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New cytokine dressings. II. Stimulation of oxidative burst in leucocytes in vitro and reduction of viable bacteria within an infected wound

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Abstract

Recently, we have developed the new dressings containing rhG-CSF or rhGM-CSF. In the present study we investigated either in vitro or in vivo biological activity of the dressings. Human whole blood samples were incubated with extracts from the collagen- or polyurethane-based dressings containing rhG-CSF or/and rhGM-CSF and phagocytic and oxidative metabolic activities were quantitated using Phagotest or Bursttest kits. The results indicate that both the number of phagocyting cells and the intensity of phagocytosis per cell, as well as the level of the oxidative burst in particular, were stimulated by one or both of the cytokines extracted. Next, the experimental skin wounds in mice were infected with 10^7 CFU of *Pseudomonas aeruginosa* strain ATCC 27853 and treated locally with the rhG-CSF-containing dressing. The analysis of the biopsies taken from the wounds indicated that in mice treated with the cytokine-containing dressing on the third day the log of CFU per biopsy was 5.0 vs. 6.2 in the control ($P < 0.001$), and on the 8th day was lower than 4 vs. 5.4 in control ($P < 0.0001$). Our findings clearly suggest that the newly designed dressings containing the incorporated CSFs can be used as effective topical cytokine-delivery system in the treatment of bacterial infections in wounds. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Cytokine dressings; rhG-CSF; rhGM-CSF; Phagocytic activity; Oxidative burst; Topical cytokine-delivery system; Murine model of infected wound

1. Introduction

Bacterial infection of wounds can significantly compromise the healing process. Recently, new methods of the infected wound care have been developed using antimicrobial dressings composed of the collagen biomaterials and antibiotics either in free or liposomal forms (Trafny et al., 1996; Grzybowski et al., 1997) or based on the polyurethane sponge containing liposome-encap-

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sulated antibiotics (Price et al., 1990). However, overuse of antibiotics may result in the development of drug resistance of bacterial pathogens (Cohen, 1992; Goldman et al., 1996). Indeed, the rational use of topical antibiotics is possible only when the special, rigorous criteria are fulfilled, as summarized in the recent review of Langford and Benrimoj (1996).

Over the last few years topical administration of cytokines to the wounds, both as the supplementation to antibiotics or as sole factors, has become a subject of the intensive investigation. In fact, reports of the encouraging effects of the local administration of various peptide cytokines and growth factors that act as physiological stimulators of the healing process, are accumulating (Andreatta-van Leyen et al., 1993; Bennett et al., 1993; Smith et al., 1994; Abraham and Klagsbrun, 1996; Martin, 1997). It appears that of the many factors tested to date granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are among the most powerful stimulators of wound repair. Indeed, topical administration of GM-CSF has been reported to enhance the healing of skin injuries, including trophic and leprotic wounds, in both animal models and human patients (Kaplan et al., 1992; Jyung et al., 1994; Pojda and Struzyna, 1994, Hui et al., 1996). Moreover, as reviewed recently by Schneider and Dashner (1998), G-CSF and, to a lesser extent GM-CSF, may be used as the valuable complementary drugs for the systemic treatment of bacterial and fungal infections.

Recently, we have developed the 'cytokine dressings' containing G-CSF or GM-CSF. As indicated in part I of the presented studies, we were able to retrieve a significant part of the incorporated cytokines from the dressings, as determined by the specific ELISA tests, during the three-step extraction procedure (Grzybowski et al., Part I, this issue).

In this part of the study we set out to evaluate the biological activity of rhG-CSF (Neupogen®) and rhGM-CSF (Leucomax®) incorporated into the collagen- and polyurethane-based dressings by: (i) evaluation of in vitro activity of the both cytokines extracted from the dressings, and (ii) examination of in vivo effectiveness of the polyurethane dressings containing rhG-CSF(Neupogen®). In the in vitro experiments the biological activity of the both CSF-s after their extraction from the dressings on the phagocytic activity of human leucocytes was shown. In the in vivo experiments we have demonstrated for the first time that the rhG-CSF-containing polyurethane sponge dressings significantly accelerated the reduction of the viable *Pseudomonas aeruginosa* cells within the experimentally infected skin wounds in mice.

