J. Med. Microbiol. — Vol. 49 (2000), 441–450
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ISSN 0022-2615

PREVENTION OF INFECTION

Use of scanning electron microscopy to investigate the prophylactic efficacy of rifampin-impregnated CSF shunt catheters

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Infection continues to be one of the major complications of cerebrospinal fluid (CSF) shunting procedures, and is caused mainly by skin-derived bacteria. Production of an extracellular biofilm plays an important role in the pathogenesis of shunt-associated infections by protecting bacteria from immune mechanisms and antibiotics. So far, removal of the original shunt and implantation of a new shunting device has been the only successful treatment for most patients. As an alternative strategy to prevent CSF infections, a rifampin-impregnated silicone catheter was designed to provide high initial and long-lasting (>60 days) release of bactericidal drug. To investigate the pathophysiological mechanism of its function, this new device was investigated both *in vitro* and in a rodent model of CSF infection by scanning electron microscopy (SEM) and bacterial culture. *Staphylococcus epidermidis* (10^8 cfu/ml) and *S. aureus* (10^4 cfu/ml) served as test strains. SEM demonstrated that, in contrast to the unloaded catheters, initial bacterial adherence on the catheter surface could be reduced to a few single cells, which did not show visible signs of proliferation. Bacterial cultures obtained simultaneously were all sterile, showing that adherent bacteria were killed immediately by the rifampin released from the catheter. Although rifampin incorporation into silicone polymers was not able to prevent initial bacterial adhesion completely, subsequent colonisation could be prevented.

Introduction

Infection is one of the major complications of cerebrospinal fluid (CSF) shunting procedures. In a meta-analysis of 19 575 patients, Aschoff *et al*. showed that the average infection rate dropped from 11.8% in the 1950s to 6.4% in the 1990s, predominantly among the very young and the elderly [1]. *Staphylococcus epidermidis* accounts for *c*. 50% of all CSF shunt infections, followed by *S. aureus* at *c*. 25% [2–4].

More than 90% of shunt infections are diagnosed within the first 12 months after surgery (the majority within the first month [5]), resulting in the assumption that intra-operative implant contamination has to be considered as a major cause of these infections.

It is generally accepted that the presence of a foreign body implant enables skin-derived bacteria to become pathogenic. After adhesion to the implant surface, coagulase-negative staphylococci produce an extracellular mucoid biofilm that embeds the growing cells [6–9]. This 'slime' appears to be a virulence factor by acting as a barrier that prevents penetration of antibiotics [10–12] or interferes with the function of lymphocytes and neutrophils [11, 13–15].

As a result of these pathogenic mechanisms, shunt removal seems to be the only successful therapeutic approach for most patients, followed by a temporary external ventricular drainage and finally the implantation of a new shunting device.

It has been shown that shunt-related infections may be reduced by modification of the operative procedure,

Received 20 April 1999; revised version received 28 Aug. 1999; accepted 4 Oct. 1999.
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shortening of operation time and peri-operative systemic use of antibiotics [16]. Nevertheless, shunt infection is still considered a major problem in most neurosurgical centres.

The key to effective prophylaxis of infection seems to be the prevention of initial bacterial adhesion or colonisation of the foreign body surface, or both, leading to the concept of antimicrobial device impregnation or coating. In the last few years, several studies have showed only in-vitro efficacy or failure to prevent clinical infection due to insufficient loading of the silicone material [17–23].

A technique capable of incorporating an antibiotic into a silicone polymer has been developed, leading to high initial and long-lasting drug release characteristics *in vitro* [24]. Preliminary investigations of the in-vivo efficacy of these rifampin-impregnated catheters, in a rodent model gave excellent results [25–27]

This report describes the use of scanning electron microscopy (SEM) and microbiological culture to investigate how surface colonisation is prevented by this rifampin-impregnated silicone catheter.

Materials and methods

Catheters

Silicone catheters, normally used as CSF shunt ventricular catheters, were supplied by Cordis® and cut into 1-cm pieces (outer diameter 2.5 mm). Rifampin was chosen because of its high physicochemical compatibility with the hydrophobic polydimethylsiloxane (silicone) matrix and its good activity against staphylococci. Rifampin was added to the swelling activated silicone matrix (in chloroform) until a concentration of 9% was reached. Removal of the solvent chloroform by controlled evaporation produced an antimicrobial device with reproducible defined surface and matrix loading. In-vitro tests showed that *c*. 30% of the incorporated rifampin was released as an initial burst within the first 24 h. Thereafter, a continuous release of rifampin (*c*. 25 μg/cm of polydimethylsiloxane/day), with only minimal further decline, has been observed for up to 60 days. At this time point, the surface concentration of rifampin was 10 μg/cm of polydimethylsiloxane [24]. *In vivo*, the rifampin concentrations in the brain tissue adjacent to the catheter were 10–50 μg/mg 6 days after implantation [28].

The incorporation process slightly changed the biomechanical properties of the silicone catheter. There was a 27% decrease in the tensile strength (σ_R) and a decrease in elasticity (ε_R) from 585% to 321%. Nevertheless, because the processed catheter could still be stretched to more than three times its original length, it is unlikely that these changes are of clinical significance [28].

