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The effect of recombinant human granulocyte/macrophage-colony-stimulating factor (rHu GM-CSF) and rHu G-CSF administration on neutrophil chemiluminescence assay in patients following cyclic chemotherapy

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Abstract Secondary infections related to neutropenia and functional defects of phagocytes are common consequences in patients treated for cancer. The hematopoietic colony-stimulating factors (CSF) have been introduced into clinical practice as additional supportive measures that can reduce the incidence of infectious complications in patients with cancer and neutropenia. The aim of this study was to determine the role of granuolcyte/macrophage(GM)-CSF and granulocyte(G)-CSF in enhancing in vivo human neutrophil function. A luminol-dependent chemiluminescence assay was developed to evaluate whether the repair in neutropenia accompanies the ability of neutrophils to function. A dose of 5 μg G-CSF kg^{-1} day^{-1} [recombinant human (rHu) G-CSF; filgrastim] or 250 μg GM-CSF m^{-2} day^{-1} (rHu GM-CSF; molgramostim) was administered subcutaneously once daily to 12 metastatic cancer patients being treated with different cytotoxic regimens. All injections of CSF were given after the initiation of neutropenia and continued until the occurrence of an absolute neutrophil recovery. rHu GM-CSF and rHu G-CSF, administered once daily at the 250 μg m^{-2} day^{-1} and 5 μg kg^{-1} day^{-1} level, were effective in increasing the absolute neutrophil count and neutrophil function, as measured by an automated chemiluminescence system.

Key words Chemotherapy · Granulocyte-colony-stimulating factor · Neutrophil · Granulocyte/macrophage-colony-stimulating factor

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Introduction

Neutropenia caused by chemotherapy-related myelosuppression is one of the most important causes of poor prognosis and mortality in cancer patients. Cytokines that enhance the differentiation of stem cells to mature neutrophils and monocytes are of the greatest value for survival [1]. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) and granulocyte-colony-stimulating factor (G-CSF) are biological agents, cytokines, that have been cloned, and recombinant forms of these glycoproteins have been produced and purified [4, 11, 16, 23]. This has created the opportunity to test the function of these agents in vivo. These growth factors are required for the proliferation and differentiation of hematopoeitic progenitor cells; in addition they also enhance the bactericidal and phagocytic activity of neutrophils brought about by receptors that exist on their surface [17]. The phagocytic functions that are affected by these cytokines include an oxidative burst by neutrophils in response to certain soluble and particulate stimuli that leads to an increase in neutrophil oxygen uptake [14, 20].

In this experimental system *N*-formylmethionyl-leucyl-phenylalanine (FMet-Leu-Phe) has been used as a chemoattractant that has been demonstrated to induce a neutrophil oxidative response [21]. Zymosan-activated-serum (ZAS) was used as a soluble stimulus to increase neutrophil oxygen uptake and oxidative burst. Zymosan is a polysaccharide cell-wall extract of yeast that is capable of activating the alternative pathway of the complement system when it is incubated in fresh serum [19].

This study examined the in vivo effects of GM-CSF and G-CSF administration on the respiratory burst metabolism of neutrophils by using a luminol-mediated chemiluminescence system. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is employed to amplify chemiluminescence signals [2]. Luminol is oxidized by some reactive species of oxygen generated during the respiratory burst. The oxidation of luminol results in the

production of an exited aminophthalate anion that relaxes to the ground state with the production of light [3]. Emittance is at around 450 nm, so that the liquid scintillation counter method is applicable in addition to single-photon counting [2].

Patients and methods

Patients

All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of 1964 Declaration of Helsinki.

Twelve patients between 30 and 73 years of age with stage II–IV solid tumors were entered in the study and informed consent was obtained for all participants. All patients were receiving combination chemotherapy and had developed chemotherapy-induced neutropenia in previous chemotherapy cycles. Patients were divided into two groups of six patients each. The groups received 5 μg kg^{-1} day^{-1} G-CSF or 250 μg m^{-2} day^{-1} GM-CSF. CSF were administered after the initiation of chemotherapy-induced neutropenia (absolute neutrophil count $< 2 \times 10^9$/l) until the occurrence of neutrophil recovery. Daily a complete blood count was performed on 2-ml EDTA-anticoagulated samples using a Coulter counter. Three different measurements of neutrophil chemiluminescence were obtained; before treatment, after the initiation of neutropenia and after neutrophil recovery. Patients' characteristics and selected chemotherapy regimens are listed in Table 1.

