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SUPEROXIDE PRODUCTION BY NEUTROPHILS IN CHILDREN WITH MALIGNANT TUMORS TREATED WITH RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR

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ABSTRACT

Background. **Human recombinant granulocyte colony-stimulating factor (rhG-CSF), widely used to combat chemotherapy-induced neutropenia, stimulates both *in vivo* and *in vitro* intra- and extracellular O_2^- production in human polymorphonuclear cells (PMNs).**

Patients and Methods. **Twelve patients with solid tumors or acute lymphoblastic leukemia were treated during induced aplasia with rhG-CSF (5 μg/kg/day). Intra- and extracellular O_2^- production by PMNs isolated from these patients after 5 days of rhG-CSF therapy was assessed following both fMLP and PMA stimulation.**

Results. **All patients showed a rise in PMN count; administration of rhG-CSF enhanced intra- and extracellular O_2^- release after fMLP but not after PMA stimulation.**

Conclusions. **rhG-CSF potentiates *in vivo* O_2^- production by PMNs stimulated with receptor-mediated agonists via G-protein (e.g. fMLP), but not by those stimulated with agonists that bypass receptors via protein kinase C (e.g. PMA).**

Key words: rhG-CSF, superoxide ion, PMNs, tetrazolium salts, malignant tumors

Colony stimulating factors (CSFs) are a group of cell-derived products responsible for the proliferation and differentiation of progenitors cells.[1,2] Human recombinant granulocyte colony-stimulating factor (rhG-CSF) may be useful for preventing or reducing the duration of neutropenia and the associated morbidity and mortality due to infections following cytotoxic chemo- and/or radiotherapy and bone marrow transplants.[3-9] rhG-CSF has also been successfully administered in patients with cyclic neutropenia[10] and congenital agranulocytosis (Kostmann syndrome).[11] Several studies indicate that rhG-CSF helps avoid dose delays, dose reductions and shortening of the cycle time; furthermore, rhG-CSF can be given safely with minimal toxic reactions.[6,7]

Recent evidence suggests that rhG-CSF stimulates the function of mature neutrophils (PMNs). In particular, it has been demonstrated *in vitro* that the antibody-dependent cellular cytotoxicity of PMNs for malignant cells is enhanced by rhG-CSF treatment. This effect may well be associated with an increased ability to produce superoxide (O_2^-) upon stimulation with the chemotactic peptide N-formyl-methionine-leucyl-phenylalanine (fMLP).[12,13] On the other hand, PMN elimination of many pathogens requires the production of reactive oxygen derivatives through the phagocyte respiratory burst pathway. In this series of reactions, molecular oxygen is initially converted to superoxide (O_2^-) and then to hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and possibly the hydroxyl radical ($OH^\bullet$).[14,15] Recent studies show that after administration of rhG-CSF

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Table 1. Characteristics of the 12 patients studied.

case	sex/age	diagnosis	previous therapy	ANC/Lx10^9/L day 0	ANC/Lx10^9/L day 5
1	F/6	Wilms	VCR, ACT-D, IL-2, VP16, CBDCA	0.7	3.5
2	F/4	Rhabdomyosarcoma	CBDCA, VP16	0.5	3.5
3	F/4	Schwannoma	VP16	0.7	4.5
4	M/9	Medulloblastoma	CBDCA, VP16	0.6	5.5
5	F/5	Astrocytoma	CBDCA, VP16	0.7	6.0
6	F/10	Medulloblastoma	CBDCA, VP16	0.6	3.5
7	F/11	Medulloblastoma	CPDD	0.6	2.5
8	M/6	Hepatoblastoma	CBDCA, ADR	0.7	6
9	M/2	Rhabdomyosarcoma	IFOS, VCR, ADR, ACT-D	0.7	6.5
10	M/5	Neuroblastoma	Rhabdomyosarcoma VP16	0.6	3.5
11	M/12	ALL	VP16, ARA-C, MIT, PDS	0.4	4.5
12	M5	ALL	ARA-C, L-ASP	0.5	5.6

VCR: vincristine; ACT-D: dactinomycin; IL-2: interleukin-2; VP16: etoposide; CBDCA: carboplatin; CPDD: cisplatin; ADR: adriamycin; IFOS: ifosfamide; ARA-C: cytarabine; MIT: mitoxantrone; PDS: prednisone; L-ASP: asparaginase.

both extracellular O_2^- release and expression of C3bi-receptors were markedly increased in seven adult malignant lymphoma patients receiving cytotoxic chemotherapy.[16]

The aim of our study was to assess the ability of PMNs collected from children affected by solid tumors or acute lymphoblastic leukemia treated with rhG-CSF to produce intra- and extracellular O_2^-.