2. Materials and methods

2.1. Preparation of the dressings and extraction of cytokines therefrom

The cytokine-containing dressings (1×1 cm) were prepared as described previously (Grzybowski et al., Part I, this issue). Briefly, rhG-CSF (recombinant human granulocyte-colony stimulating factor) in the form of the commercial preparation Neupogen® (Hoffman-La Roche) or rhGM-CSF (recombinant human granulocyte/macrophage-colony stimulating factor) in the form of the commercial preparation Leucomax® (Sandoz Pharma) were incorporated into the dressings prepared using either the collagen sponge Biokol (Stalmed, Poland) or polyurethane sponge Ligasano® (Ligamed Medikal Produkte, Germany) as the base. The final concentration of the cytokines was 0.5 μg/cm^2 for the in vitro and 15 μg/cm^2 for the in vivo experiments. These concentrations resulted from the loading level of the cytokines used. All the dressings were prepared in sterile conditions and stored at 4°C. Some of them were freeze-dried. Altogether, five types of the cytokine-containing dressings have been prepared (Table 1).

Extraction of the cytokines was performed for 2 days at 37°C using the three-step extraction procedure with polyurethane sponge collectors, as previously described (Grzybowski et al., Part I, this issue). This procedure resulted in the release of about 25 or 50% of the incorporated G-CSF or GM-CSF (respectively), as determined by the spe-

cific ELISA tests. The extracts were stored for several days at 4°C without any noticeable loss of the cytokine concentrations.

2.2. Evaluation of the leukocyte phagocytic activities

2.2.1. Estimation of the fraction of phagocyting cells and the amount of phagocytosed material per cell

To examine the effect of cytokines on the phagocytic potential of human peripheral blood leukocytes (neutrophils and monocytes) the specific Phagotest kit (Orpegen Pharma, Germany) was used. Briefly, the G-CSF and GM-CSF extracts were initially diluted to the concentration of 50 ng/ml, as determined by the ELISA diagnostic kit (Grzybowski et al., Part I, this issue). Twenty μl of such solutions were added to 100 μl of the heparinized whole blood obtained from a healthy volunteer or a diabetic patient with recurrent staphylococcal infections and the samples were incubated for 1 h at 37°C and 5% CO_2. Then, 20 μl of the suspension of the opsonized FITC-labeled *Escherichia coli* or PBS (control) were added to the blood samples which after a short incubation in ice bath were supplemented with 100 μl of the 'Quenching Solution'. After washing with the 'Washing Solution', the erythrocytes were disrupted by adding 2 ml of the 'Lysing Solution' to the blood samples. Finally, 200 μl of the 'Staining Solution' was added to the samples and percent of the phagocyting cells as well as the intensity of the fluorescence per cell were evaluated as described below.

2.2.2. Determination of the oxidative burst

To estimate the effect of the extracted cytokines on the oxidative metabolism of peripheral blood granulocytes and monocytes in vitro the specific Bursttest (Phagoburst®) kit was used. For examination of the stimulation of the oxidative burst in human peripheral blood leukocytes the cytokine extracts were added to the heparinized blood samples, as above; the samples were obtained from two healthy donors, one diabetic patient with chronic staphylococcal infections and one renal failure patient. After incubation for 60 min at 37°C, 5% CO_2, 20 μl of either opsonized, nonlabeled *E. coli* suspension, PMA (phorbol 12-myristate-13-acetate) solution or fMLP (*N*-formylmethionyl-leucyl-phenylalanine) solution included in the kit were added to the samples and incubated for 10 min at 37°C in the water bath. After that, 20 μl of 'Substrate Solution' (dihydrorhodamine 123; DHR123) was added and incubated for another 10 min at 37°C. At the end of the incubation the blood samples were lysed and fixed with 2 ml 'Lysing Solution', then washed and supplemented with 200 μl DNA 'Staining Solution'. Measurements of the cell suspensions were done using the flow cytometer, as described below, within 30 min after 10 min incubation with the 'Staining Solution'.