Chemicals

The biosynthetic recombinant human (rHu) GM-CSF preparation (molgramostim) was obtained from Schering Plough (USA) and the rHu G-CSF preparation (filgrastim) was from Roche (Switzerland). Luminol, zymosan A, histopaque 1119, Hanks' balanced salt solution (HBSS) and fMet-Leu-Phe were purchased from Sigma Chemical (St. Louis, M.). fMet-Leu-Phe was used at a final concentration of 10 μmol/l and a stock solution of 1.5 mM luminol was prepared and stored at 4 °C.

Zymosan-activated serum preparation

ZAS was used as a stimulus to enhance oxidative bursts in neutrophils. A 5-mg sample of zymosan was resuspended in 1 ml pooled normal human serum. The mixture was incubated in a 37 °C water bath for 60 min. After incubation, the zymosan was

Table 1 Patient characteristics and treatment regimens. Drug doses are given in miligrams per square meter of body surface area. The days on which the drugs were given on each cycle are shown in parentheses. *G-CSF* granulocyte-colony-stimulating factor, *GM-CSF* granulocyte/macrophage-colony-stimulating factor

Patient age (years), sex	Cancer histology, stage	Cytotoxic regimen: dose (mg m^{-2})	Present chemotherapy cycle	CSF dose and schedule	Duration of neutropenia (day)
1. 30, male	Malignant schwannoma, stage IV	Cisplatin 35 (1 → 5) ifosfamide 1200 (1–5)	4	G-CSF (filgrastim) 5 μg kg^{-1} day^{-1}	8
2. 43, female	Colon cancer, stage III	5-Flurouracil 425 (1 → 5) leucovorin (1–5)	4	G-CSF (filgrastim) 5 μg kg^{-1} day^{-1}	4
3. 67, female	Head and neck cancer, stage II	Cisplain 75 (1), 5-fluorouracil 250 (1 → 4)	3	G-CSF (filgrastim) 5 μg kg^{-1} day^{-1}	4
4. 73, female	Breast cancer, stage IV	Cyclophosphamide 500 (1), methotrexate 40 (1), 5-fluorouracil 600 (1)	1	G-CSF (filgrastim) 5 μg kg^{-1} day^{-1}	4
5. 67, female	Breast cancer, stage IV	Cyclophosphamide 500 (1), methotrexate 40 (1), 5-fluorouracil 600 (1)	1	G-CSF (filgrastim) 5 μg kg^{-1} day^{-1}	2
6. 63, female	Breast cancer, stage IV	Cyclophosphamide 500 (1), doxorubicin 45 (1), 5-fluorouracil 600 (1)	5	G-CSF (filgrastim) 5 μg kg^{-1} day^{-1}	5
7. 51, male	Pancreatic cancer, stage III	Gemcitabine 2000 (1)	2	GM-CSF (molgramostim) 250 μg m^{-2} day^{-1}	4
8. 33, female	Malignant shawannoma, stage IV	Ifosfamide 2500 (1–2), etoposide 120 (3 → 5)	1	GM-CSF (molgramostim) 250 μg m^{-2} day^{-1}	4
9. 55, female	Breast cancer, stage III	Cyclophosphamide 500 (1), methotrexate 40 (1), 5-fluorouracil 600 (1)	1	GM-CSF (molgramostim) 250 μg m^{-2} day^{-1}	9
10. 46, male	Small-cell lung carcinoma, stage IV	Cyclophosphamide 1000 (1), doxorubicin 40 (1), vincristine 1 (1)	1	GM-CSF (molgramostim) 250 μg m^{-2} day^{-1}	7
11. 67, female	Breast cancer, stage IV	Cisplatin 120 (1), paclitaxel 135 (1)	3	GM-CSF (molgramostim) 250 μg m^{-2} day^{-1}	3
12. 60, male	Small-cell lung carcinoma, stage IV	Cyclophosphamide 1000 (1) doxorubicin 40(1), vincristine 1 (1)	1	GM-CSF (molgramostim) 250 μg m^{-2} day^{-1}	5

removed by centrifugation (1500 g) for 20 min and ZAS remained [12]. A 0.5 ml sample of ZAS was divided amongst sterile test-tubes and stored at −20 °C. When used, 0.5 ml ZAS was diluted in 2 ml HBSS into a final concentration of 1 mg/ml.

