JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 628–634

Incidence of infection in implanted polyurethane tubing segments serially injected with staphylococci

P. B. VAN WACHEM. M. J. A. VAN LUYN, E. H. BLAAUW
Groningen University, Laboratory for Cell Biology and Electron Microscopy, Oostersingel 69/2, 9713 EZ Groningen, The Netherlands

D. RAATJES
University Hospital, Department of Hospital Epidemiology, Oostersingel 59, 9713 EZ, Groningen, The Netherlands

P. T. CAHALAN*, M. HENDRIKS
Medtronic Bakken Research Center BV, PO Box 1220, 6201 MP Maastricht, The Netherlands

One of the major clinical complications in the biomedical application of synthetic materials is the incidence of implant-associated infections. Such infections are very often induced by Staphylococcus aureus. To obtain information on tissue reactions and minimal bacterial challenge needed to create an infection related to untreated implant surfaces, we injected polyurethane tubing segments with a series of *Staphylococcus aureus*. The segments were subcutaneously implanted in rats. Implantation periods varied from 2, 5 and 10 days to 3 weeks. Specimen were evaluated using light and transmission electron microscopy. At least 0.25×10^4 of Staphylococci aureus were needed to clearly recognize that bacteria had been injected in the polyurethane tubing segments. The evidence was *indirect*, showing high infiltration and activation of neutrophils and macrophages, but not bacteria. Furthermore, 0.25×10^6 *S. aureus* were needed to induce a persistent specific inflammatory reaction with high concentrations of lymphocytes, i.e. mainly plasma-cells, at 3 weeks. The results indicate that this model functioned well to obtain the wanted information. Results are discussed with respect to (a-) specific inflammatory reactions occurring with (bacterial-challenged) biomaterials. Ultimately, our goal is to develop infection-resistant materials, for which the *in vivo* model developed may be used to qualify the processed materials

1. Introduction

Synthetic materials are widely used in biomedical applications, such as in the construction of devices used for treatment of disease, e.g. catheters, hemoperfusion devices, or as permanent implants to replace defective tissue or organ function, e.g., pacemakers, heart valves. Although a wide variety of materials are available, currently there is no material that is completely satisfactory for long-term use [1].

One of the major clinical complications is the incidence of implant-associated infections, mostly induced by *Staphylococcus* (*S.*) *aureus* and coagulase negative staphylococci [2–5]. Implant-associated infections are most often irreversible and necessitate implant removal in order to eliminate the infectious complications (6).

It is generally accepted that bacterial adherence, followed by formation of biofilm-enclosed microcolonies enhances the persistence of these infections, even in the presence of the host defence mechanisms and antibiotics [7–14]. Additionally, the presence of a foreign body provides a hindrance to appropriate tissue regeneration and healing, resulting in an impaired host defence mechanism, thus facilitating the establishment of infection [15–19].

By preventing the initial bacterial adherence to the implant, surface infection incidence may be reduced, or even prevented. Furthermore, promoting a better tissue response, leaving the natural host defence mechanism intact, may improve the clinical performance of implanted materials.

In the present study we developed an *in vivo* model to obtain information on tissue reactions and minimal bacterial challenge needed to create an infection related to untreated implant surfaces. For this purpose we implanted polyurethane tubing segments, injected with a series of *S. aureus*, in rats. Ultimately, our goal is to develop infection-resistant materials, for which the developed *in vivo* model may be used to qualify the processed materials.

0957–4530

2. Materials and methods

2.1. Materials

Polyurethane (PU) tubing material was made from 2363-55D Pellethane® resin (Dow Chemical, Midland, Michigan, USA) by Medtronic (Minneapolis, Minnesota, USA). The material was rinsed twice for 1 min in an ultrasonic bath with isopropylalcohol (ACS reagent) obtained from Aldrich Chemie (Bornem, Belgium), air-dried and sterilized with ethylene oxide.

2.2. Bacteria

A strain of *S. aureus* (code PW230693), obtained from a patient with a PU-catheter-related infection, was used. *S. aureus* were routinely cultured on blood-agar plates (4% sheep erythrocytes; from Oxoid Ltd., Hampshire, UK).

S. aureus cultured for 18 h at 37 °C in brain heart infusion-broth (from Oxoid) were washed three times with phosphate-buffered saline (PBS), pH 7.4 and resuspended in PBS. The bacterial suspension was spectrophotometrically adjusted to an optical density of 1.0 at 540 nm (PYE Unicam PU 8600 UV/VIS spectrophotometer from Philips, Eindhoven, The Netherlands), containing $\pm 10^9$ colony-forming units (CFU) per ml.

Serial ten-fold PBS-dilutions were made with *S. aureus* concentrations of 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9/ml. From these dilutions 1 µl, which exactly fitted into 3 mm PU-tubing segments, was used for injection. This resulted in a series of PU-tubing segments with 10^1, 10^2, 10^3, 10^4, 10^5 or 10^6 *S. aureus* per segment.