Bacterial contamination of catheters in vitro

Experimental design. Unchanged and rifampin-impregnated silicone catheters were investigated in two identical but separate experiments under sterile conditions. A 500-ml flask was sealed with a plastic plug, in which four small plastic rods with stainless steel needle-shaped tips were inserted. One-cm pieces of two longitudinally divided silicone catheters were pinned to each steel tip. Aeration was achieved through an additional filter-covered plughole. The whole system was placed in a water bath and incubated at 37°C.

Bacterial suspension. An overnight culture of *S. epidermidis* strain RP62a (ATCC no. 35984) was diluted with nutrient broth to a concentration of 1.75×10^5 cfu/ml. Subsequently, 300 ml of this bacterial suspension were added to each flask. The suspension was shaken continuously with a magnetic stirrer at 15 rpm.

Test performance. The sterile catheters were immersed in the bacterial suspension. After 30 min, 3 h, 12 h and 24 h, respectively, one catheter was removed from each test system. To remove non-attached bacteria, the catheters were gently rinsed with 20 ml of a NaCl 0.9% solution. A 4-mm piece was cut off and rolled over a blood agar plate, according to the method of Maki *et al*. [29], to detect the presence of viable bacteria adherent to the foreign body surface. Antibiotic susceptibility of positive cultures was determined by measurement of the minimal inhibitory concentration (MIC). Each time a catheter was removed, the bacterial concentration of the suspension was measured (cfus) with a Spiralometer (Spiral Systems, Cincinnati, OH, USA) and screened for the development of rifampin-resistant strains. The remaining 6-mm pieces were prepared for SEM.

Bacterial contamination of catheters in an animal model

A newly developed intracranial animal infection model was used [25–27], and 55 New Zealand White rabbits were randomised into five groups:

(1) control group, sterile implanted unimpregnated catheters;
(2) unloaded catheters, infected with *S. aureus*, (1×10^4 cfu/ml);
(3) unloaded catheters, infected with *S. epidermidis*. (1×10^8 cfu/ml);
(4) rifampin-impregnated catheters, infected with *S. aureus*, (1×10^4 cfu/ml);
(5) rifampin-impregnated catheters, infected with *S. epidermidis*, (1×10^8 cfu/ml).

Test strains included a biofilm-producing *S. epidermidis* strain RP62a (ATCC no. 35984), and a *S. aureus* strain (5aW) obtained from a clinical specimen. As reported previously, the threshold dose for achieving a reproducible and reliable foreign body CNS infection was found to be 1×10^4 cfu/ml for *S. aureus* and 1×10^8 cfu/ml for *S. epidermidis* [25].

After implantation of the respective catheter through a fronto-parietal burrhole under stereotactical guidance, either 100 μl of a suspension of *S. epidermidis* (1×10^8 cfu/ml) or *S. aureus* (1×10^4 cfu/ml) were injected through its lumen into the surrounding brain tissue (three additional holes were cut into the walls of each catheter prior to implantation). The animals were killed on the sixth postoperative day. Each catheter was removed under sterile conditions; a 7-mm section was plated directly on to blood agar base (Oxoid) and also added to some brain heart infusion (BHI) broth. The remaining 3-mm section was prepared for SEM.

This protocol was reviewed and approved by the local state authorities (Regierungspräsidium Karlsruhe). All procedures were performed in accordance with the Animal Care Guidelines of the University of Heidelberg.

Scanning electron microscopy

The catheters obtained from the in-vitro experiments were fixed with glutaraldehyde 2.5% in 0.1 M sodium phosphate buffer (pH 7.3) for 24 h. Afterwards, they were briefly rinsed with distilled water to prevent crystalline artefacts. Specimen dehydration and critical-point drying were not used. To examine both the inside and the outside of each catheter, the longitudinally divided samples were mounted on to aluminium stubs. The samples were then coated with gold in a Polaron SEM sputter coater (2.4 kV, 20 mA, 3.5 min) and examined with a Philips PSEM 500 scanning electron microscope.

The rabbit brain implants were rinsed with NaCl 0.9% solution and fixed with glutaraldehyde 2.5% in 0.1 M sodium phosphate buffer (pH 7.3) for 24 h. The implants were sequentially dehydrated for 10 min in 50%, 70%, 90% and 96% ethanol solutions and then twice for 20 min in absolute ethanol to prevent crystalline artefacts. After critical-point drying, mounting on stubs and gold sputtering, SEM was performed as described above.

Results

Original silicone catheters without bacterial challenge

The SEM examination of the catheter surfaces showed a very rough appearance. The inside surface was interspersed with grooves and fissures while the outside had the appearance of a 'lunar landscape', with smooth bumps and irregular holes (Fig. 1a).

Rifampin-impregnated silicone catheters without bacterial challenge

The inner and outer surfaces of the catheters were covered with rifampin crystals of different shapes and sizes (5–40 μm), that originated from the impregnation process (Fig. 1b). This surface appeared to be even rougher than that of the original catheters. No difference could be found between the inside and outside of the catheter.

