Chronic Neutropenia and Defect in Superoxide Generation of Granulocytes in Two Patients: Enhancement of Bactericidal Capacity and Respiratory Burst Activity by Treatment with Recombinant Human Granulocyte Colony-Stimulating Factor

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ABSTRACT

We have identified two unrelated girls with chronic neutropenia [absolute neutrophil counts (ANC) 10–870 and 10–940/ μ L in patients 1 and 2, respectively] and severe defect in superoxide anion generation by granulocytes. Formyl-methionyl-leucyl-phenylalanine-induced superoxide release was 1.2 ± 0.9 and $1.9 \pm 1.9\%$ (mean $\pm$ SEM, $n = 3$) of normal controls', mean value in patients 1 and 2, respectively. However, granulocytes from both patients released a normal amount of superoxide upon stimulation with phorbol myristate acetate. Patient 2 exhibited characteristic features of Duane syndrome, a rare disorder of eye movement. Treatment of the patients with recombinant granulocyte colony-stimulating factor led to significant clinical improvements and reduction of infectious complications and to increases in the ANC, to 400–2100/ μ L in patient 1 and to 500–3000/ μ L in patient 2. Treatment with 5 μ g/kg/d resulted in increased intracellular killing of opsonized *Staphylococcus aureus* by granulocytes and an enhancement of superoxide release upon stimulation with formyl-methionyl-leucyl-phenylalanine in both patients up to 11.1 ± 6.0 and $13.5 \pm 7.0\%$ (mean $\pm$ SEM, $n = 5$) of normal controls', mean value in patient 1 and patient 2,

respectively. These data suggested that recombinant human granulocyte colony-stimulating factor treatment enhanced resistance to bacterial infection by stimulation of superoxide generation and increasing the bactericidal capacity of peripheral blood granulocytes. (*Pediatr Res* 37: 50–55, 1995)

Abbreviations

ANC, absolute neutrophil counts
FMLP, formyl-methionyl-leucyl-phenylalanine
G-CSF, granulocyte colony-stimulating factor
rhG-CSF, recombinant human G-CSF
CGD, chronic granulomatous disease
KRPD, Krebs-Ringer phosphate buffer containing dextrose
NHS, normal human serum
 O_2^- , superoxide anion
SOD, superoxide dismutase
PHA, phytohemagglutinin
PMA, phorbol myristate acetate
CFU, colony forming unit
[Ca²⁺]_i, cytoplasmic free calcium

Human G-CSF, a 177-amino acid glycosylated protein, is one of the growing number of recognized cytokines involved in the regulation of hematopoiesis (1, 2). G-CSF preferentially stimulates the growth and development of neutrophils (3). Administration of rhG-CSF to patients with myelodysplastic syndrome, aplastic anemia, and chemotherapy-induced neutropenia was found to increase the absolute neutrophil count, and

to reduce infectious complications (4–7). Recent observations suggest that patients with chronic neutropenia may also benefit from rhG-CSF treatment (8–11). Previously, the treatment of these patients relied on supportive care alone.

The underlying disorder in chronic neutropenia, such as Kostmann's syndrome, cyclic neutropenia, and idiopathic chronic neutropenia, has only partially been characterized. Previous work has shown that the defect of myelopoiesis in these disorders is more likely due to reduced cellular responsiveness to G-CSF or another regulatory defect at the level of the stem cell than to G-CSF deficiency (11, 12).

rhG-CSF exerts a variety of *in vitro* and *in vivo* effects on neutrophils such as up-regulation of CD11b and CD64 recep-

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tors, priming for enhanced release of reactive oxygen radicals and arachidonic acid metabolites, and increased antibody-dependent cellular cytotoxicity (13–17). However, the precise mechanism of action of rhG-CSF in patients with neutropenia is not known.

We report two patients with chronic neutropenia and deficiency of superoxide generation of granulocytes. The unique finding in these patients was the severe respiratory burst defect of granulocytes stimulated with FMLP, which was similar to that of cells from patients with CGD. We found that treatment of these patients with rhG-CSF resulted in marked increases of the bactericidal capacity and superoxide generation by granulocytes isolated from the patients blood.