2.3. Flow cytometry analysis

Fractions of phagocytic cells, intensity of fluorescence of the labeled phagocytized bacteria per cell and percent of cells with oxidative burst were measured in the blood samples with use of the FACSCalibur" (Becton-Dickinson, USA) flow cytometer equipped with the laser beam emitting

Table 1
Types of the cytokine dressings examined in this study

Type of dressing matrix (code)	Cytokine incorporated	Cytokine content (μg/cm²)	Form of the dressing	Type of experiment
Collagen sponge (CS-FD)	GM-CSF	0.5	Freeze-dried	In vitro
Polyurethane sponge (PS-FD)	GM-CSF	0.5	Freeze-dried	In vitro
Polyurethane sponge (PS-FD)	G-CSF	0.5	Freeze-dried	In vitro
Polyurethane sponge (PS-W)	G-CSF	0.5	Wet	In vitro
Polyurethane sponge (PS-W)	G-CSF	15	Wet	In vivo

the blue-green 488 nm light. For the analyses of blood leukocytes from the samples (at least 15 000 cells per sample) the CellOuest" software (BDIS, California) was utilized. The fluorescence was estimated using histograms from the FL2 channel.

2.4. Investigation of stability of the cytokine dressings

Two types of the cytokine dressings were subjected to stability control: (i) collagen sponge containing GM-CSF, freeze-dried (code CS-FD), and (ii) polyurethane sponge containing G-CSF, freeze-dried (code PS-FD), were stored for 3 months at 4°C.

The dressings were undertaken to extraction: immediately after preparation, after 2 weeks, after 1, 2, and 3 months. In the separate experiment the freshly prepared freeze-dried dressings of the same types were sterilized by irradiation (25 kGy) and extracted a few days later. All the extracts were then examined using Bursttest.

2.5. The murine model of the infected wound

The superficial nonlethal infection was performed according to Stepinska et al. (1995). Briefly, 40 C3H/BL6 mice weighting 20–25 g were anesthetized with diethylether inhalation. Pieces of skin 1.5×1.5 cm were cut on the back of the animal but one of the edges of the flap remained connected to the dorsal skin. Next, 0.05 ml of *Pseudomonas aeruginosa* ATCC 27853 suspension was injected between the very thin skin muscle and the paraspinus muscle.

The mice were divided into two groups, and infected wounds were dressed with two types of the dressings-with or without (control) the rhG-CSF. Each dressing was placed on the infected muscle and covered with the flap of the skin which was sutured to the surrounding tissue.

2.6. Bacteriological examinations of biopsies

The mice were sacrificed by transection of the spinal cord under ether anesthesia on the 1st, 2nd, 3rd, and 8th day after infection. Next, the dressings were removed and superficial muscles were excised from the infected area to yield about 100 mg of the tissue. The excised material (biopsies) was homogenized and used for determination of *P. aeruginosa* colony forming units (CFU) by plating.

2.7. White blood cell counts

In a separate set of the experiments white blood cell counts (WBCs) were checked in the following groups of the wounded mice: (i) control, noninfected mice whose wounds were covered with the dressings that did not contain rhG-CSF, (ii) infected mice with wounds covered with the dressings that did not contain rhG-CSF, (iii) noninfected mice with wounds covered with the rhG-CSF-containing dressings, and (iv) infected mice with wounds covered with the rhG-CSF-containing dressings. On the 1st, 2nd, 3rd, and 8th day after infecting the wounds blood samples were collected to heparinized tubes from the extraorbital sinuses of the anaesthetized mice and the numbers of leukocytes per ml were estimated using the haematologic Sysmex analyzer.

