suspended in 10 ml 0.9% NaCl. The fecal flora contained anaerobic bacteria (70%), *E. coli* (20%), lactobacilli (7%) and streptococci (3%).
**Biomaterials**

Discs (diameter 8 mm, 0.5 mm thick) without sharp edges were made of commercially available silicone rubber (SR), polyethylene (PE), polypropylene (PP), poly(tetrafluoroethylene) (PTFE), poly(ethylene terephthalate) (PET), poly(methyl methacrylate) (PMMA), polyurethane (PU) (pellethane 2363-75D) and glass. The discs were cleaned in a 2% RBS 25 detergent solution under simultaneous sonication and thoroughly rinsed in demineralized water, sterilized in 70% ethanol and finally washed with sterile Millipore-Q water.

**Implantation**

Each rat received only four subcutaneous biomaterial discs as space was limited. After induction of inhalation anesthesia with N\textsubscript{2}O/O\textsubscript{2} (3/2) and halothane, the backs of the rats were shaved and disinfected with 0.5% chlorhexidine in 70% ethanol. Four 1 cm incisions were made, two on either side of the middle line, at least 3 cm apart. Subcutaneous pockets of at least 2 cm deep were created. The 4 different implants were inserted as deep as possible. The incision was then closed with degradable suture material. The surgical instruments used were disinfected after each surgical action.

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**Figure 1.** Design of intravenous injection experiments, involving a total of 28 rats, for practical reasons subsequently divided in 5 groups, depending on the infecting organisms. Number of rats per group are indicated between brackets.

**Intravenous inoculation experiment**

Figure 1 shows the experimental design used for i.v. inoculation. Half of the 28 rats received a SR, PTFE, PP and PE disc while the other half received a PU, PET, PMMA and glass disc.
After 4 weeks, 0.5 ml of bacterial suspension was injected in the tail vein. From each biomaterials group 12 rats were injected with a different one of the following 12 bacterial suspensions: 3 x 10^7; 10^8; 3 x 10^8 or 10^9 CFU ml^{-1} S. aureus or S. epidermidis or 10^8; 3 x 10^8; 10^9 or 3 x 10^9 CFU ml^{-1} P. aeruginosa. Three other rats received suspensions with 3 x 10^8; 10^9 or 3 x 10^9 CFU ml^{-1} of their own fecal flora, while the last one (i.e. 3 x 10^9 CFU ml^{-1} fecal flora) was also injected in one other rat, thus receiving foreign fecal flora.

**Stimulated bacterial translocation experiment**

Figure 2 shows the experimental design used to stimulate bacterial translocation.

![Figure 2. Design of bacterial translocation experiments, involving a total of 20 rats, divided in four groups: rats on vitamin A deficient diet (-vit A), rats on liquid diet and rats with an intraperitoneal implant with or without vitamin A deficiency. Number of rats per group are indicated between brackets.](image)

The rats in this experiment all received a disc of SR, PTFE, PET and PMMA. 5 rats were fed normal rat chow, while the other 15 were fed vitamin A free rat chow (Hope Farms, The Netherlands), starting directly after s.c. implantation, as vitamin A deficiency has been reported to occur 4 weeks after the onset of the diet [6]. 4 weeks after implantation the diet of 5 vitamin A deficient rats was changed to total liquid elemental nutrition (Nutrison powder, Nutricia, Zoetermeer, The Netherlands), which contained vitamin A, made according to the manufacturers descriptions with sterile demineralized water in sterile drinking bottles. Also 4 weeks after s.c. implantation a proteograft patch (dimensions 3.3 x 3.3 cm, similar to Dacron velour material, Braun, Oss, The Netherlands) was intraperitoneally implanted in 5 vitamin A deficient rats and in the 5 rats on normal rat chow. To this end the rats were anesthetized with
N₂O/O₂ (3/2) and halothane and their anterior side was shaved and disinfected. A 5 cm incision was made in the skin longitudinally over the middle line. Then a 4 cm incision was made in the abdominal wall. The implant was inserted close to the gut taking care it was not irritating the bladder or the liver. The abdominal wall was closed and the skin was closed separately. The rats received postoperatively pain killers (0.1 mg kg⁻¹ Temgesic a day) for 1 week.

