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ORIGINAL PAPER

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# Granulocyte colony-stimulating factor for the treatment of biomaterial-associated staphylococcal infections in-vitro

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**Abstract** Staphylococcal infections are a common and severe complication after the implantation of a prosthesis. We developed an in-vitro model for biomaterial-associated infections and studied the effects of human recombinant granulocyte colony-stimulating factor (rhuG-CSF; filgrastime) on the eradication of bacteria from the surface of biomaterial. Latex beads (25 $\mu$m) were incubated with $10^7$ colony forming units of either a slime producing (DSM 3269) or non-slime producing strain (ATCC 14990) of *Staphylococcus epidermidis*. Infected particles were consecutively confronted with effector cells, derived from heparinized whole blood samples taken from healthy volunteers, after stimulation with rhuG-CSF (5,000 IU/ml, 10,000 IU/ml). Control blood specimens were not stimulated or conditioned with normal saline. The results indicate that stimulation with rhuG-CSF induced an increased rate of phagocytosis and lead to a more rapid reduction of adhering bacteria from the surface of the beads. Therefore, the in-vitro data suggest that patients with prosthesis infection may profit from an additional treatment with rhuG-CSF.

**Keywords** rhG-CSF · Biomaterial · *Staphylococcus epidermidis* · Phagocytosis

## Introduction

Prosthesis infections are a major clinical complication due to an increasing use of biomaterials for diagnostic and therapeutic purposes [7, 9, 17]. The pathogenesis of biomaterial-associated infections (BAI) is multifactorial and still poorly understood; it has been suggested to include microbial virulence, environmental preconditions and characteristics of the material itself [6, 13]. Predominantly staphylococci (coagulase-negative and coagulase-positive) are involved in BAI [9, 11, 14]. The bacterial colonization and formation of a biofilm coat is facilitated by the production of microbial slime and the specific bacterial adhesion to host proteins that were adsorbed on the polymer [1, 9, 11, 14]. In addition, host defence mechanisms are compromised by toxic components released from the implant, resulting in the deactivation of effector cells and a decrease of intracellular killing. Once established, BAI is resistant to antibiotic therapy, thus, in most cases, the implant has to be removed [6]. However, host defence against invading pathogens is directed by cytokines, of which colony-stimulating-factors (CSF) are of major interest [2, 5, 13].

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that regulates the proliferation and differentiation of myeloid progenitor cells as well as the efflux of cells out of the bone marrow [3, 8, 16]. Moreover, this growth factor stimulates the functional activities of mature leukocytes. Finally, G-CSF and other CSFs have been reported to enhance the defence of bacterial and fungal infections in normal or immunocompromised hosts and support the efficacy of antibiotic therapy [3, 4, 8, 12, 13, 16, 19].

Therefore, we speculate that CSF may also increase the antimicrobial potency of the immune system in the presence of implanted biomaterial. To test this hypothesis, we developed an in-vitro model and determined whether the stimulation of polymorphonuclear leucocytes (PMN) with G-CSF increases the phagocytosis of bacteria from the surface of the biomaterial.

## Materials and methods

### Bacterial strains

A slime-producing strain (DSM 3269, Deutsche Stammsammlung von Mikroorganismen, Göttingen, Germany) and a non-slime-

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producing strain of coagulase-negative *Staphylococcus epidermidis* (ATCC 14990, American Type Culture Collection, Manassas, USA) were used.

### Fluorescence labeling of bacteria

Bacterial strains were cultured in Todd Hewitt yeast bouillon (Oxoid, Basingstoke, Hampshire, England). The bacterial concentration (colony forming units, cfu) of the suspension was counted by spectrophotometry (Ultrospec III, Pharmacia Biotech, Amersham, USA) at 600 nm. The bacteria were washed with phosphate buffered saline (PBS Dulbecco, pH 7.2, Biochrom, Berlin, Germany) and labeled with biscarboxyethylcarboxyfluorescein-pentaacetoxymethyl-ester (BCECF/AM: final concentration 1 $\mu$m/l, 30 min, 37°C) (Molecular Probes, Eugene, Ore., USA). BCECF emits green fluorescence after excitation at 488 nm. The bacteria were washed twice with PBS at 3,000 *g* (5 min) and were resuspended in PBS ($10^9$ cfu/ml). All experiments with labeled bacteria were performed in darkness.