After implantation, the number of viable CFU was determined by spreading 0.1 ml portions from the serial ten-fold dilutions of bacterial suspensions on blood-agar plates, followed by 18 h of incubation at 37 °C and CFU-counting.

2.3. Implantation

Male AO-rats of approximately three months age were ether-anaesthetized and subcutaneous pockets were made to the right and left of two midline incisions on the back. PU-tubing was implanted as 3 mm segments either as-received for controls, or injected with the series of 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 *S. aureus*, in the pockets at a distance of about 1 cm from the incisions. Implantation periods were 2, 5, and 10 days and 3 weeks.

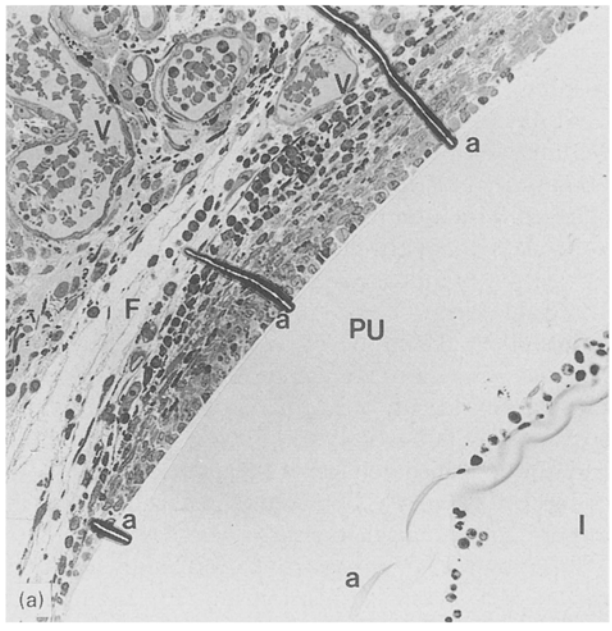
2.4. Microscopy

After a certain implantation period, specimens were carefully dissectioned from the surrounding tissue and immersion-fixed in 2% (v/v) glutaraldehyde in PBS (pH 7.4) for at least 24 h at 4 °C, postfixed with 1% OsO_4 and 1.5% $K_4Fe(CN)_6$ in PBS, dehydrated in graded alcohols and embedded in Epon 812.

Semi-thin sections (1 µm) for light-microscopical evaluations were stained with toluidine blue. Ultra-thin sections (70 nm) were cut and stained with uranyl acetate and lead citrate and examined with a Philips EM 201 transmission electron microscope, operated at 40 kV.

3. Results

CFU-counting was performed to check the viable number of injected *S. aureus* into PU-tubing segments. From these calculations it was found that ± 25% of the *S. aureus* had been viable. Thus, for example, 10^6



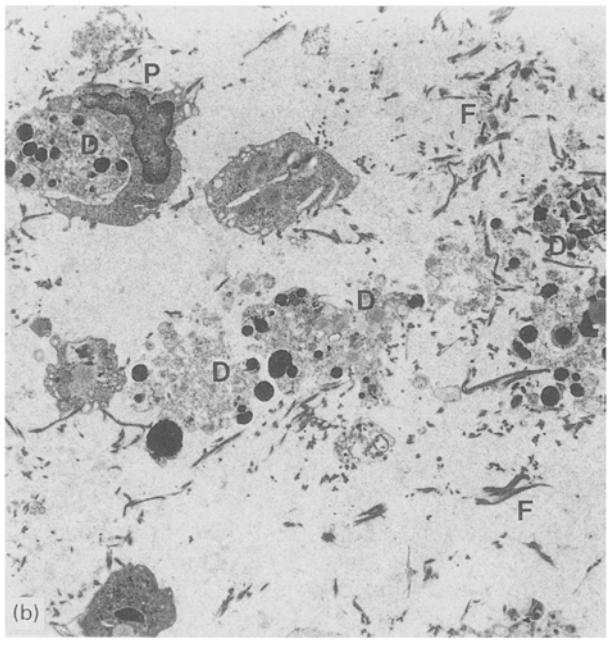


Figure 1 (a) Light micrograph (160×) of part of a control PU tubing segment, implanted for 2 days. At this time mainly macrophages and granulocytes have migrated from blood vessels (V) and adhered to the material (PU). On the outside, areas with relatively extended cell adhesion as well as with hardly any cell adhesion (arrow) can be observed. A fibrin network (F) is often present. Intraluminally (I) cell numbers are low. Artifacts (a) are the result of differences in hardness between PU and tissue, occurring after mounting of the cover slip. (b) Transmission electron micrograph (3000×) of the lumen of a control PU tubing segment, implanted for 2 days, showing (Organelles of) degenerated cells (D) and phagocytosis (P) by a macrophage within a fibrin network (F).