Patients and Methods

Patients

Twelve patients (6 males and 6 females) with histologically and immunologically confirmed solid tumors or acute lymphoblastic leukemia were studied between July 1993 and February 1994. The patients ages ranged from 18 months to 14 years. rhG-CSF was administered to patients with absolute PMN counts (ANC) of less than 0.7×10^9/L as a daily intravenous bolus injection for five days (5 μg/kg per day), starting 24 hours after drug-induced aplasia. Bacterially synthesized recombinant human G-CSF (Neupogen) was supplied by Dompè Biotec.

The study was carried out on patients after 5 days of therapy with rhG-CSF. The following hematologic parameters were essential for admission: ANC of more than 2.0×10^9/L; adequate cardiac, renal and hepatic function. All patients were afebrile, and had previously received chemotherapy alone or in combination with radiotherapy. The characteristics of the 12 participants are shown in Table 1. Normal reference values were obtained from 40 healthy donors, aged 20-40 years.

Reagents

Dulbecco's buffer (without calcium chloride and magnesium chloride), pH 7.4. Phorbol myristate acetate (PMA) 1 μg/mL in Dulbecco's buffer (Sigma Chemical Co., St.Louis, MO), nitroblue tetrazolium (NBT) 0.2% in Dulbecco's buffer (Sigma), N-formyl-methionine-leucyl-phenylalanine (fMLP) 10^{-7} mol/L (Sigma), Histopaque 1077 and 1119 (Sigma), EDTA K3 (Sclavo Diagnostics), cytochrome c from horse heart 150 nmol/mL (Sigma), superoxide dismutase (SOD) (EC 1.15.1.1) from horseradish 40 μg/mL (Sigma), Hank's balanced salt solution (Flow).

Samples

Blood (5 mL in EDTA K3) was drawn from the subjects by venipuncture. PMNs were isolated by double gradient as previously described[17,18] and approximately 5.0×10^6/mL cells with 98% purity were obtained, as measured by an electronic cell counter (Technicon H1 J). Cell viability was tested by trypan blue staining.

Evaluation of superoxide production by PMNs

Extracellular O_2^- (nM×10^5 PMNs) was assayed spectrophotometrically by superoxide dismutase inhibitable cytochrome c reduction, and continuous assay was performed in a Beckman DU 65 spectrophotometer (a double wavelength spectrophotometer) equipped with thermostated cuvette holder (37°C), as described previously.[19]

The cell suspension in Hank's solution was added to a 1 mL cuvette containing 80 μmol/L cytochrome c to obtain a final volume of 0.995 mL. Final cell concentration was 5×10^6 cells/mL. Reduction of cytochrome c was measured at 550 nm with a reference wavelength at 540 nm. fMLP (10^{-7} mol/L) or PMA (1 μg/mL) was added to the reaction mixture to obtain a final volume of 1 mL, while the time course of cytochrome c reduction was followed on the recorder. Intracellular O_2^- production was assayed densitometrically by the Nitroblue Tetrazolium Densitometric Kinetic Test (NBT DK Test).[20] This method is based on the reduction of intracellular NBT in activated PMNs, which was evaluated by transmittance (T%) variations due to intracellular O_2^- production.[20]

The cell suspension in Dulbecco's buffer (250 μL) was placed in an aggregometer (Aggrecorder II Daiichi Kagaku Co.), and the determination was made by using a diode (LED) with maximum wavelength at 650 nm as light source. The reaction occurred at 37°C and at an agitation speed of 500 rpm. Nearly a minute after the registration was begun, 500 μL of a mixture consisting of 250 μL of 0.2% NBT in Dulbecco's buffer and 250 μL of 1 μg/mL PMA in Dulbecco's buffer or 250 μL of fMLP 10^{-7} mol/L were added in order to achieve a rapid increase in T%, which regularly reached a value of 65%. After a short lag time, a decrease in T% due to NBT reduction occurs; the reaction was followed for 25 min.

Results

Effects of rhG-CSF administration on absolute PMN count

As shown in Table 1, infusion of rhG-CSF resulted in an increase in the mean ANC in all patients. The mean ANC after 5 days of therapy with rhG-CSF was 4.59×10^9/L, range 2.5-6.5×10^9/L. The periods of neutropenia with counts <1.0×10^9/L and <0.5×10^9/L during the course of rhG-CSF treatment were 3.8±4.9 and 1.9±2.5 days, respectively.

Effects of rhG-CSF administration on intra- and extracellular superoxide production

To assess *in vivo* activation of PMN function by rhG-CSF, we studied intra- and extracellular release of O_2^- in human PMNs stimulated with either fMLP or PMA. As shown in Table 2, enhanced intra- and extracellular O_2^- release was already detected after rhG-CSF administration in human PMNs stimulated with fMLP. In contrast, there was no enhancement of intra- or extracellular O_2^- production in PMNs stimulated with PMA after rhG-CSF administration (Table 2, Figures 1-3).