METHODS

Isolation of granulocytes. A maximum of 4 mL of heparinized (10 U/mL) venous blood was obtained from the patients several times before and during treatment with rhG-CSF. Granulocytes were separated from blood as described (18). Cells were washed and resuspended to a concentration of 5×10^6 /mL in KRPD. The granulocyte suspension contained more than 95% neutrophils (band-form and segmented neutrophils) as checked by May Grünwald-Giemsa staining in cytocentrifuge preparations. For selected experiments, granulocytes were isolated from patients with CGD or from healthy children or adults. Special attention was paid to avoid lipopolysaccharide contamination throughout the isolation procedure (19).

Bacteria. *Staphylococcus aureus* (type 42D) was cultured overnight at 37°C in Nutrient Broth (Oxoid, London), harvested by centrifugation at $1500 \times g$ for 10 min, washed twice with KRPD, and finally resuspended in KRPD containing 0.1% gelatin to a concentration of 5×10^6 bacteria/mL (20).

Preopsonization. NHS was prepared from five healthy adults and stored at –70°C. Bacteria were preopsonized by incubation of 5×10^6 *S. aureus*/mL with 10% serum for 30 min at 37°C under rotation (4 rpm), followed by centrifugation and washes in KRPD at 4°C. The bacteria were finally resuspended to a concentration of 5×10^6 /mL (18, 20).

Measurement of O_2^- release. The release of O_2^- from granulocytes was quantitated as the SOD-inhibitable reduction of ferricytochrome *c* (type III; Sigma Chemical Co., St. Louis, MO) (21, 22). FMLP (10^{-7} M) or PMA (500 ng/mL) was added to phagocytic cells (10^6 /mL) in KRPD buffer with 80 μ M cytochrome *c*, with or without 50 μ g/mL SOD. After 5 to 30 min of incubation at 37°C, the reaction was stopped by shaking the tubes in crushed ice followed by centrifugation at 4°C for 5 min. Supernatants were transferred to microtiter plates (200 μ L/well; Nunc, Fernruf, Denmark) and reduction of ferricytochrome *c* was measured spectrophotometrically at 550 nm by using a computerized ELISA reader (Molecular Devices, Menlo Park, CA).

Measurement of phagocytosis. Phagocytosis of *S. aureus* was measured by incubating 50 μ L of a phagocytic cell suspension containing 5×10^6 granulocytes/mL with an equal volume of a suspension of 5×10^6 bacteria/mL in the presence of 10% NHS for 60 min at 37°C under rotation (4 rpm). At 0

and 60 min time points, 25- μ L aliquots of the mixture were removed and added to ice-cold KRPD (475 μ L) containing 0.01% human albumin (Sigma Chemical Co.). The number of viable extracellular bacteria was determined by colony counts (18). The percentage of phagocytosis of *S. aureus* by granulocytes was determined as the decrease in the number of viable extracellular bacteria (18).

Measurement of intracellular killing. Equal volumes of granulocyte suspension (5×10^6 cells/mL) and preopsonized *S. aureus* suspension (5×10^6 bacteria/mL) were mixed and incubated at 37°C under rotation (4 rpm). Total volume was 100 μ L. After 3 min of incubation, phagocytosis was stopped by shaking the tubes in crushed ice, and noningested bacteria were removed by differential centrifugation and washes in ice-cold KRPD (18). Next, the phagocytic cells containing ingested bacteria were resuspended to a concentration of 2.5×10^6 /mL and incubated at 37°C under rotation (4 rpm). Aliquots of the suspension (25 μ L) were removed at 0 and 60 min of incubation, and the granulocytes were lysed by adding (75 μ L) ice-cold distilled water containing 0.01% albumin and pipetting the suspension for 1 min. The percentage of bacteria that had been killed was determined by colony counting in duplicate (18).

Hematopoietic progenitor cell assay. CFU were determined in soft agar cultures of bone marrow samples aspirated for diagnostic purposes (23). Triplicate cultures of bone marrow cells ($1-2 \times 10^6$ mononuclear cells/mL) were plated in Petri dishes. The source of CSF was 0.2 mL of conditioned medium of 4×10^6 human blood mononuclear cells. PHA-stimulated leukocyte-conditioned media were prepared from healthy adult donors. Cells were cultured for 5 d in McCoy's medium containing 15% autologous serum, 17 μ g PHA/mL and 5 μ g levamisole/mL.