**Harvesting**

After induction of anesthesia with N₂O/O₂ (3/2) and halothane, the back of the rats was shaved and disinfected. The subcutaneous implants were explanted and stored in 5 ml sterile reduced transport fluid (RTF). Swabs were taken of the pockets and streaked onto blood agar. Subsequently, the anterior side of the rats was shaved and disinfected. The abdominal cavity and chest were opened through a midline incision, and 0.1 ml of ventricular blood, a swab of the inside of the abdominal wall, the intraperitoneal implant when appropriate, a halved kidney, the halved spleen and a section of the liver were streaked onto blood agar plates. In the BT experiment also a section of the lungs was taken and streaked onto blood agar, and the mesenteric lymph nodes (MLN) were harvested and homogenized in 5 ml RTF. The rats were terminated by a cut in the heart. The MLN suspension and the biomaterials in RTF were sonicated on ice for 5 min to remove the attached bacteria, and cultured on blood agar. The blood agar plates were incubated aerobically at 37°C. The subcutaneous implants from the rats injected with fecal microflora and from the rats in the BT experiment, including the intraperitoneal implant, were also anaerobically cultured. Also the MLN were anaerobically cultured. The plated samples were considered infected if more than 2 of the same colonies were found on the agar plate (corresponding to more than 100 CFU per biomaterial disc). Bacteria harvested were characterized by colony morphology and Gram-staining.

**Results**

**Intravenous inoculation experiment**

The two rats that had received the highest dose of *S. aureus* and the rat receiving the highest dose of its own fecal flora, were terminated within 4 days because of severe illness and excluded from the study. The rat receiving foreign fecal flora died after 17 days due to sepsis, but was not excluded from the study.
Late hematogenous biomaterial-centered infections

Table 1 shows the numbers of positive organs and biomaterials cultures, together with the number of CFU isolated from the discs. Out of the 100 implanted discs 92 showed no infections. Moreover, most of the infected biomaterial discs revealed different bacterial strains than used for injection. These were staphylococci on both PE discs, two staphylococcal strains on one PU disc and a staphylococcal and streptococcal strain on the other PU disc. The SR disc was infected with two different strains of Gram-positive rods.

Table 1. Biomaterial-centered infections in intravenous injection and bacterial translocation model, including culture positive organs and different s.c. implanted biomaterials found infected and the numbers of CFU per biomaterial disc. Numbers between brackets present: (number of positive organs or discs/number of animals involved). Note that 168 out of the 180 implanted biomaterial discs were sterile.

<table>
<thead>
<tr>
<th>Intravenous injection model</th>
<th>Bacteria</th>
<th>Positive organs</th>
<th>Positive biomaterial discs</th>
<th>CFU (x10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. epidermidis HBH2102</td>
<td>Kidney (8/8)</td>
<td>PU⁺ (1/4)</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>S. aureus ATCC 12600</td>
<td>Kidney (8/8)</td>
<td>PU⁹ (1/4); SR⁹ (1/4)</td>
<td>1.4; 29</td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa AK1</td>
<td>None</td>
<td>PE⁹ (2/4)</td>
<td>100; 100</td>
</tr>
<tr>
<td></td>
<td>Own fecal flora</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foreign fecal flora</td>
<td>All organs</td>
<td>SR (1/1); PP (1/1); PE (1/1)</td>
<td>15; 2.5; 1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial translocation model</th>
<th>Animal condition</th>
<th>Positive organs</th>
<th>Positive biomaterial discs</th>
<th>CFU (x10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin A deficiency</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liquid diet</td>
<td>None</td>
<td>SR⁸ (1/5); PMMA⁸ (1/5)</td>
<td>0.45; 100</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal implant and vitamin A deficiency</td>
<td>MLN (3/5); Kidney (1/5); Peritoneum (1/5); Intraperitoneal implant (1/5)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal implant</td>
<td>MLN (4/5); Kidney (1/5); Liver (1/5); Spleen (1/5); Blood (1/5); Peritoneum (1/5)</td>
<td>PET⁸ (1/5); PTFE⁸ (1/5)</td>
<td>4.5; 10</td>
</tr>
</tbody>
</table>

Colony form did not correspond to the injected strains.
Colony form and Gram-staining did not correspond with translocated bacteria.

Stimulated bacterial translocation experiment
The results for the BT experiment are also shown in Table 1. Bacterial translocation to the MLN and some organs was only observed in the intraperitoneal implant group and found to
be due to Gram-negative bacilli (*E. coli*) and Gram-positive branched rods (Actinomyces spp.). Out of the 80 implanted discs 76 showed no infections. Moreover, the translocating species were not found on the infected biomaterial discs. A PET disc showed two different strains of staphylococci, while the other infected discs revealed one staphylococcal strain.