Statistical analysis

Comparision between data was carried out using Spearman's formula. The results obtained with the NBT DK test and SOD inhibitable cytochrome c reduction in patients under rhG-CSF therapy were compared with those obtained in healthy donors under different stimulation ($p<0.02$; $r= -0.9$). Data are presented in Table 2.

Discussion

The present study confirms that rhG-CSF administration increases the absolute PMN count in children with acute lymphoblastic leukemia and solid tumors, as previously

Table 2. In vivo effects of rhG-CSF therapy on intra- and extra-cellular O_2^- production by PMNs.

NBT DK test PMA	*NBK DK test fMLP*	*Cyt. C reduction PMA*	*Cyt. C reduction fMLP*
T%	T%	nMx10^5 PMNs	nMx10^5 PMNs
14.9±5.6	8.0±2.1	37.13±7.40	55.7±7.47
n.r. 13.1±10.8	n.r. 28.0±3.0	n.r. 35.11±7.31	n.r. 31.22±6.45 p<0.02 r=–0.9

n.r. normal range

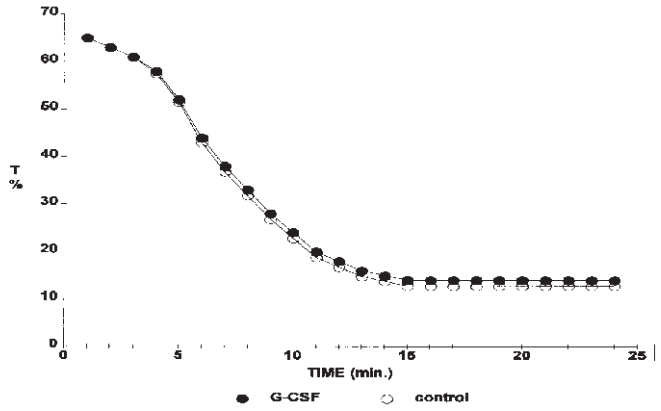

Figure 1. Intracellular O_2^- production (NBT DK TEST) following PMA stimulation in patients treated with rhG-CSF and in controls (see text).

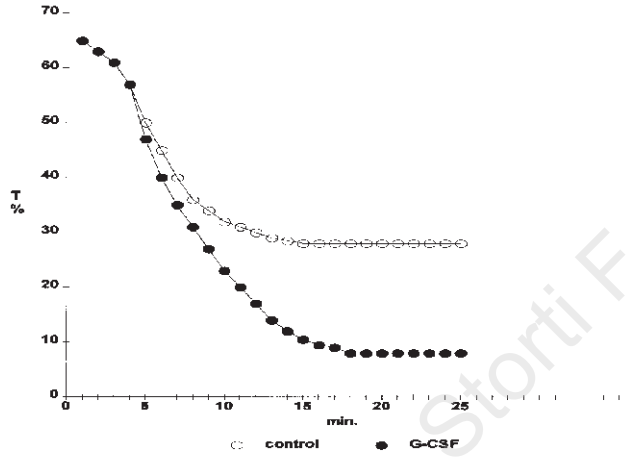

Figure 2. Intracellular O_2^- production (NBT DK TEST) following fMLP stimulation in patients treated with rhG-CSF and in controls (see text).

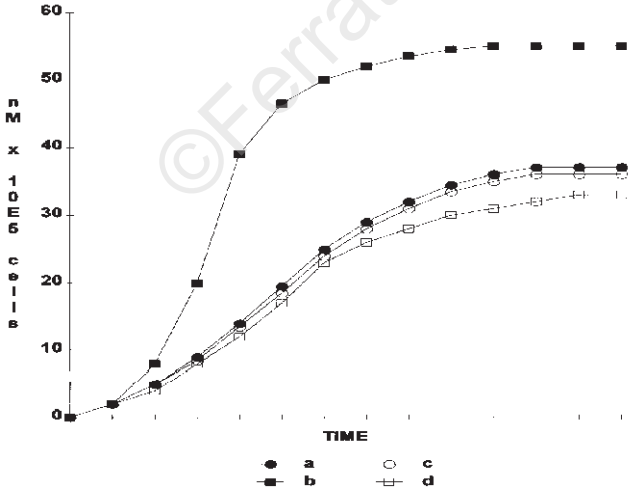

Figure 3. Extracellular O_2^- production (cytochrome c reduction) following PMA stimulation (a), and fMLP stimulation (b) in patients treated with rhG-CSF; mean value of controls (patients not treated with rhG-CSF) following PMA stimulation (c), and mean value of controls (patients not treated with rhG-CSF) following fMLP stimulation (d) (see text).

described.[3-8] rhG-CSF is also capable of enhancing the responsiveness of mature cells of the immune system.[22] The goal of the present study was to assess the functional ability of PMNs that are increased after rhG-CSF administration to produce O_2^- through different activation pathways. Our experiments showed that PMNs are also primed *in vivo* by rhG-CSF; in particular, both