CASE REPORTS

Patient 1. This 7-y-old girl had been repeatedly hospitalized because of bacterial infections of the skin, middle ear, gingiva, lung, and bone since the age of 2 mo. By her sixth birthday, she had spent 514 d in hospital. At the age of 2 y, bilateral mastoidectomy had to be performed in the course of chronic, purulent otitis media and mastoiditis. Recurrent otitis media caused predominantly by *S. aureus* and *Pseudomonas aeruginosa* led to severe hearing loss, impaired speech development, and mental retardation. Deep-seated skin infections and paronychia caused by *S. aureus* required surgical management on two occasions. At the age of 5 y, she developed cellulitis in the fourth digit of the right leg, which led to osteomyelitis of the involved toe and required amputation of the distal phalanx. The chronic gingivostomatitis resulted in gingival hypertrophy and premature loss of both deciduous and permanent teeth. The chronic infections were complicated by hypoproteinemia, anemia, and failure to thrive. Kidney and liver function tests and ultrasonography of the liver and spleen were normal. Bone marrow morphology disclosed maturation arrest at the promyelocyte-myelocyte stage but indicated an increased normoblastic erythropoiesis and thrombocytopoiesis. The diagnosis of chronic neutropenia with defective activity of the granulocyte

respiratory burst was made at the age of 5 y, when O_2^- generation by granulocytes was measured repeatedly (Fig. 1A).

The parents of this patient are unrelated and healthy. Her 4-y-old sister has had recurrent upper respiratory tract infections without detectable immunodeficiency (data not shown).

Patient 2. This 15-mo-old girl presented with frequent episodes of fever, purulent otitis media, tonsillitis, bronchitis, and pneumonia beginning during the first 4 mo of life. At the age of 1 y, bilateral mastoidectomy had to be performed in the course of chronic otitis media. Kidney and liver function tests and abdominal ultrasonography were normal. Bone marrow morphology findings indicated maturation arrest at the promyelocyte-myelocyte stage and an increased number of monocytes and eosinophilic granulocytes, and an increased normoblastic erythropoiesis. The diagnosis of chronic neutropenia with an associated respiratory burst defect was made by measurements of O_2^- release by granulocytes at the age of 13 mo (Fig. 1B).

The patient's brother had myelodysplastic syndrome and died at the age of 8 y; her sister of 9 y and her unrelated parents are healthy.

She exhibits slight esotropia, impairment of abduction, and retraction on adduction of the right globe, which are characteristic features of Duane syndrome, a rare disorder of eye movement (24).

RESULTS

Clinical responses. Therapy with rhG-CSF was well tolerated without toxicity and led to considerable clinical improvement and reduction of infectious complications over periods of 6 and 3 mo in patients 1 and 2, respectively. The severe gingivostomatitis in patient 1 resolved dramatically after 3 wk of rhG-CSF therapy without administration of antibiotics. Contrary to delayed recoveries from infections before treatment in patient 2, otitis media and pneumonia resolved rapidly by administration of rhG-CSF ($5 \mu\text{g/kg/d}$) and antibiotics. After 7–8 wk of rhG-CSF treatment, the concentration of serum Ig decreased markedly in both patients, which is compatible with resolution of chronic infection (Fig 2).

Peripheral blood counts and CFU. The initial numbers of granulocytes were 1.0 and $0.3 \times 10^9/\text{L}$ in patients 1 and 2, respectively (Fig. 3). After 7–10 d of treatment, the ANC increased. After achieving a peak, the ANC decreased in both patients. In patient 1, increasing the daily dose of rhG-CSF resulted in transient increases in ANC following by a gradual decrease in neutrophil number. Data in Figure 3 indicate the lack of definite relationships between ANC and the dosage of rhG-CSF.

Adrenalin stimulation tests ($0.01 \text{ mg adrenalin/kg}$, s.c.) were performed in both patients and showed 31 and 35% increases of ANC, respectively, over a period of 2 h. Hydrocortisone stimulation test ($2 \text{ mg hydrocortisone/kg}$, i.v.) showed 40 and 47% increases of ANC, respectively, over a period of 24 h. These data suggested that neutropenia in either of these patients was not due to increased margination of granulocytes or storage pool defect (25).