In-vitro experiments

Unloaded catheters. The surfaces of catheters removed from the bacterial suspension after 30 min were already densely populated with bacteria (Fig. 2a); *c.* 5×10^5 cells were attached per mm^2. Surface irregularites appeared to be the preferential sites of attachment and individual organisms showed first signs of biofilm production. After 3 h, the surface was almost completely covered with a bacterial monolayer. In some areas a high degree of biofilm production could be detected. Heavy colonisation with multiple layers of staphylococcal cells, combined with a pronounced production of exopolymer products, occurred after 12 h (Fig. 2c). After 24 h the catheter surface was covered with a thick layer (10–30 μm) of slime-embedded bacteria (Fig. 2e). Cell division was detectable throughout the 24-h incubation period. The concentration of *S. epidermidis* in the suspension increased continuously to 5×10^7 cfu/ml after 24 h.

Cultivation of the catheter surfaces revealed viable *S. epidermidis* RP62a colonies in all specimens.

Rifampin-impregnated catheters. At 30 min, 3 h and 12 h after exposure, very few single staphylococcal cells were seen on the inner and outer catheter surfaces, attached between or on the rifampin crystals (Fig. 2b and d). At 30 min, one-to-three bacteria could be seen per mm^2 and after 12 h this number increased to 300–400. In all specimens, the bacteria appeared as single cell bodies without signs of cell division. Biofilm production was not detected at any time. All bacterial cultures obtained simultaneously from the catheter surface were sterile. Because of the release of rifampin from the catheter surface, the bacterial concentration of the suspension decreased continually, reaching 400 cfu/ml after 12 h. After incubation for 24 h, various bacterial colonies were visible on the inner and outer catheter surfaces, even next to or on the rifampin crystal residues. The bacteria were obviously alive, showing signs of cell division and enclosing themselves within a slime matrix (Fig. 2f). At this time a positive bacterial culture could be obtained and the bacterial concentration in the suspension rose to 2×10^7 cfu/ml. Sensitivity testing of *S. epidermidis* RP62a obtained