2.8. Statistical analysis

The significance of the differences between groups was evaluated by the Student's t- test.

3. Results and discussion

In the first part of our study (Grzybowski et al., Part I, this issue) we have documented that both the CSFs used can be extracted in a significant amount from the spongy experimental dressings. However, the ELISA test employed for that part of the study was based on the recognition of the antigen epitopes only. The epitopes of the either rhG- or rhGM-CSF were not destructed during the preparation and extraction procedures. In the study presented here we demonstrate that the two above-mentioned cytokines extracted from our experimental dressings possess an in vitro biological activity. We also present in this study an in vivo efficacy of the polyurethane dressing containing rhG-CSF.

To our knowledge, the results of this study are the first to describe the stimulatory effects of the extracts of the dressings containing rhG-CSF (Neupogen®) or rhGM-CSF (Leucomax®). In the first step of our investigation the effects of the cytokine extracts on phagocytic parameters of peripheral blood human leukocytes obtained from a diabetic patient with recurrent staphylococcal infections were studied. This particular donor was chosen because the baseline (control) numbers of leukocytes phagocyting *E. coli* obtained from normal, healthy individuals range between 65 and 95% for monocytes and 95–99% for granulocytes (Brosche and Platt, 1995). In fact, the mean fraction of the active phagocytes (nonstimulated by cytokines) in the whole blood (i.e. the natural mixture of granulocytes and monocytes) of our diabetic patient did not exceed 65%. In spite of this, only a moderate (i.e. never exceeding 90%) increase in the numbers of active phagocytes within the tested cell populations could be detected after incubation of the patient's blood samples with all four extracts of the cytokines. This is possibly due to a development of a state of anergy induced in circulating leukocytes of the diabetic by the recurrent bacterial infections. All the extracts obtained from CS-FD or PS-FD dressings contained GM-CSF, and from CS-FD or PS-W dressings containing G-CSF stimulated the blood leukocytes' phagocytic activity to a comparable extent. Moreover, the extracts from the CS-FD or PS-W dressings containing rhG-CSF and to a lesser extent obtained from the dressings PS-FD containing GM-CSF stimulated the fluorescence intensity inside of the leucocytes used for the test (see Table 1 for codes of the dressings).

In the next step of the study the oxidative burst in the leukocytes was tested as the end result of incubation of the whole blood samples with the cytokine extracts. Indeed, respiratory burst which begins with the formation of superoxide followed by H_2O_2 and other reactive oxygen species (ROS) is a crucial arm of the phagocytes' microbiocidal activity (Smith and Weidemann, 1993). In order to quantitate the production of ROS in the blood leukocytes tested, we used the commercial Burst-test kit employing as substrate dihydrorhodamine 123 (DHR123) which is one of the most sensitive

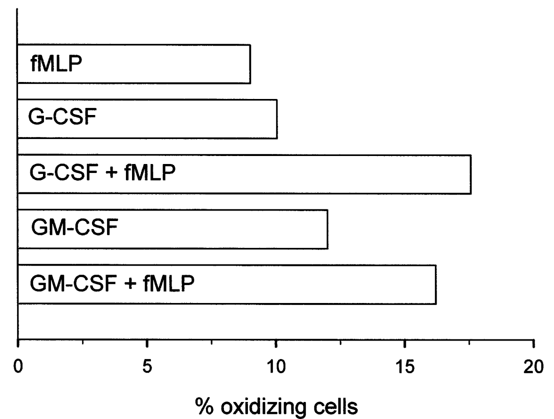

Fig. 1. Stimulation of the oxygen burst by rhG-CSF and rhGM-CSF extracted from the polyurethane dressing-wet (non-lyophilized) and freeze-dried, respectively, in the peripheral blood leukocytes obtained from a diabetic patient with chronic bacterial infection. The results present the average of three experiments.