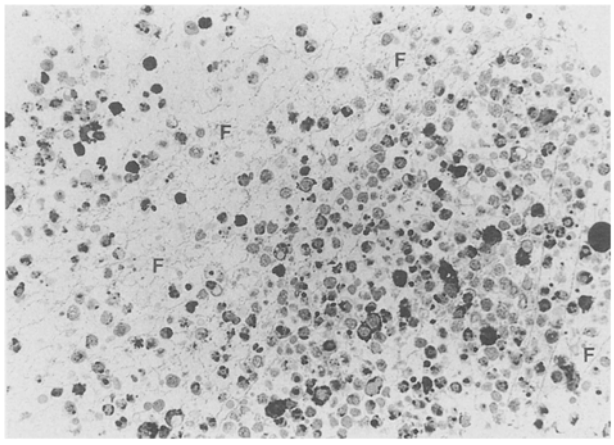
S. aureus in a segment afterwards was found to give 0.25×10^6 viable *S. aureus*. In following sections we will continue to mention the original series.

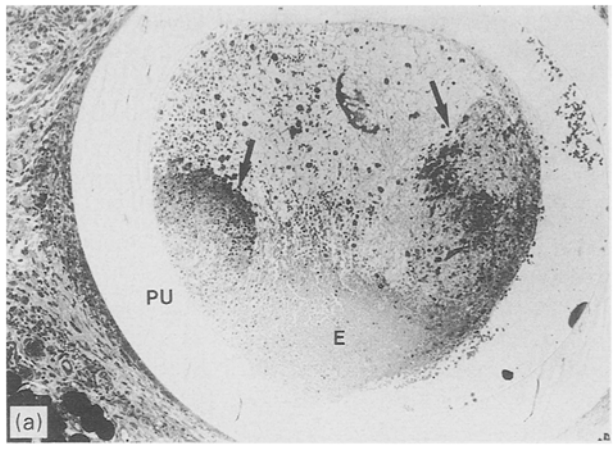
Non-infected control PU-tubing segments at 2 and 5 days induced adhesion of neutrophils and macrophages both to the inside and outside of the tubing segment (Fig. 1). On the outside, areas with relatively extended cell adhesion as well as with hardly any cell adhesion could be observed (Fig. 1a). The morphology of the adhering cells was normal. A fibrin network was often present. Cell numbers on the inside, thus intraluminally, were very low. Morphology of these cells was generally normal, although also some cell degeneration and phagocytosis occurred (Fig. 1b). Also here, fibrin networks were sometimes present.

At day 5, on the outside some indications of giant cell formation were observed, while active encapsulation started by way of the presence of fibroblasts and collagen formation.

At day 10 neutrophils were no longer present. Formation of a quiet capsule with fibroblast activity and giant cells proceeded (Fig. 2). Encapsulation also occurred on the inside by way of the ingrowth of fibrotic tissue into the lumen. At week 3 a small capsule with usually one or two layers of macrophages/giant cells, followed by some layers with fibroblasts and newly-formed collagen and surrounded by loose connective tissue, was observed. The fibrotic ingrowth into the lumen had then clearly proceeded.

Infection with *S. aureus* was performed by injection of 1 µl of *S. aureus* suspension into PU tubing segments. At days 2 and 5 clear evidence of infection was found intraluminally, with 10^4 or more *S. aureus* (Fig. 3). This evidence consisted of a high infiltration of granulocytes, i.e. mostly neutrophils, and macrophages, the highest infiltration being with the 10^6 *S. aureus* specimen (Fig. 4a and b). Here also fibrin formation and erythrocytes, as indicators of hemorrhages, were widely present (Fig. 4b). The granulocytes were highly activated, as judged from pseudopods and intracellular glycogen accumulations (Fig. 5a and b). Phagocytotic actions of degenerated cellular material, probably of both host and bacterial origin, by macrophages and neutrophils were often observed. Bacteria as such were not recognized, but sometimes indicated from inclusions in several neutrophils (Fig 6).





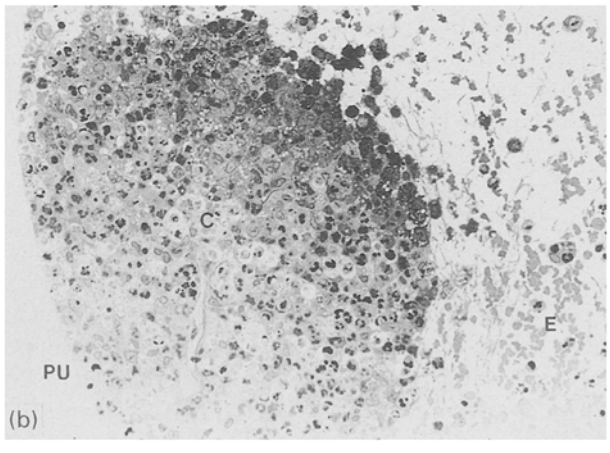


Figure 4 Light micrographs of a PU tubing segment injected with 10^6 *S. aureus*, implanted for 5 days. (a) (63 ×) shows the segment (PU) in cross-section. The lumen of the segment was completely occupied with fibrin and cells. Areas with mainly granulocytes and macrophages (arrows) as well as with mainly erythrocytes (E) can be observed. (b) (250 ×) shows a magnification of a clotted (C) area with mainly granulocytes and macrophages next to the area with loose erythrocytes (E).