Soft agar cultures of bone marrow before treatment with rhG-CSF yielded no colonies in patient 1 and $14 \pm 7 \text{ CFU}/10^5$

Figure 1. Release of O_2^- by 2×10^5 granulocytes before treatment (open columns) and during treatment (hatched columns) with varying doses of rhG-CSF for 5 or more preceding d on the same dose. The heights of the columns represent data of single, one-tube measurements. Blood was drawn before administration of the daily dose of rhG-CSF. Cells were stimulated with 10^{-7} M FMLP for 5 min. Release of O_2^- by granulocytes from normal controls was $62 \pm 17 \text{ nmol}$ (mean $\pm$ SEM; $n = 6$).

Figure 2. Serum Ig concentrations were measured 3–5 d before treatment (open columns) and 7–8 wk after treatment (hatched columns) with rhG-CSF.

Figure 3. ANC in patients treated with rhG-CSF. Vertical bars and numbers indicate the number of days since start of treatment and doses of rhG-CSF, respectively.

bone marrow cells in patient 2, compared with 28.4 ± 5.1 CFU/ 10^5 bone marrow cells in controls (mean $\pm$ SEM, $n = 33$).

Phagocytosis and intracellular killing of *S. aureus* by granulocytes. Phagocytosis and killing of fully opsonized *S. aureus* by granulocytes were measured before and during treatment with rhG-CSF. The extent of ingestion of bacteria before treatment was comparable to that of controls in both patients (Table 1). There were no differences in the phagocytosis of *S. aureus* by granulocytes isolated before or during rhG-CSF treatment. However, granulocytes from either patient were unable to kill bacteria efficiently before treatment (Table 1); significant improvement in the bactericidal activities of granulocytes could be detected during rhG-CSF treatment ($p < 0.001$ in both patients). The daily dose of rhG-CSF did not correlate with the killing capacities of granulocytes, and administration of rhG-CSF at $20 \mu\text{g/kg/d}$ did not result in proportionally increased killing by granulocytes compared with killing at doses of 5 g/kg/d .

Respiratory burst activation in granulocytes. We measured FMLP-induced O_2^- release by granulocytes in an attempt to define the biochemical basis of greater killing of *S. aureus* by these cells from rhG-CSF-treated patients. As shown in Figure

Table 1. Phagocytosis and intracellular killing of *S. aureus* by granulocytes from neutropenic patients

Patient	<i>S. aureus</i> ingested*		<i>S. aureus</i> killed†	
	Before treatment (%)	During treatment (%)	Before treatment (%)	During treatment (%)
1	96 ± 8	86 ± 8	3 ± 2	62 ± 9
2	88 ± 7	89 ± 4	5 ± 3	69 ± 9

All data represent mean $\pm$ SEM ($n = 3$ or more) and are expressed as percentages of normal controls' mean value ($n = 6$) before and during treatment with $5 \geq \mu\text{g/kg}$ rhG-CSF for 5 or more preceding days.

* Determined as the percentage decrease of the number of viable extracellular microorganisms after a 60-min incubation of granulocytes and bacteria in the presence of 10% normal human serum.

† Determined as the percentage decrease of the number of viable intracellular microorganisms after a 60-min incubation of granulocytes containing ingested bacteria.

1, administration of rhG-CSF for at least 5 d at doses of $5 \mu\text{g/kg/d}$ resulted in a 6–9 times larger amount of O_2^- released compared with values before treatment. No further increase in O_2^- release by granulocytes was found at daily doses of 10 or $20 \mu\text{g/kg}$ rhG-CSF in patient 1. However, when the daily dose of rhG-CSF was decreased to $1 \mu\text{g/kg/d}$ for more than 1 wk in patient 1 or treatment was ceased for 3 wk in patient 2, the amount of O_2^- released by granulocytes decreased to values measured before treatment (Fig. 1). Incubation of granulocytes with FMLP over a period of 30 min did not result in a significantly higher amount of superoxide compared with data obtained with 5 min of incubation (data not shown). Contrary to findings with FMLP, the PMA-induced O_2^- release by granulocytes from patient 1 and patient 2 was 81 ± 12 and $94 \pm 11\%$ of normal controls', mean value, respectively (mean $\pm$ SEM, $n = 3$).