**Discussion**

In this study, the susceptibility of subcutaneously implanted biomaterials to late hematogenous infection was determined in rats, 4 weeks after biomaterial implantation. Two routes of hematogenous spreading of bacteria were used: either single i.v. injection of bacteria as a model for transient bacteremia, or by promoted bacterial translocation from the digestive tract. None of the respective biomaterial discs in the non-septic rats became infected by i.v. injected bacterial strains. Five biomaterial discs revealed bacteria that were probably originating from perioperative contamination. The 4 infected biomaterial discs in the increased BT group showed exclusively staphylococcal strains, suggesting that translocation from the intestinal tract was not the source of infection, since staphylococci are numerous on the skin, but scarcely found in the gut flora [12]. Furthermore no staphylococci were found in the MLN, indicating absence of staphylococcal translocation. Most probably, these bacteria also originated from perioperative contamination. Perioperative contaminations are likely to occur in animal experiments as usually no ultra-clean operating rooms are used and antibiotic prophylaxis is not common [13,14]. All infected biomaterials, were relatively hydrophobic, while glass, a hydrophilic material, was not involved in any biomaterial-centered infection. This corresponds with earlier *in vitro* findings that surface growth of staphylococci is slow on glass as compared with the other materials [15].

To our knowledge, the use of late hematogenous infection models with subcutaneously implanted biomaterials has not been reported in rats before. In mice [16], i.v. injection of $1 \times 10^7$ *S. aureus* did not yield infection of subcutaneously implanted biomaterials, 1 month after implantation, while a higher dose $1 \times 10^8$ *S. aureus* killed the mice. However, in rabbits, 6 to 8 weeks after total joint replacement, Blomgren and Lindgren [17] successfully induced hematogenous infections in 40% of the cases through i.v. injection of around $10^9$ *S. aureus* (note this is a 10 to 100 fold higher dose than used in our study). Hematogenous infection directly after implantation yielded infection in 80% of the animals [18]. Also Southwood *et al.* [19] showed a similar decrease in hematogenous infection rate in
rabbits from 40% immediately after surgery to 10% after 3 weeks of implantation. Vascular grafts in dogs were infected by i.v. challenge of $10^8$ *S. aureus* 3 to 6 months after implantation, yielding infections in 10% to 80% of the animals, depending on the type of graft [20]. Interestingly, a similar study in rats revealed that the infection rate of caval vein grafts was reduced from 100% to zero during 2 weeks of implantation as a result of increased endothelialization, while the infection rate of aorta grafts was still 100% after these 2 weeks [21].

Evidently, hematogenous biomaterial-centered infections can be induced directly after implantation, but are much more difficult to achieve after prolonged implantation time. Essentially, the occurrence of biomaterial-centered infections is a race for the surface [22] between infecting microorganisms and host cells. When infecting organisms arrive long-time after implantation on a biomaterial surface, the race is won in most cases by host cells and the biomaterial surface is out of reach for adhering organisms. Yet, many late biomaterial-centered infections in man, most notably those associated with orthopedic implants are said to be hematogenous in origin [1,2,14,23], although it is also suggested that the hematogenous route of infection will only occur in immuno-compromised patients. For example, 40% to 100% of all late hematogenous orthopedic implant infections are found in patients with rheumatic arthritis [24] using immuno-modulating drugs. Late infections associated with dental procedures have been reported mostly in diseased patients with drug or irradiation induced immuno-suppression, insulin dependent diabetes mellitus or hemophilia [25]. At this point it must be noted that in clinical practice infection is often assumed to be of hematogenous origin without attempts to obtain any proof, for instance, by culturing blood or joint fluids [1]. As many strains, including *S. aureus* and Gram-negative rods, can survive intracellularly in epithelial and scar tissues, therewith circumventing the host’s immune system for prolonged periods of time [26], it is suggested more and more that many biomaterial-centered infections assumed to be of hematogenous origin actually results from delayed appearance of perioperatively introduced bacteria. These suggestions would be in line with the results of this study, demonstrating that it is virtually impossible to create a biomaterial-centered infection in rats by the hematogenous route, although of course differences between the immune system of rat and man may be of crucial importance here.
References