fluorogenic probes for flow cytometric measurements (Smith and Weidemann, 1993; Vowells et al., 1995). To test if the leukocytes obtained from different donors respond in the present study to the classical weak and strong stimulators of the oxidative metabolism in cells, the samples were obtained from two healthy donors, one diabetic patient with recurrent staphylococcal infections and one renal failure patient and incubated with *N*-formyl-Met-Leu-Phe (fMLP), phorbol 12-myristate-13-acetate (PMA) or unlabeled *E. coli.* The results obtained indicate that the latter two factors exhibited maximum stimulation in cells from all the donors, whereas fMLP triggered oxidative burst only in relatively small fractions of the leukocytes tested (data not shown), and its activity can be substantially elevated when the responding cells are primed by cytokines for an enhanced respiratory burst. Thus, in the following experiments blood cells obtained from one healthy donor and the diabetic patient were primed with fMLP and then incubated with extracts from the GCSF and GM-CSF-containing dressings.

As indicated in Fig. 1 and Fig. 2, G-CSF and GM-CSF extracted from polyurethane-based dressings (non-lyophylized or lyophylized, respec-

tively) were effective in triggering oxidative burst in the whole blood samples obtained from either the diabetic or normal, healthy individuals. These differences between G-CSF + fMLP or GM-CSF + fMLP vs. fMLP for the blood samples of the healthy donor (presented in Fig. 2) were verified as statistically significant ($P < 0.0001$). Importantly, when the two cytokine extracts were simultaneously added to the blood sample from the latter donor, stimulation of the oxidative metabolism was almost twice as high as those with each cytokine tested alone (Fig. 2). This very high difference between G-CSF + fMLP + GM-CSF + fMLP vs. GM-CSF + fMLP ($P < 0.00001$) points on the strong additive effect of both CSF-s when they are used simultaneously.

Similar results-high effectiveness of the biological activity of the cytokines extracted were obtained when the lyophilized cytokine dressings: either polyurethane-based with rhG-CSF (PS-FD) or collagen-based with rhGM-CSF (CS-FD) were stored at 4°C for 2 or 3 months, respectively. Moreover, rhGM-CSF incorporated to the CS-FD type of the dressing was not susceptible to the electron-irradiation (25 kGy). While, the rhG-CSF incorporated to the PS-FD type of the dressing almost completely lost their activity after irradiation. This shortest time of rhG-CSF stability (only 2 months) within freeze-dried dressing and susceptibility of this cytokine to the electron-irradiation evidence a higher lability of the rhG-CSF molecule as compared to rhGM-CSF.

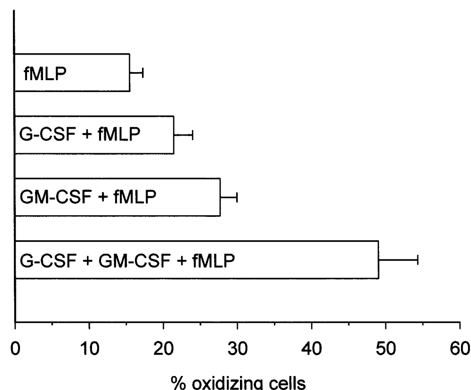

Fig. 2. Stimulation of the oxygen burst by rhG-CSF and rhGM-CSF extracted from the polyurethane dressing-wet (non-lyophilized) and freeze-dried, respectively, in the peripheral blood leukocytes obtained from a healthy donor. The results present the average (± S.D >) of six to nine experiments.