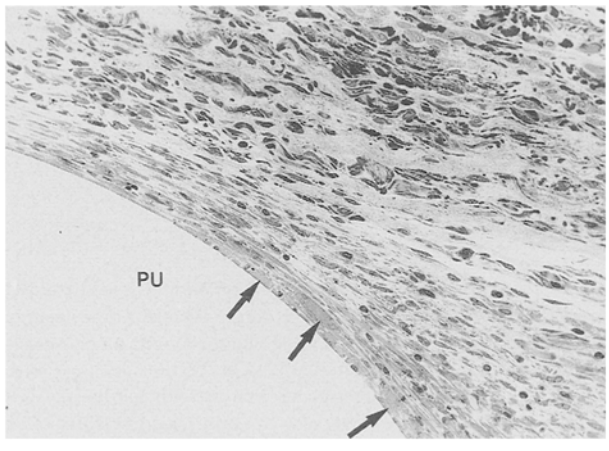
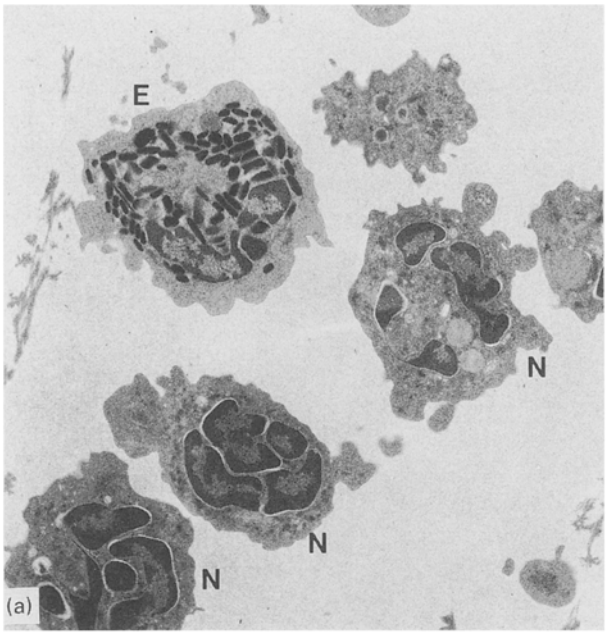


Figure 2 Light micrograph (160 ×) of a control PU tubing segment, implanted for 10 days, showing the presence of a quiet capsule at the outside of the material (PU). Giant cells (arrows), fibroblasts and newly formed collagen are present. Collagen bundles further from PU are larger and more mature.



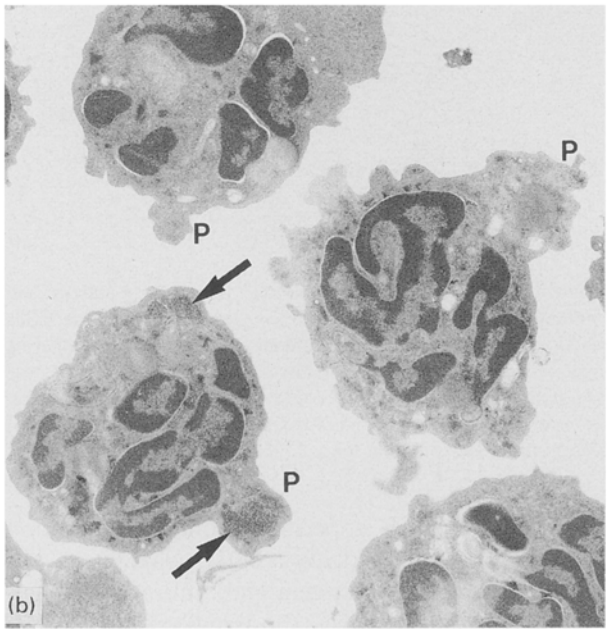


Figure 5 Transmission electron micrographs of the lumen of a PU tubing segment, inoculated with 10^5 *S. aureus* and implanted for 2 days. (a) (4500 ×) showing activated granulocytes, i.e. three neutrophils (N) and one eosinophil (E) containing the specific eosinophilic granules. (b) (10 000 ×) showing neutrophilic granulocytes with segmented nucleus, psuedopods (P) and glycogen accumulations (arrows).