Preparation of polymorphonuclear leukocytes (PMN)

Fresh heparinized venous blood was obtained from patients before treatment, after the initiation of neutropenia and after neutrophil recovery. Whole blood was layered by gravity over histopaque-1119 (density 1.119 g cm^{-3}; Sigma) and centrifuged at 700 g for 30 min at room temperature (18–26 °C). The mononuclear cells were removed and the granulocyte cell layer was transferred to a tube labeled "granulocytes". This method provided enhanced PMN recovery. The purity of PMN was found to be more than 95% by Wright staining. Purified PMN were washed three times with phosphate-buffered saline (PBS) and resuspended in an appropriate volume of HBSS. The cells counted electronically were found to be more than 95% viable by trypan blue exclusion.

Measurement of chemiluminescence

Luminol-dependent chemiluminescence of neutrophils was measured with a Packard liquid scintillation counter in the single-photon counting mode. Four different reaction mixtures were prepared and placed in scintillation vials. Briefly, each vial contained HBSS, 0.4×10^6 PMN/ml and 0.15 mM luminol in a total volume of 3 ml. ZAS at 200 μg/ml and fMet-Leu-Phe at 10 μmol/l were added to the third and fourth vial respectively. The first vial was used as a background control and included only HBSS and luminol. Four groups of assays were carried out simultaneously in the same run. The change in chemiluminescence per minute (cpm) was continuously monitored for 2 h at 27 °C by measurements made at 15-min intervals.

Statistical analysis

The data were analyzed by Student's two-tailed *t*-test by a Microsoft Excel software program on an IBM computer. $P < 0.05$ was considered to be significant.

Results

A total of 12 patients, mean age 55 years (range 30–73), of both sexes completed the study. The mean baseline white blood cell (WBC) count was $6.26 \times 10^9 \pm 1.31$ (mean ± SD) $\times 10^9$/l in the G-CSF group compared to $(6.51 \pm 1.21) \times 10^9$/l in the GM-CSF group. The duration of neutropenia during which G-CSF and GM-CSF were administered was a mean of 4.5 days (range 2–8) and 5.3 days (range 3–9) respectively. G-CSF medication significantly raised the white blood cell count ($P = 0.006$) while GM-CSF treatment showed no significant change from the baseline counts. The neutrophil response was significantly increased in groups treated with G-CSF or GM-CSF as compared with the baseline counts ($P = 0.005$); ($P = 0.008$) respectively (Table 2).

When compared to baseline measurements, G-CSF treatment enhanced the respiratory burst activity in patient neutrophils without a secondary stimulus ($P < 0.005$) (Fig. 2, Table 4) while GM-CSF treatment resulted in no significant effect (Fig. 1, Table 3).

GM-CSF and G-CSF medication markedly increased the ZAS- and fMet-Leu-Phe-stimulated oxidative burst in patient neutrophils when compared to baseline and neutropenic measurements (Fig. 1, Table 3 and Fig. 2, Table 4) respectively. In both G-CSF- and GM-CSF-treated groups, fMet-Leu-Phe showed a greater ability to stimulate the neutrophil oxidative burst than ZAS, as shown in Figs. 1, 2 and Tables 3, 4.

The data also revealed that neutropenia was accompanied by a decrease in the oxidative activity per neutrophil. The maximum chemiluminescence times were 30–60 min for GM-CSF-stimulated neutrophils and 30–45 min for G-CSF-stimulated neutrophils. fMet-Leu-Phe was found to shorten the maximum chemoluminescence times for both G-CSF- and GM-CSF-stimulated neutrophils (data not shown).

Discussion

GM-CSF and G-CSF have been demonstrated to prime neutrophils to release reactive oxygen species in vitro in response to a secondary stimulus by fMet-Leu-Phe and ZAS [8, 9, 18, 22]. This study investigated the in vivo

Table 2 Mean white blood cell (*WBC*) and neutrophil counts during GM-CSF and G-CSF treatment

CSF	Phase	WBC count ($\times 10^9$/l), mean ± SD	*n*	Neutrophil count ($\times 10^9$/l), mean ± SD	*n*
GM-CSF	Baseline	6.51 ± 1.21	6	4.54 ± 1.59	6
	Neutropenia	2.54 ± 1.02	6	1.43 ± 0.41	6
	Neutrophil recovery	7.85 ± 2.39[*1]	6	5.78 ± 1.95[*4,*5]	6
G-CSF	Baseline	6.26 ± 1.31	6	4.05 ± 1.20	6
	Neutropenia	3.22 ± 0.89	6	1.98 ± 0.60	6
	Neutrophil recovery	22.38 ± 9.94[*2,*3]	6	20.13 ± 9.19[*6,*7]	6