Granulocytes from two patients with X-linked CGD, who were free of acute infection, were also studied. The O_2^- released by CGD cells under the experimental conditions used in this study was 1.8 ± 0.6 and $2.4 \pm 1.8\%$ (mean $\pm$ SEM, $n = 3$ in both patients) compared with normal controls', mean value ($n = 7$). Negligible killing of *S. aureus* by CGD cells could be detected over 60 min of incubation (2.5 ± 1.4 and $5.2 \pm 2.1\%$ of normal controls', mean value in patient 1 and patient 2, respectively ($n = 3$), whereas phagocytosis of opsonized staphylococci was comparable to that of age-matched controls (data not shown).

DISCUSSION

We report two patients with chronic neutropenia and severe deficiency of O_2^- release by granulocytes. Bone marrow morphology in one of the patients (patient 2) fulfilled one of the criteria of Kostmann's syndrome, namely maturation arrest at the promyelocyte-myelocyte stage, but the number of peripheral blood granulocytes exceeded $200/\mu\text{L}$, which could not be expected in this syndrome (26). Another unusual finding in this patient was the association of chronic neutropenia with Duane syndrome. This syndrome, a rare congenital defect of eye movement, can occur alone or in association with other disorders (24). To our knowledge, occurrence of chronic neutrope-

nia in association with Duane syndrome has not been published.

Granulocytes from both patients exhibited a severe impairment of activation of the respiratory burst upon stimulation with FMLP. It has been reported that a mild to moderate decrease of O_2^- generation can occur in patients with chronic neutropenia (8, 27). Granulocytes from our patients showed comparable FMLP-induced superoxide generation and functional activities to those of cells from CGD patients (Fig. 1). Contrary to CGD cells (28), however, granulocytes from our neutropenic patients could be induced with PMA to release O_2^- , a finding that ruled out the possibility that the patients had CGD. Based on these observations we believe that our patients with chronic neutropenia and severe defect of O_2^- generation have a distinct disease entity.

In selected experiments, we studied O_2^- generation of the patients', granulocytes stimulated with preopsonized staphylococci (21). The experiments gave similar results to those obtained with FMLP (the amount of O_2^- generated by 2.5×10^5 cells over a period of 30 min was 6.5 ± 3.4 and $8.2 \pm 4.3\%$ of normal controls', mean value in patient 1 and patient 2, respectively $n = 3$). These data suggest that in the patients granulocytes exhibit a signal transduction defect when surface receptors are involved in the stimulation of the respiratory burst.

In vivo effect of rhG-CSF on the microbicidal activity of granulocytes in patients with chronic neutropenia has not been reported to our knowledge. We report that rhG-CSF treatment in patients with chronic neutropenia and defective superoxide generation may result in an enhanced capacity of granulocytes to kill viable *S. aureus*. These functional changes may be especially important in patients whose ANC increases only moderately during rhG-CSF treatment, and may help our understanding of the mechanism of action of rhG-CSF in patients with chronic neutropenia.

Stimulus-response coupling in phagocytic cells stimulated with FMLP or opsonized particles involves receptor binding, an increase in $[Ca^{2+}]_i$ and release of O_2^- and other reactive oxygen intermediates (29, 30). Granulocytes from patients with chronic neutropenia have been reported to have abnormal regulation of signal transduction pathways such that their granulocytes do not respond to FMLP with an increase in $[Ca^{2+}]_i$ (31). Treatment with rhG-CSF has been reported to restore the ability of granulocytes in such patients to respond to FMLP stimulation with an increase in $[Ca^{2+}]_i$ (32). These findings and the observations described are in agreement and suggest that an impairment of a calcium transient and O_2^- generation may contribute to impaired host defense in patients with quantitative neutrophil deficiency.

Recent observations suggest that patients with chronic neutropenia have no defect in G-CSF production or in expression of G-CSF receptor binding sites on neutrophil granulocytes, but a defective response of neutrophil precursors to endogenous or exogenous G-CSF may play a role (14, 32). Our findings further emphasize the importance of the effect of G-CSF treatment on functional and metabolic activities of peripheral blood granulocytes in neutropenic patients.

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