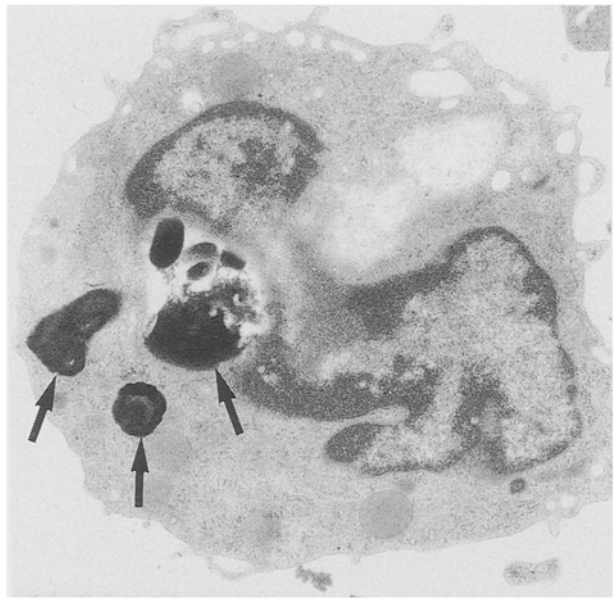


Figure 6 Transmission electron micrograph (15 000 ×) of a neutrophil containing inclusions which may represent remnants of phagocytosed *S. aureus* (arrows) in the lumen of a PU tubing segment, injected with 10^5 *S. aureus* and implanted for 2 days.

At day 10, apart from the 10^6 *S. aureus* specimen, with all PU-tubing segments, encapsulation by way of fibrous tissue formation around and at the inside, had started. PU-tubing segments injected with 10^1, 10^2 or 10^3 *S. aureus* showed a tissue reaction which was very similar to non-infected PU, i.e. there seemed to be no delay in encapsulation as a result of *S. aureus* injection. The 10^4 or higher *S. aureus* specimen showed a couple of days delay. Fibrous tissue formation occurred also in the 10^6 *S. aureus* specimen, but here evidence of infection was found by way of a slightly increased presence of loose lymphocytes in the connective tissue around the PU-tubing segment. Intraluminally, fibrotic tissue was present, which in the centre contained macrophages with abundant phagocytosed substances, while macrophages around the ingrowing tip had active pseudopods and contained many lipid droplets.

At week 3 the 10^1–10^4 *S. aureus* specimens had been surrounded by a quiet capsule, similar to the control. Fibrotic ingrowth had proceeded to the centre of the tubing segments. These specimen showed no evidence of bacterial challenge.

In contrast, the 10^5 *S. aureus* specimen contained some increase of loose lymphocytes in the surrounding connective tissue. This was similar to the presence of loose lymphocytes in the 10^6 *S. aureus* specimen at day 10.

The 10^6 *S. aureus* specimen at week 3 had been loosely encapsulated (Fig. 7a) and, similar to the other specimen, contained a fibrotic ingrowth, in this case also with phagocytosing macrophages (Fig. 7b). At one side clear areas with high concentrations of lymphocytes, some hemorrhages and fibrin formation were found in the loose connective tissue surrounding the encapsulated tubing segment (Fig. 8a and b). The encapsulating layer at this side was less quiet, i.e. apart from giant cells, it contained a few loose lymphocytes, while some neutrophils and macrophages seemed freshly arrived. As clearly discriminated by TEM, most of the lymphocytes concerned plasma cells (Fig. 9a and b), with the characteristic increased amount of rough endoplasmic reticulum. Also macrophages, which had been actively phagocytosing were present (Fig. 9b).

4. Discussion

PU as control material induced reactions normally observed after implantation of non-cytotoxic, non-degradable implant materials with a smooth surface.