### Colonization of biomaterial

Latex beads of 25 $\mu$m diameter were used (SD 26 styrene-divinylbenzene suspension; Sigma-Aldrich-Chemical, Deisenhofen, Germany). A total of $4\times10^8$ labeled bacteria were incubated with 100 $\mu$l of the bead-suspension (beads:bacteria 1:70). After 30 min incubation in a thermal mixer (Eppendorf, Hamburg, 37°C, 1,400 rpm), colonized biomaterial was washed with PBS at 3,000 *g* (5 min) and the pellet was resuspended in 1 ml PBS. The colonization density achieved was confirmed by florescence microscopy (Leitz Dialux 22 EB, Wetzlar, Germany). Colonized beads were immediately used for confrontation experiments.

### Preparation of polymorphonuclear leukocytes

Whole blood of healthy volunteers (aged 20–40 years) was heparinized with 10 IU/ml (LiqueminN, Roche, Grenzach-Wyhlen, Germany). Test samples were exposed to rhuG-CSF (Neupogen, Amgen, Munich, Germany) (5,000 IU/ml or 10,000 IU/ml; 10 min; Hybridisierungsofen OV5, Biometra, Göttingen, Germany), or to an aliquot of normal saline, before entering the confrontation assay.

### Confrontation assay

The confrontation of bacteria, beads and effector cells was performed in 1 ml polypropylene tubes. Fresh 200 $\mu$l whole blood samples ($10^6$ PMN; cytokine-treated or control) were added to each tube and incubated with 50 $\mu$l suspension of free bacteria ($10^8$ cfu/ml), native beads or colonized beads. Confrontation was carried out under gentle centrifugation (1,400 rpm) for 10, 30, 60, 120 or 180 min (37°C). The reactions were terminated by placing the tubes on ice. A total of 2 ml of FACS lysing solution (Becton Dickinson, Heidelberg, Germany) were added and fragments of erythrocytes were removed at 300 *g* (5 min, 5°C). The pellet was washed twice (NaCl 0.9%) and resuspended in 300 $\mu$l normal saline.

### Flow cytometry

A fluorescence activated cell sorter (FACScan, Becton Dickinson, San Jose, USA) emitting an argon laser beam at 488 nm (Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) was used. The instrument settings for the quantification of phagocytosis were: forward scatter (FSC) threshold 52 (for detection of PMN sized particles); detector set at E00, 414, 672, and 690 for forward scatter (FSC), SSC (for detection of granulocytes granularity), fluorescence 1 (FL1, green; for detection of BCECF-labeled bacteria), and fluorescence 2 (FL 2, red), respectively. To measure the elimination of bacteria from the surface of the beads (i.e. the decrease in mean fluorescence intensity), the instrument settings were E00, 321, 635, and 753 for FSC, SSC (for detection of beads), FL 1, and FL 2. Signals were gated from 10,000 cells per tube. Data were processed using CellQuest (version 3.1) software (Becton Dickinson Immunocytometry Systems, Heidelberg, Germany). The fluorescence distribution was displayed as two-color contour plot analysis or as single histograms for SSC and FL 1. Up-take of bacteria by effector cells was quantified as the phagocytic index: number of fluorescent PMN to number non-flourescent PMN (%). Elimination of bacteria from the surface of the beads was expressed by the decline of mean fluorescence intensity of beads. Assuming a first order linear regression, the elimination kinetic was expressed as a sigma-minus-plot of the elimination curves (within the first 30 min of confrontation). The negative decline of regression lines were compared.

### Statistics

The Chi-squared and Barlett test procedures were used. Sigma-minus-plots were performed to describe the kinetics in phagocytosis rate and elimination of bacteria from the surface of beads. Mean data were compared using the Mann-Whitney U-test (Winstat, version 3.1, Kalmia).

## Results

### Fluorescence labeling

The efficacy of fluorescence labeling of ATCC 14990 and DSM 3269 bacteria with BCECF/AM according to the present procedure was more than 95%. Fluorescence activity was stable for more than 1 h.

### Colonization of biomaterials

Due to the fact, that polystyrene beads (data not shown) could not be colonized with bacteria, we decided to use latex beads for further experiments. The efficacy for colonization was high with 73% (non-slime-producing ATCC 14990) to 85% (slime-producing strain DSM 3269) of beads being colonized and only 15% (DSM 3269) to 22% (ATCC 14990) of bacteria remaining free in suspension (Fig. 1).