[*1] $P < 0.05$ compared to neutropenia
[*2] $P < 0.05$ compared to baseline
[*3] $P < 0.05$ compared to neutropenia
[*4] $P < 0.05$ compared to baseline
[*5] $P < 0.05$ compared to neutropenia
[*6] $P < 0.05$ compared to baseline
[*7] $P < 0.05$ compared to neutropenia

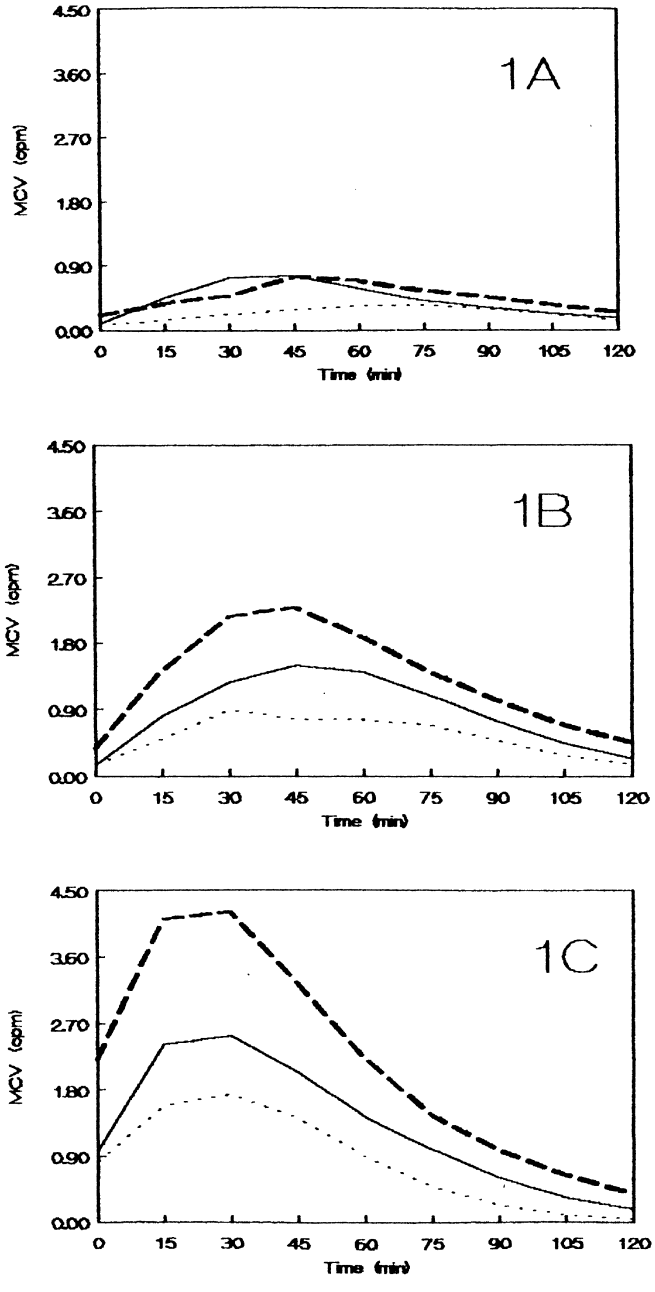

Fig. 1 The effect of granulocyte/macrophage-colony-stimulating factor administration on neutrophil respiratory burst. The change in chemiluminescence per minute (cpm) was continuously monitored for 2 h. The results are expressed as the maximum chemiluminescence value (MCV) $\times 10^{-6}$, $n = 6$. ... Neutropenia, ___ baseline, - - - neutrophil recovery. **A** Oxidative burst in patients' neutrophils after GM-CSF medication. **B** Oxidative burst in patients' neutrophils stimulated with zymasan-activated serum (ZAS) after GM-CSF medication. **C** Oxidative burst in patients' neutrophils stimulated with fMel-Lei-Phe after GM-CSF medication