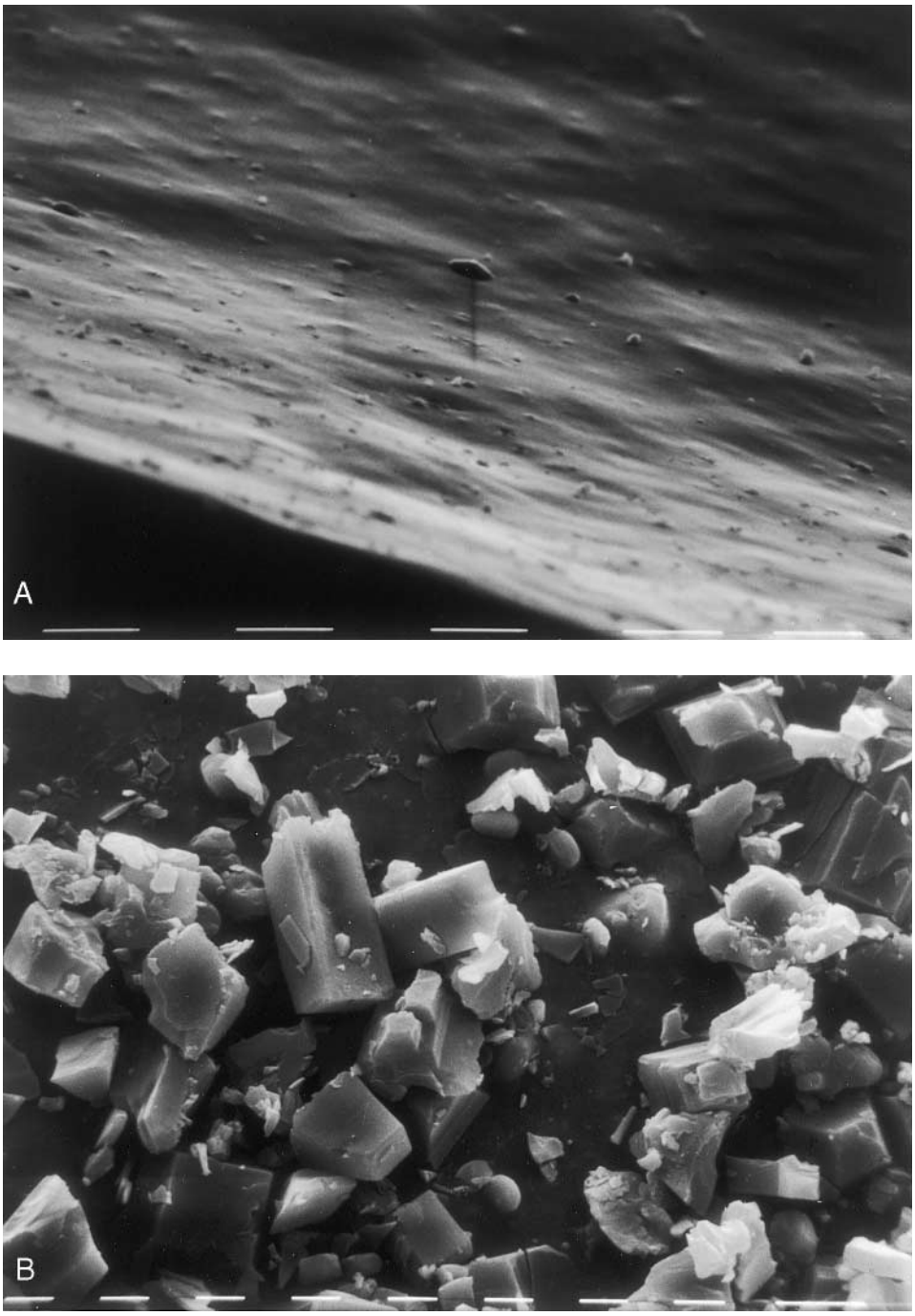

Fig. 1. SEM of (**a**) the surface of an unused silicone ventricular catheter (Cordis®) (bar = 10 μm) and (**b**) the outer surface of a rifampin-impregnated catheter (bar = 10 μm). Note the surface irregularities of the unused original catheter, which tend to act as sites for bacterial adherence. The rifampin-impregnated catheter surface has a very rough appearance due to the rifampin crystals of different sizes and shapes (5–40 μm).

from the suspension and the catheter surface revealed the development of rifampin resistance.

Animal model

SEM investigations of all catheters showed a wide variety of deposits on the catheter surfaces, 60–90% of which were covered with leucocytes, erythrocytes and fibrin-like amorphous material.

Animals with unimpregnated catheters. Both *S. epidermidis* and *S. aureus* colonies were detected between the deposits. The bacteria were usually covered with an amorphous slime matrix and were difficult to visualise. *S. epidermidis* colonies seemed to be embedded in a smooth slime matrix (Fig. 3a), whereas *S. aureus* colonies were surrounded by a more 'crumbly' extracellular material. Cultivation of the catheters showed the presence of *S. epidermidis* in 100% of the cases and *S. aureus* in 92% of the cases.

Animals with rifampin-impregnated catheters. Similar to the in-vitro results, single *S. epidermidis* and *S. aureus* cells were rarely detected and bacterial colonies