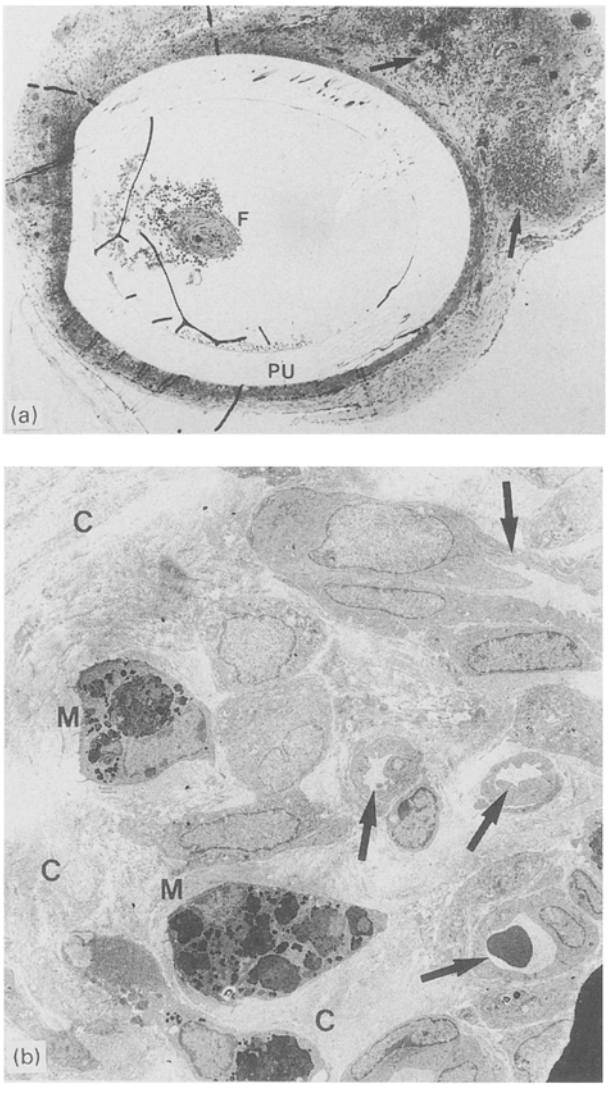


Figure 7 PU tubing segment, injected with 10^6 *S. aureus* and implanted for 3 weeks. (a) Light micrograph (400 ×) showing the centre of the segment (PU) in cross-section. The lumen shows the tip of ingrowing fibrotic tissue (F). On the outside a rather thick reactive layer can still be observed, while in the surrounding loose connective tissue evidence of lymphocytes and hemorrhages (arrows) are present (b) Transmission electron micrograph (2000 ×) of ingrowing fibrotic tissue showing actively phagocytosing macrophages (M) and capillaries (arrows) in between newly formed collagen (C).

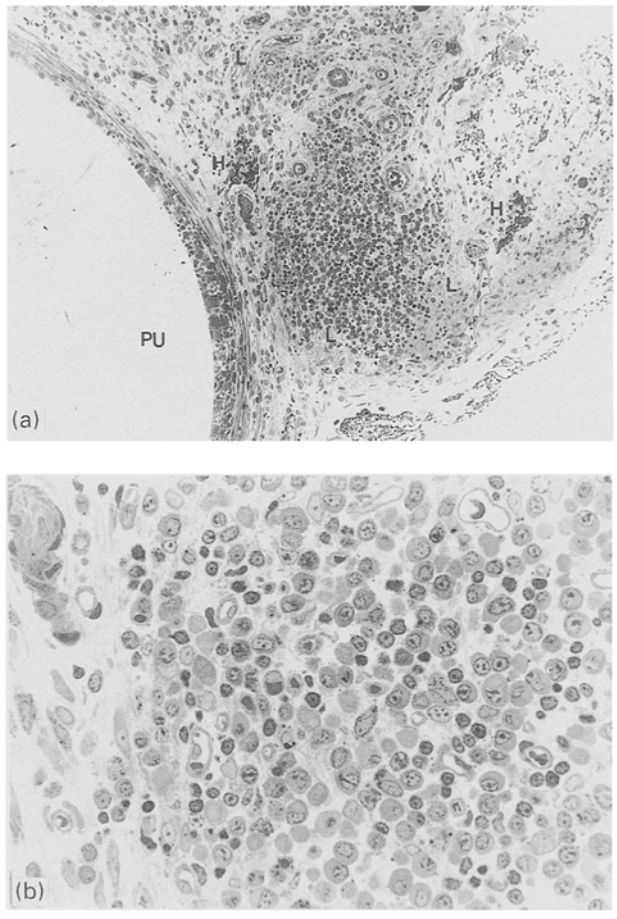


Figure 8 PU tubing segment, injected with 10^6 *S. aureus* and implanted for 3 weeks. (a) Light micrograph (100 ×) of the tissue reaction to the outside of the material (PU) showing an adhering layer which is, at the side of areas with lymphocytes (L) and hemorrhages (H) in the surrounding connective tissue, still rather reactive. (b) Light micrograph (400 ×) of an area with lymphocytes present in the surrounding loose connective tissue.

PU was finally surrounded by a small capsule. Although intraluminal circumstances in lead segments seemed to be slightly unfavourable, as judged by the low incidence of intraluminal cell degeneration, these results justify the conclusion that PU is a very biocompatible material. This agrees with many other observations [20].

Results obtained with infected PU tubing segments indicate that at least 0.25×10^4 *S. aureus* are needed to clearly recognize that bacteria had been injected in the PU-tubing segments. The evidence is indirect, showing high cellular infiltration and activation, but not bacteria. Furthermore, 0.25×10^6 *S. aureus* were needed to induce a persistent inflammatory reaction with high concentrations of lymphocytes, i.e. mainly plasma cells, at 3 weeks.

These results are explained as follows. At the start of the wound healing process, immediately after implantation, an acute a-specific inflammatory reaction [21–23] starts with activation of the coagulation cascade, the complement system and infiltration of neutrophils and macrophages. Cytokines released by different cells play important signalling roles.