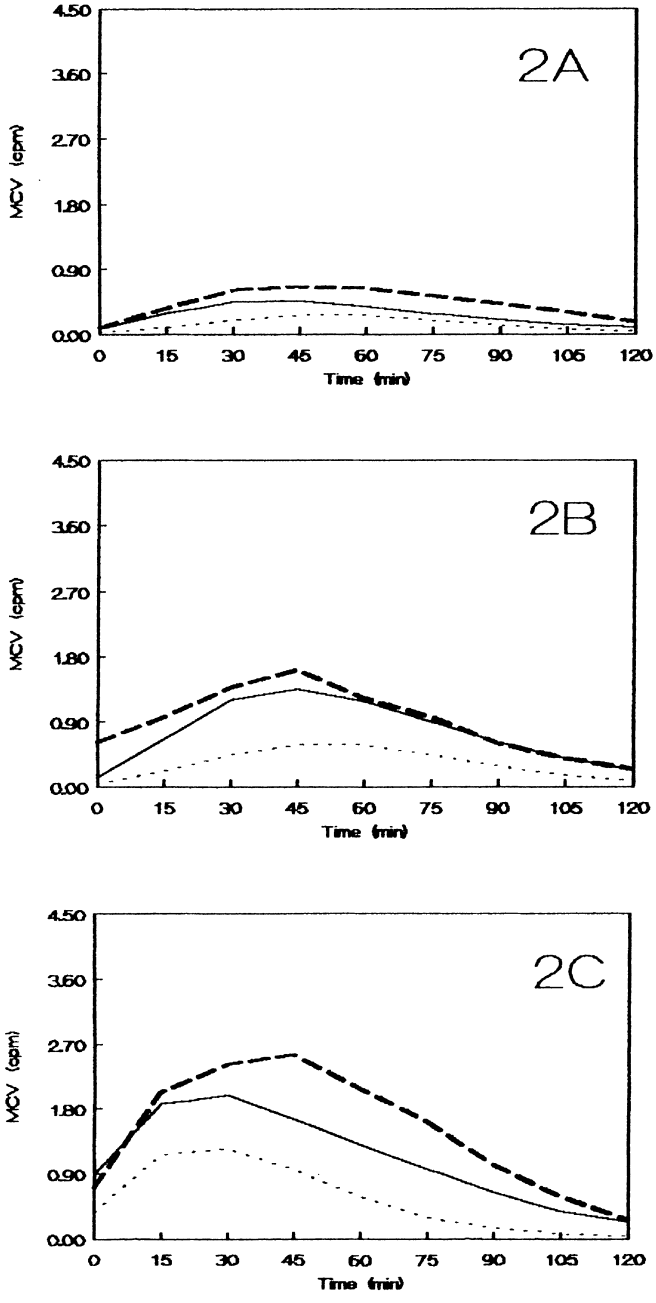

Fig. 2 The effect of G-CSF administration on neutrophil respiratory burst. The change in chemiluminescence per minute (cpm) was continuously monitored for 2 h. The results are expressed as MCV $\times 10^{-6}$, $n = 6$. ... Neutropenia, ___ baseline, - - - neutrophil recovery. **A** Oxidative burst in patients' neutrophils after G-CSF medication. **B** Oxidative burst in patients' neutrophils stimulated with ZAS after G-CSF medication. **C** Oxidative burst in patients' neutrophils stimulated with fMet-Leu-Phe after G-CSF medication

role of GM-CSF and G-CSF in patients following chemotherapy. Chemiluminescence assays were performed to investigate a potential correlation between in vivo and in vitro effects. Similar to the in vitro effect, the systemic administration of GM-CSF and G-CSF augmented the neutrophil respiratory burst in the presence of a secondary stimulus. The priming of the oxidative burst demonstrated that patients' neutrophils retained the ability to respond to in vitro stimulation by fMet-Leu-Phe and ZAS. When compared to baseline measurements, G-CSF treatment enhanced the respiratory burst activity in patients' neutrophils without a secondary stimulus while GM-CSF treatment could not. This

Table 3 Maximum chemiluminescence value (MCV) in patient neutrophils stimulated with zymosan-activated serum (*ZAS*) and fMet-Leu-Phe after GM-CSF medication

Groups	Phase	MCV(cpm), mean ± SEM	*n*
Only GM-CSF medication	Baseline	762 753 ± 13 064	6
	Neutropenia	348 960 ± 8528[*1]	6
	Neutrophil recovery	767 708 ± 17 766	6
GM-CSF medication with ZAS stimulation	Baseline	1 488 496 ± 25 359[*2]	6
	Neutropenia	903 989 ± 25 500	6
	Neutrophil recovery	2 301 323 ± 43 321[*3]	6
GM-CSF medication with fMet-Leu-Phe stimulation	Baseline	2 414 721 ± 47 824[*4]	6
	Neutropenia	1 752 260 ± 25 796	6
	Neutrophil recovery	4 216 548 ± 53 100[*5]	6