Some authors suggested that topical administration of GM-CSF can increase the rate of wound healing as a result of macrophage stimulation (Jyung et al., 1994), while others indicated that the principal target of the cytokine action is a polymorphonuclear leukocyte (Sullivan et al., 1993). In fact, although macrophage can be regarded as a central modulator of various cell functions during repair of injured tissues (Browder et al., 1988; Danon et al., 1989; DiPietro, 1995), granulocytes are important eliminators of bacteria that may enter and colonize wound sites (Simpson and Ross, 1972, Smith and Weidemann, 1993).

In their recent study, Baran and co-workers have demonstrated that in contrast to monocytes which died by apoptosis early (2–4 h) after the commencement of phagocytosis of various bacteria in vitro, the life-time of granulocytes was prolonged rather than inhibited by their contact with and phagocytosis of the microorganisms (Baran et al., 1996). These findings suggest that triggering of the microbiocidal activities in granulocytes of prolonged viability by GM- and/or G-CSF in vivo may importantly contribute to reduction in the number of bacteria within an infected wound.

In fact, the dressings designed and prepared by us may constitute an effective system of the topical delivery of cytokines to wounds and thus represent a potential powerful therapeutic strategy in wound care. The dressings used in our study seems to be stable enough for further in vivo experiments. The encouraging results of treatment of both the acute and chronic skin injuries (Kaplan et al., 1992; Jyung et al., 1994; Pojda and Struzyna, 1994; Hui et al., 1996) provide support for and substantiate this latter conclusion.

From the two CSF-s used in our studies, rhGM-CSF can stimulate exclusively human leucocytes. However, rhG-CSF can stimulate both human and murine cells in vitro and in vivo, and for this reason in the next part of the experiment only the rhGC-SF (Neupogen®)-containing

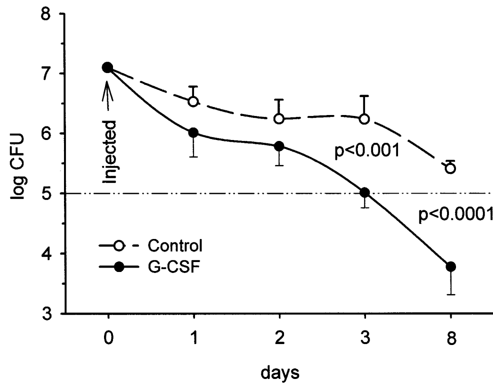

Fig. 3. Reduction of bacterial cell number in wounds experimentally infected with *P. aeruginosa*. The wounds were dressed with the polyurethane sponge dressings with or without (control) the incorporated rhG-CSF. The results present the means ± S.D. calculated from five repetitions.

polyurethane dressings were tested for their efficacy in fighting bacterial infections in vivo using the murine skin infected wounds. This model of skin wound, which was developed in our laboratory (Stepinska et al., 1995), was already proved by us to be a valuable tool for investigating antimicrobial activities of the antibiotic-containing dressings in vivo (Trafny et al., 1996; Grzybowski et al., 1997).

As indicated in Fig. 3, on the first and on the second day of observation the numbers of CFUs in the biopsies from the wounds covered with the rhG-CSF-containing dressings were lower than in the control groups of mice. However, these differences were not significant. The significant differences began to appear on day third post-infection. In fact, on the 3rd day after the injection of the *P. aeruginosa* cells into the wound the log CFU number in the biopsies equaled to five in the rhG-CSF-treated group of mice, as compared to 6.2 in the control group ($P<0.01$). This difference was even more pronounced on day 8 post-infection when the log CFU number in the biopsies from the experimental group of mice declining to below 4 per biopsy as compared to 5.4 in the control ($P<0.001$). In view of the fact that the strain of mice used in the present study ([C3H/BL6]F1) is characterized by the efficient immunological system, the described by us significant antimicrobial effect of the rhGC-SF-containing dressings in these mice is of special importance. Indeed, the efficiency of the dressings housing rhG-CSF in fighting the *P. aeruginosa* infection in vivo appeared to be comparable to that of the previously employed by us collagen dressings with incorporated the liposome-encapsulated antibiotic, polymyxin B (Trafny et al., 1996).