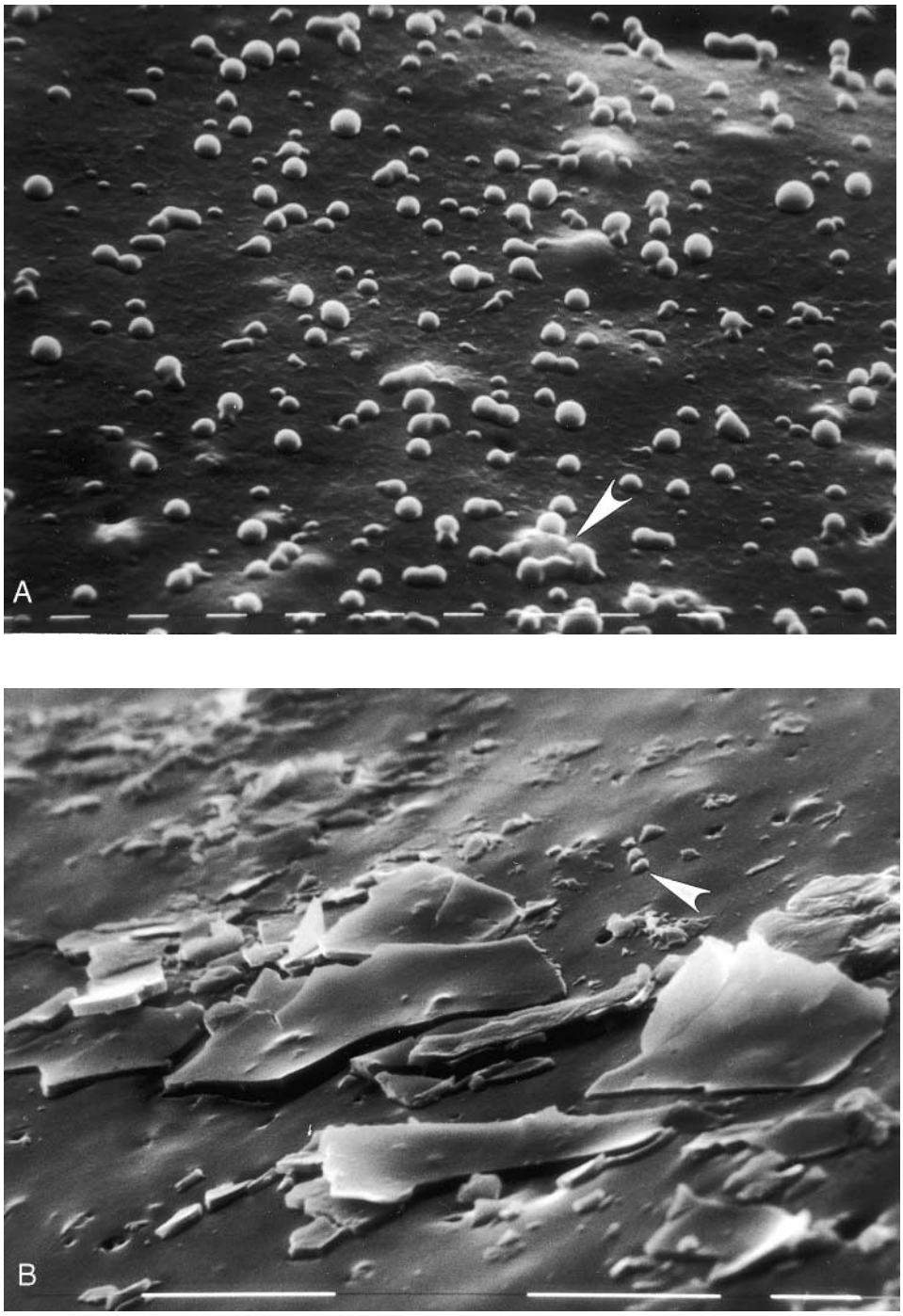

Fig. 2. SEM of unimpregnated catheters and rifampin-impregnated catheters exposed *in vitro* to *S. epidermidis* for 30 min, 12 h or 24 h. (**a**) At 30 min, the unimpregnated catheter already shows a surface densely populated with bacteria and the first signs of slime production (arrow) (bar = 1 μm). (**b**) In contrast, on the rifampin-impregnated catheter very few single round bacteria (arrow) were discovered without signs of cell division or slime production (*c.* 1–3/mm^2) (bar = 10 μm). (**c**) At 12 h, the surface of the unimpregnated catheter is almost completely covered with a bacterial monolayer and in some areas marked slime production is visible (arrow) (bar = 1 μm). (**d**) This is in striking contrast to the rifampin-impregnated catheter, where only few bacteria (200–400/mm^2) without any visible signs of proliferation were detected (arrow) (bar = 10 μm). (**e**) At 24 h after bacterial exposure, the surface of the unimpregnated catheter was covered by a thick layer of slime-embedded bacteria (bar = 10 μm). (**f**) Colonies of bacteria with slime matrices can also be recognised on the rifampin-impregnated catheter (bar = 10 μm).

could not be seen at all. The single bacteria resembled 'ping-pong balls' and showed no signs of cell division or biofilm production (Fig. 3b). The fact that all catheters were sterile after culture suggested that the visible cells had been rendered non-viable by the released rifampin.

Discussion

In recent years, more insight has been gained into the basic mechanisms of bacterial adhesion and colonisation of foreign body surfaces. After adherence to an implant surface, mediated primarily by bacterial