Early after implantation, the reaction is clearly visible in specimens with 10^4 and higher *S. aureus*. The low level, and thus hardly visible, a-specific reaction occurring in the specimens with 10^1–10^3 *S. aureus* show that it can obviously cope with these bacterial challenges. The a-specific reaction occurring in the 10^4 *S. aureus* challenge can also cope, as observed at day 10. However, in the case of 10^5 and especially 10^6 *S. aureus*, a specific immunologic reaction is induced. This reaction is probably initiated by macrophages, which have been in contact with *S. aureus*, and thereafter migrate towards nearby lymph nodes [21–23]. Here, interactions between macrophages and T-lymphocytes (T_O-cells) result in helper functions of other T-lymphocytes (T_{h2}-cells) inducing proliferation and maturation of B- cells. This results in immunoglobulins (Ig)-producing plasma cells. Ig forms at the implantation site complexes with (remnants of) *S. aureus*, thus inducing new macrophages to phagocytose [21–23]. This sequence of events takes approximately one week, which explains why accumulation

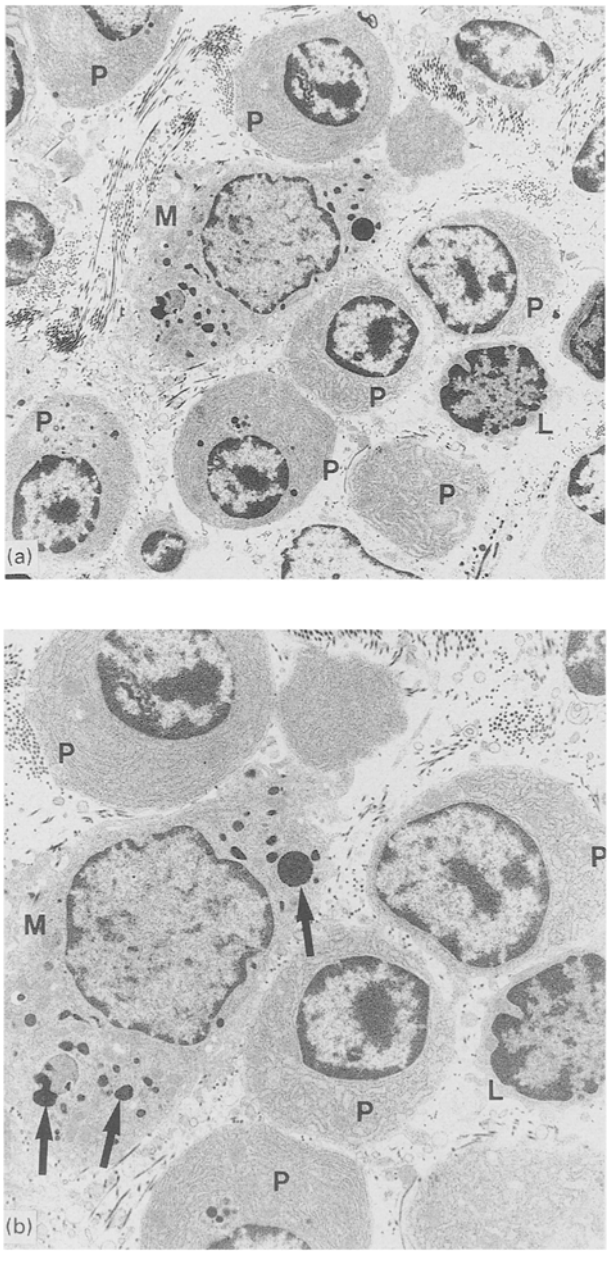


Figure 9 PU tubing segment, injected with 10^6 *S. aureus* and implanted for 3 weeks. (a) Transmission electron micrograph (3000 ×) showing plasma cells (P), a macrophage (M) with phagocytosed substances, and a T-lymphocyte, observed in the area with lymphocytes in the loose connective tissue around the implant. (b) Magnification (4500 ×) showing the rough endoplasmic reticulum largely present in the characteristic circle-like appearance in plasma cells (P), and phagocytosed cellular (bacterial?) substances (arrows) in the macrophage (M). T: T-lymphocyte.

of lymphocytes was observed not earlier than at day 10 (in the 10^6 *S. aureus* specimen). The 10^5 *S. aureus* challenge is obviously so low that only a low specific immuno-reaction is observed at 3 weeks. The lymphocytes present in the surrounding loose connective tissue have probably migrated from nearby lymph nodes; however, it is not impossible that also local proliferation has occurred.

During the whole implantation period, cytokines, released from neutrophils, macrophages and/or lymphocytes, are responsible for the observed microvascular thrombo-hemorrhagic changes as a result of their effects on the endothelium [23]. Since *S. aureus* is coagulase-positive and thus may have formed coagulase, this may represent an additional factor for excessive fibrin formation [24].

No clear direct evidence for the presence of *S. aureus* was observed, not even with the highest bacterial challenge. It was expected to find (remnants of) bacteria either at the material surface or within cells. The fact bacterial adherence to PU was not observed, is in contrast with many reports, suggesting that adhesion and microcolony/biofilm formation represent a first step in biomaterial-centred infection [7–14]. Furthermore, it is surprising that such high challenges were needed to obtain clear (indirect) reactions, since it is claimed that the presence of a biomaterial significantly enhances infection incidence [15–19]. We meanwhile have performed experiments with higher bacterial challenges (10^9 *S. aureus*). Inclusions, as shown in Fig. 6 were often observed, which makes it very likely that these concern remnants of *S. aureus*. Apart from the 10^6 *S. aureus* challenge obviously still resulting in a rather low (no excessive presence of bacteria) reaction in this model, also the day 2 -explantation chosen as the earliest explantation time may have been late; at that time, most bacteria seem to have been dealt with by complement and neutrophils and macrophages.