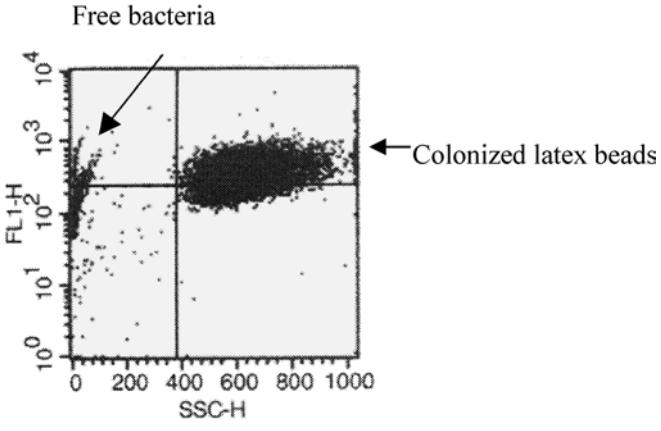


**Fig. 1** Dot-plot analysis of colonized latex beads

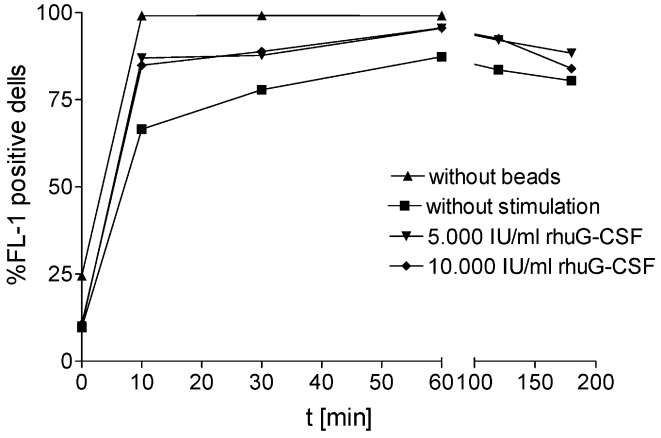


**Fig. 2** Kinetics of Fl 1-positive polymorphonuclear leukocytes indicate an increase of phagocytosis of BCECF-labeled, non-slime producing ATCC 14990 bacteria by cytokine-stimulated PNM

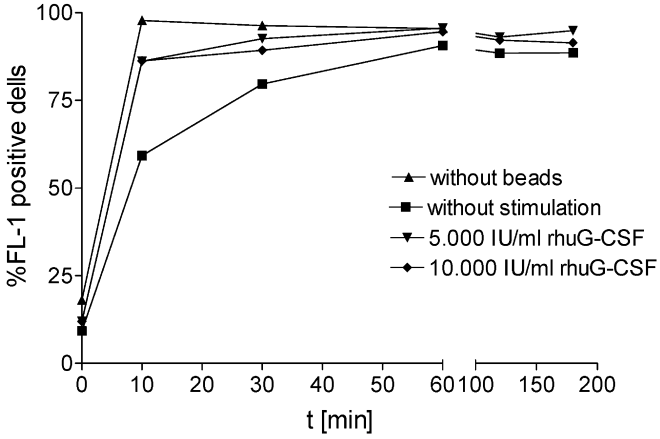


**Fig. 3** Kinetics of the percentage of Fl 1-positive polymorphonuclear leukocytes indicate an increase of phagocytosis of BCECF-labeled, slime producing DSM 3269 bacteria by cytokine-stimulated PNM

### Confrontation experiments

Ten minutes after confrontation, 99% of native, non-cytokine activated PMN (native) were already loaded with fluorescent bacteria, with free bacteria (i.e. in the absence of beads) in suspension (ATCC 14990 and DSM 3269). Slime production of the strain did not influence the phagocytic index.

Latex beads of 25 $\mu$m diameter were not phagocytised by PMN.

### Protracted phagocytosis in the presence of biomaterial

In contrast, less PMN were loaded with fluorescent bacteria in the presence of latex beads: only 59–65% of PMN phagocytised after 10 min, and 76–78% after 30 min (Figs. 2, 3). However, after 60 min nearly all PMN were phagocytising.

### Activation of PMN by rhuG-CSF

Treatment of native PMN with 5,000 IU/ml rhuG-CSF increased the rate of phagocytising effector cells by up to 85–86% within the first 10 min and 89–92% 30 min after confrontation with colonized bacteria of both strains ($P = 0.01714$–$0.04645$). Activation with 10,000 IU/ml rhuG-CSF did not further increase phagocytosis.