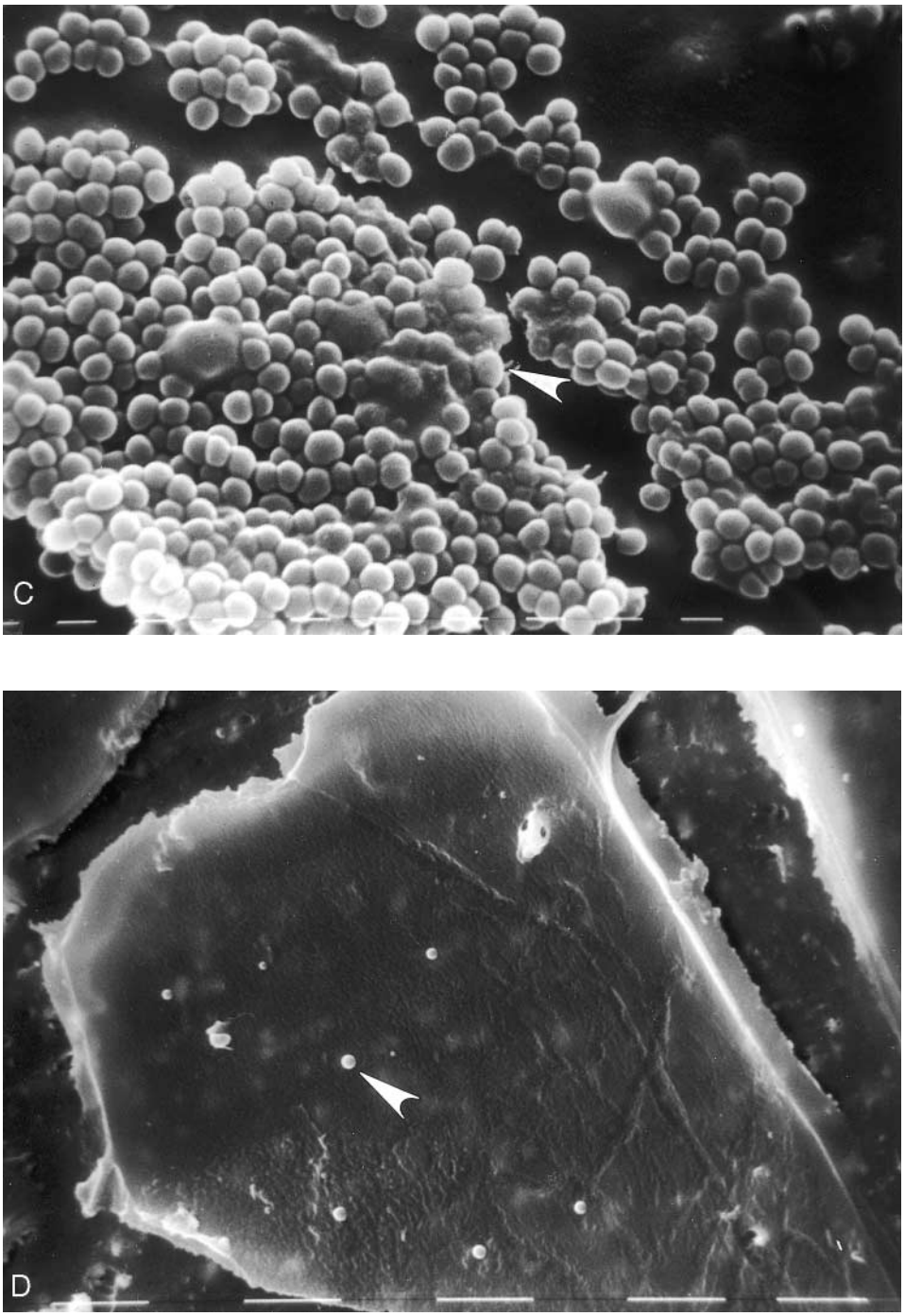

Fig. 2. (*Continued*)

adhesins of a proteinaceous or carbohydrate nature, or both, several bacteria (especially staphylococci), are able to produce extracellular slime matrices. This biofilm seems to protect the bacteria from host defence mechanisms by specific and non-specific interference with the immune system [11, 13–15]. In addition, it acts as a barrier to antibiotics [10–12, 23].

Intracerebral implants face especially difficult conditions. Because of the blood-brain barrier, intracerebral antibiotic concentrations are reduced and host immune defence mechanisms are limited initially [30]. Therefore, incorporation of antibiotics on to or into the implants themselves, to provide initial and long-lasting bactericidal antibiotic concentrations at their surface, has been investigated by several research groups as a possible preventive approach [4, 19–23]. However, the inability to achieve sufficient antibiotic loading of the silicone polymer was the main cause of failure to prevent shunt infections.

In 1989, Bayston *et al*. described a new impregnation procedure capable of conferring antimicrobial protection against coagulase-negative staphylococci for up to 28 days [17]. The most effective catheter design was achieved with a combination of rifampin and clindamycin at a concentration of 0.2%. The main aim of this impregnation process was protection against luminal