In conclusion, our results indicate that the injection of a series of *S. aureus* suspensions into PU tubing segments implanted in rats represents a good model to obtain information on tissue reactions and on the minimal bacterial challenge needed to create infections. Serial injection of bacteria with higher bacterial challenge, will therefore also be used for cell biological evaluations of infection-resistant materials. Currently, efforts are being made to develop materials that reduce or prevent the occurrence of implant-related infection. Research efforts are directed at the role of the implant itself, and consequently on modification of the material surface to protect it from being colonized by bacteria. The first option is the development of materials that provide a controlled release of antimicrobial agents at the site of implantation (25–27). However, other options will also be examined, including surface modification techniques to improve the overall tissue response to the implanted material [28], and thus leave the natural host defence mechanism intact and competent to resolve most infectious complications.

Acknowledgements

The authors wish to thank Dr. H. G. de Vries-Hospers of the Dept. for Medical Microbiology for her kind advice and use of facilities, and Mr P. van der Sijde and Mr D. Huizinga for the photography.

References

1. J. BLACK, "Biological performance of materials: fundamentals of biocompatibility", 2nd edn (Marcel Dekker, New York, 1992).
2. A. L. BISNO, and F. A. WALDVOGEL, "Infections associated with indwelling medical devices" (ASM, Washington, DC, 1989).
3. F. J. SCHOEN, ASAIO, **33** (1987) 8.

4. J. DANKERT, A. H. HOGT, and J. FEIJEN, *CRC Crit. Rev. Biocompat.* **2** (1986) 219.
5. A. H. HOGT, J. DANKERT, and J. FEIJEN, *J. Biomed. Mater. Res.* **20** (1986) 533.
6. A. F. VON RECUM and E. BARTH, E., *J. Invest. Surg.* **2**, (1989) 351.
7. W. P. REED, and R. C. WILLIAMS, *J. Chron. Dis.* **31** (1978) 67.
8. J. W. COSTERTON, *Rev. Infect. Dis.* **6** (suppl. 3) (1984) S608.
9. H. ANWAR, et al, *Antimicr. Agents Chemother.* **36** (1992) 1347.
10. A. G. CRISTINA, C, D. HOBGOOD, L. X. WEBB, and Q. N. MYRVIK, *Biomaterials* **8** (1987) 423.
11. N. K. SHETH, T. R. FRANSON, and P. G. SOHNLE, *Lancet* **2** (1985) 1266.
12. G. M. JOHNSON, W. E. REGELMANN, E. D. GRAY, G. PETERS, and P. G. QULE, *Zbl. Bakt. Suppl.* 16 (1987) 33.
13. G. D. CHRISTENSEN, L. M. BADDOUR, and W. A. SIMPSON, *ibid.* **16** (1987) 103.
14. N. K. SHETH, P. G. SOHNLY, and T. R. FRANSON, **Suppl 16** (1987) 177.
15. S. D. ELEK, and P. E. CONEN, *Brit. J. Exp. Pathol.* **38** (1957) 573.
16. A. G. GRISTINA, *Science* **237** (1987) 1588.
17. W. ZIMMERLI, F. A. WALDVOGEL, P. VAUDAUX, and U. E. NYDEGGER, *J. Infect. Dis.* 146 (1982) 487.
18. W. ZIMMERLI, P. D. LEW, and F. A. WALDVOGEL, *J. Clin. Invest.* **73** (1984) 1191.
19. J. M. ANDERSON, *Cardiovasc. Pathol.*, **2** (1993) 33S.
20. H. PLANCK, G. EGBERS and I. SYRÉ, (eds) "Polyurethanes in biomedical engineering" (Elsevier Science Publishers BV, Amsterdam, 1984).
21. A. K. ABBAS, A. H. LICHTMAN, and J. S. POBER, "Cellular and molecular immunology" (W.B. Saunders, Philadelphia, PA, 1991).
22. J. BROSTOFF, G. K. SCADDING, D. K. MALE, and I. M. ROITT, "Clinical immunology" (Gower Medical Publishing, London 1991).
23. J. B. VAN DER MEER, and M. C. J. M. DE JONG, *Neth. J. Med.* **40** (1992) 244.
24. F. A. WALDVOGEL, "Principles and practice of infectious diseases", 3rd edn (Churchill Livingston, New York, 1990) pp. 1489.
25. B. JANSEN, "Progress in biomedical polymers", edited by C. G. Gebelein and R. L. Dunn (Plenum Press, New York, 1990) pp. 347.
26. B. JANSEN, J. SCHIERHOLZ, F. SCHUMACHER-PERDREAU, G. PETERS, and G. PULVERE, *Adv. Biomater.* **9** (1990) 117.
27. G. COLOMB, and A. SHPIGELMAN, *J. Biomed. Mater. Res.* **25** (1991) 937.
28. A. G. GRISTINA, Science **237** (1987) 1588.