### Increased elimination of bacteria from the surface of beads

Adhering bacteria were eliminated more rapidly if PMN were treated with rhuG-CSF: a decline of 29–36% of fluorescence intensity of latex beads was already noticed 30 min after confrontation with cytokine-activated PMN, compared to 180 min in the case of native PMN. We observed a faster elimination of the slime-producing

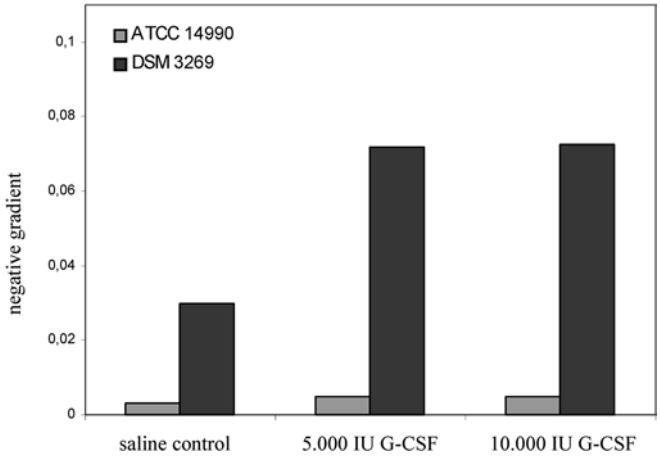


**Fig. 4** Sigma-minus-plot analysis of the reduction of adhering bacteria from the surface of the biomaterial

strain (DSM 3269) from the surface of the beads than of the non-slime-producing strain (ATCC 14990).

Moreover, regression analysis indicates that the activation of native PMN with 5,000 IU/ml rhuG-CSF caused a similar elimination kinetic to 10,000 IU/ml rhuG-CSF and was superior to controls (Fig. 4). However, the sigma-minus plot analysis demonstrated a more pronounced eradication of slime-producing bacteria from the surface of beads by cytokine-stimulated PMN compared to non-slime producing bacteria.

## Discussion

Nosocomial morbidity and mortality of BAI is predominantly mediated by *Staphylococcus epidermidis* infections [2, 5, 6, 9, 13, 14, 17]. These microorganisms are able to attach to the surface of the biomaterial as well as to biomaterial-bound host proteins. Further colonization increases the anchorage and production of

biomatrices, which represent a protective barrier against host defence mechanisms [1, 7, 9, 11, 14]. Once an infection has occurred, it is often resistant to antibiotic therapy and to the host defence system, thus, in most cases, the implant has to be removed. To control BAI, the anti-bacterial activity of local phagocytes seems to be of major importance, especially at an early stage of development of the infection. Stimulation of phagocytosis and microbicidal activity of PMNs by G-CSF in general have been documented for various microorganisms [4, 8, 12, 13, 16, 18, 19]. This was confirmed for different strains of free staphylococcal bacteria in the present investigation. In concert with other mechanisms, such as respiratory burst, the increased phagocytic activity was suggested to depend on a different expression of phagocyte integrins [18].

Only a few studies have attempted to counteract BAI by immunomodulation of the host response. Rozalska et al. [13] showed that granulocyte-macrophage colony-stimulating factor- (GM-CSF) coated implants reduced bacterial survival around an infected biomaterial in neutropenic mice, but abscess formation could not be prevented in the long term. Henke et al. [5] observed that the downregulation of MHC class II antigens in macrophages close to infected biomaterial was associated with enhanced prostaglandin $E_2$ ($PGE_2$) and interleukin (IL)-1 production. Blocking $PGE_2$ and IL-1 with indometacin and anti-IL-1, increased the bacterial clearance [5]. Boelens et al. [2] showed that subcutaneous injections of interferon-gamma, which is an activator of mononuclear cell functions, could prevent the intracellular persistence of *Staphylococcus epidermidis* in the vicinity of an implanted biomaterial in mice.

We speculated that G-CSF could effectively improve the anti-bacterial activity of phagocytes in the presence of an infected biomaterial. To test this hypothesis, we first developed an in vitro model, that was optimized and standardized by several pre-test investigations (data not shown in detail). We induced a standardized high colonization rate of latex beads and, as expected, recognized a slightly increased colonization for slime-producing bacteria. Moreover we found that the phagocytic index of native PMN initially decreased to about 40% in the presence of beads, irrespective of the bacterial strain, and it took more than six times longer until all PMN were phagocytising.

As a major result, treatment of PMN with rhuG-CSF significantly increased the phagocytic index (plus 25%). But it did not recover full activity during the initial phase of confrontation. Nevertheless, the elimination of bacteria from the surface of beads was approximately six times more rapid by cytokine-stimulated PMN. Finally, no dose-response relation was observed above a concentration of 5,000 IE/ml rhuG-CSF.

In conclusion, the present paper demonstrates for the first time that rhuG-CSF accelerates the phagocytosis and elimination of bacteria from the surface of colonized latex beads. The preclinical data recommend an early supportive therapy of infected prostheses with rhuG-CSF, e.g. in conjunction with antibiotic treatments. Finally, with the present in-vitro model, future studies can easily be performed to investigate the properties of other biomaterials, different bacteria and the influence of new drugs on BAI.

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