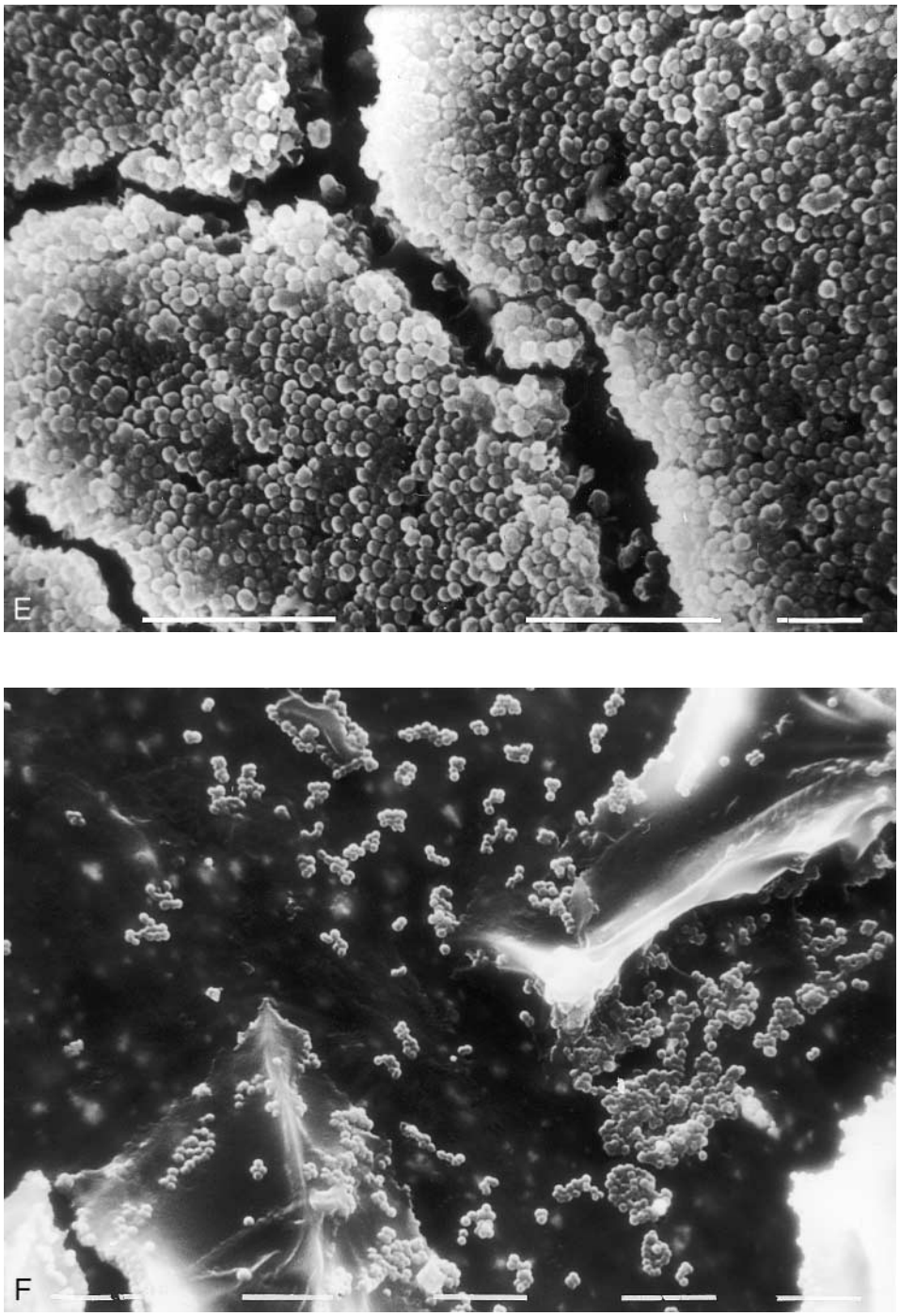

Fig. 2. (*Continued*)

colonisation, but the question of whether this catheter can also reduce the risk of external infections was not investigated. A follow-up in-vitro study showed an extension for up to 56 days in the protective activity of purely internal shunt colonisation [31]. However, to date, the efficacy of this catheter system *in vivo* has not been reported.

A somewhat different rifampin impregnation procedure was developed by Schierholz *et al*. [24]. This method allows a high initial burst with an adjustable concentration, followed by long-lasting, continuous release over >60 days. The underlying concept of this catheter system is different from that of Bayston *et al*. [17]. Besides the prevention of bacterial adhesion or colonisation of the foreign body surface, the intention is to achieve a high therapeutic concentration of antibiotic (rifampin) in the CSF and the surrounding brain tissue (initial burst), in order to kill all bacteria in the contaminated surgical area immediately after wound closure. To achieve this goal, the antibiotic load of the silicone catheter had to be as high as possible; 9% (w/w) was achieved in this catheter system. *In vitro*, a significant reduction of bacterial adhesion was observed with a one million-fold difference [24]. In the cerebrospinal infection model,