[*1] $P < 0.005$ compared to baseline and neutrophil recovery in the group receiving only GM-CSF medication group
[*2] $P < 0.05$ compared to neutropenia in the group receiving GM-CSF medication with ZAS stimulation
[*3] $P < 0.005$ compared to baseline and neutropenia in the group receiving GM-CSF medication with ZAS stimulation
[*4] $P < 0.05$ compared to neutropenia in the group receiving GM-CSF medication with fMet-Leu-Phe stimulation
[*5] $P < 0.005$ compared to baseline and neutropenia in the group receiving GM-CSF medication with fMet-Leu-Phe stimulation

Table 4 Maximum chemiluminescence value (MCV) in patient neutrophils stimulated with ZAS and Fmet-Leu-Phe after G-CSF medication

Groups	Phase	MCV (cpm) mean ± SEM	*n*
Only G-CSF medication	Baseline	458 662 ± 13 064[*1]	6
	Neutropenia	270 718 ± 8528	6
	Neutrophil recovery	668 600 ± 17 766[*2]	6
G-CSF medication with ZAS stimulation	Baseline	1 359 279 ± 13 533[*3]	6
	Neutropenia	588 868 ± 19 118	6
	Neutrophil recovery	1 626 885 ± 42 073[*4]	6
G-CSF medication with fMet-Leu-Phe stimulation	Baseline	1 987 011 ± 30 772[*5]	6
	Neutropenia	1 257 533 ± 19 670	6
	Neutrophil recovery	2 568 135 ± 45 197[*6]	6

[*1] $P < 0.05$ compared to neutropenia in the group receiving G-CSF medication
[*2] $P < 0.005$ compared to baseline and neutropenia in the group receiving only G-CSF medication
[*3] $P < 0.05$ compared to neutropenia in the group receiving G-CSF medication with ZAS stimulation
[*4] $P < 0.005$ compared to neutropenia in the group receiving G-CSF medication with ZAS stimulation
[*5] $P < 0.05$ compared to neutropenia in the group receiving G-CSF medication with fMet-Leu-Phe stimulation
[*6] $P < 0.005$ compared to baseline and neutropenia in the group receiving G-CSF medication with fMet-Leu-Phe stimulation

phenomenon may be explained by the fact that G-CSF has a more restricted action on blood progenitor cells, which are closer to mature effector cells, than did GM-CSF [15]. These data may offer an explanation for why GM-CSF, with standard-dose chemotherapy, has been less beneficial than G-CSF in significantly reducing the incidence of febrile neutropenia [13]. Although the use of GM-CSF resulted in faster neutrophil recovery, significant effects on the incidence of febrile neutropenia, time in hospital and the duration of courses of intravenous antibiotics have not been observed in many studies [6, 13]. This is in contrast with G-CSF [5].

The present results confirmed that neutropenia was accompanied by a decrease in the oxidative activity. This effect can be related to the myelotoxicity of standard chemotherapy regimens and may increase the risk in patients with chemotherapy-associated neutropenia.

Daily administration of 5–10 μg/kg GM-CSF does not alter the white blood cell nadir in patients who have significant leukopenia and neutropenia. Only the duration of leukopenia and neutropenia is shortened [10]. Hoeckman et al. [7] has also observed a reduction in the efficacy of GM-CSF in cyclic chemotherapy. The present data have also confirmed that GM-CSF administration could not increase the white cell count above baseline while G-CSF treatment led to a significant rise in the white blood cells. The neutrophil response was significantly increased in both G-CSF- and GM-CSF-treated groups as compared with the baseline counts.

Recent reports have not recommended CSF administration in afebrile neutropenic patients and there is also little justification for the use of CSF to increase the dose-intensity of chemotherapy. In response to previous data, general therapeutic applications are being developed for

hematopoietic growth factors in clinical practice. They will undoubtedly play a critical role as prophylactic agents in patients at a high risk of developing infection. The patients in the groups treated with G-CSF and GM-CSF are obviously heterogeneous with regard to diagnosis, chemotherapy regimens, metastatic sites, age and gender in this study. All of these parameters might effect the response of immune effectors. Future trials assessing the function of growth factors like G-CSF and GM-CSF comparing these confounding variables are urgently needed in order to obtain better criteria for the use of these growth factors in various clinical settings.

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