An effect of exogenic cytokines delivered into an infected wound is recognized insufficiently so far. Bacterial pathogens infecting wounded tissues can influence the cytokine regulatory network either by induction or by inhibition of some cytokine synthesis. Many cytokines (including some CSFs) can bind to bacterial cells producing stimulation of their growth. Also, bacterial proteases can degrade cytokines by proteolysis (for review see Wilson et al., 1998).

The antimicrobial effect of rhG-CSF does not necessarily mean that this cytokine will accelerate healing of the all types wounds in vivo. In fact, Jyung and co-workers were not able to detect any significant effect of the recombinant rat G-CSF (rrG-CSF) on the healing process of the wounds in rats whereas rrGM-CSF led to the markedly enhanced healing (Jyung et al., 1994). However, these authors utilized the noninfected, incisional wound model and measured the breaking strength of the healing site as the indicator of the healing process. It cannot be excluded therefore that their preparation of G-CSF would exert its bactericidal effect within the infected wound and lead thereby to the accelerated tissue repair.

Also, in contrast to the results of Jyung et al. (1994) who found that topical treatment of wounds with G-CSF led to a significant elevation of the circulating white blood cells in rats, we were not able to detect any significant changes in the peripheral blood WBC of the rhG-CSF-treated as well as of all the three remaining groups of mice (Fig. 4). One possible explanation is that the dose of the cytokine applied by Jyung and co-workers directly to the wound (30 μg) was sufficient to exert its systemic effect, i.e. enhance proliferation and/or release of the leukocytes from the bone marrow. Moreover, one can conclude that in their model of wound the cytokine penetrated well into the circulation. In our study the

initial concentration of rhG-CSF in the dressings used by us was as high as 15 μg/cm^2 In fact, this amount should be decrease to about 4 μg/cm^2 because of about 25% rate of the cytokine release observed previously (Grzybowski et al., Part I, this issue). In our experiments the amount of the cytokine that probably penetrated into the circulation was probably too small to cause any detectable elevation of the number of circulating leukocytes.

To our knowledge, this is the first report of the antimicrobial topical effect of rhG-CSF (Neupogen®) contained in the polyurethane sponge in vivo. In conclusion, taking into account our findings (from both of the in vitro and in vivo investigation) we can suggest that one or several cytokines incorporated into dressings and applied directly to infected wounds may provide a new, powerful tool for fighting of the infection being an alternative way to a therapy with antibiotics.

Thus, in turn, topical application of the cytokines may be also helpful in approaching the strategic purpose in wound healing biology which has been defined recently by Martin (1997), as aiming to more perfect skin regeneration which should be fast, effective and without any loss of the tissue.

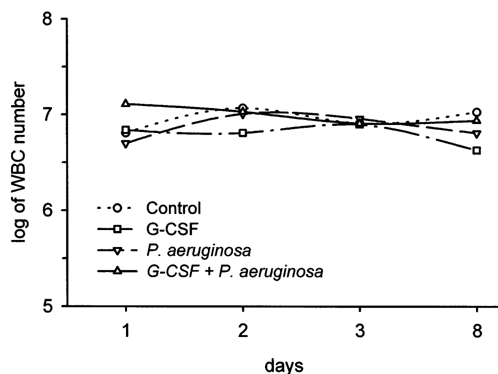

Fig. 4. Peripheral blood leukocyte counts (WBC) per 1 ml in mice with the experimental wounds in four groups of animals: (i) infected with *P. aeruginosa* and dressed with polyurethane sponge without the cytokine; (ii) only treated with the rhG-CSF-containing polyurethane dressings; (iii) only infected with *P. aeruginosa* and dressed with polyurethane sponge without the cytokine; (iv) infected with *P. aeruginosa* and treated with the rhG-CSF containing polyurethane dressings. The results present the means calculated from four repetitions.

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