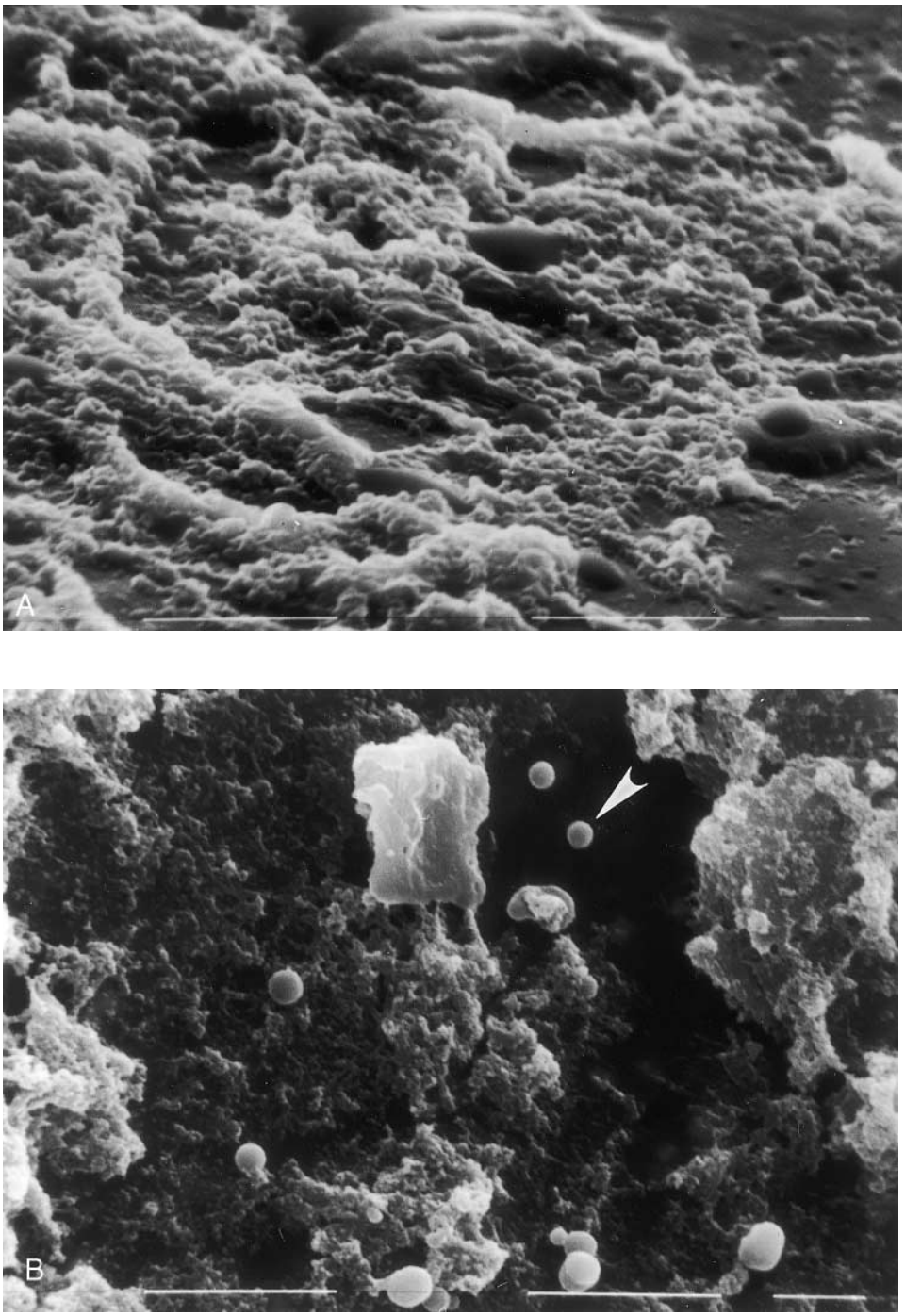

Fig. 3. Unimpregnated and rifampin-impregnated catheters investigated in the animal infection model (6 days postoperatively). (**a**) The unimpregnated catheter was challenged with 1×10^7 cfu of *S. epidermidis* RP62a (bar = 10 μm) and staphylococcal colonies covered with an amorphous slime matrix can be detected between the multiple deposits. **(b)** In contrast, on the rifampin-impregnated catheter challenged with 1×10^3 cfu of *S. aureus*, only a small number of bacterial cells are visible as single cells with no colony formation (arrow) (bar = 10 μm).

preliminary results showed that intracerebral and intraventricular infections could be treated effectively and the colonisation of the catheter surface prevented efficiently in all animals examined [26]. To understand the reasons for this result, the present study evaluated this CSF shunt catheter system by SEM and bacterial culture methods *in vitro* as well as in the rodent infection model.

By challenging the unmodified native catheters with *S. epidermidis*, previous in-vitro observations made by Peters *et al*. [9] were confirmed. With the increase in exposure interval (30 min, 3 h, 12 h and 24 h), a progressive colonisation of the catheter surface was detected, especially at sites of surface irregularities. First signs of biofilm production appeared after only 30 min and multilayer bacterial colonies (up to 40 μm) covered the whole catheter surface after 24 h.

In contrast, on the extremely rough crystal-covered

surface of the rifampin-impregnated catheters, bacteria were rarely visible between or on the residues of the rifampin crystals during the first 12 h, despite this predisposition for initial adherence. No signs of cell division, slime production or colony formation were visualised. This was presumably due to the bactericidal effect of rifampin exposed on the polymer surface. The fact that all simultaneously obtained bacterial cultures were sterile confirmed that the adherent bacterial cells were killed. Thus, although the desired prevention of initial bacterial adhesion was not achieved completely, clinically relevant colonisation of the catheter surface was completely prevented.

Rifampin is well known for the development of single-step resistance [32]. In contrast to the specimens obtained at 30 min, 3 h and 12 h, SEM of the rifampin-impregnated catheter removed at 24 h showed an increased number of adhering bacteria surrounded by a typical biofilm. Their viability was verified by cultivation. At the same time, an increased bacterial concentration was observed in the test suspension. Rifampin sensitivity testing of cultivated bacteria from both the catheter surface and the suspension revealed the development of resistance to rifampin. In the in-vitro infection model, the catheter surface area was small relative to the volume of the surrounding bacterial solution. This obviously led to rifampin concentrations below the MIC with progression of the experiment; thus, rifampin resistance could develop. These observations clearly demonstrate that the prevention of bacterial colonisation is dependent on the bactericidal effect of the incorporated rifampin and show the necessity for higher antibiotic loading or combination with another antibiotic to exclude the possibility of the development of resistance.

However, the development of resistance to rifampin did not occur in the cerebrospinal infection model, because of the large catheter surface area in relation to the surrounding CSF volume and brain tissue, leading to much higher concentrations of rifampin. The inoculated staphylococci were eradicated completely by the bactericidal effect of high local rifampin concentrations, as shown by the negative results of bacterial culture.

SEM analysis of catheters used in the in-vivo experiments revealed that leucocytes, erythrocytes and fibrin-like amorphous substances covered 60–90% of the catheter surfaces. Consistent with previous findings on contaminated CSF shunts [33], intravenous catheters [34, 35] or pacemakers [36], the *S. epidermidis* or *S. aureus* colonies detected on the unloaded catheter surfaces were predominantly embedded in extracellular slime matrices. In contrast, on the inner and outer surfaces of the rifampin-impregnated catheters, very few single staphylococcal cells were detected in areas where the surface was directly visible. On the inside of the catheter, residues of the rifampin crystals were still visible, whereas on the outside they had disappeared almost completely, probably due to the catheter's permanent contact with the surrounding brain tissue and CSF. In agreement with the in-vitro experiments, the bacteria discovered did not show any signs of viability.

The incorporation of a combination of rifampin with other antibiotics into a silicone polymer to minimise the risk of development of resistance against rifampin is now under investigation [37].

We are indebted to S. Camp for her help in proofreading this paper. This research was supported by grant nos. 01 KI 8816/2 and 01 KI 9453 to A. A. and J. A. H. from the Bundesministerium für Forschung und Bildung, BMBF.

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