**Improvement of Superoxide Production in Monocytes From Patients With Chronic Granulomatous Disease by Recombinant Cytokines**


Cytokines have been shown to modulate the respiratory burst of polymorphonuclear leukocytes and monocytes from normal controls. We have examined whether monocytes from children with chronic granulomatous disease (CGD) can be primed by cytokines other than interferon-γ (IFNγ), which has been demonstrated to improve the production of reactive oxygen species in vivo and in vitro. Monocytes isolated from peripheral blood were cultured without and with IFNγ (500 U/mL), tumor necrosis factor-α (500 U/mL), interleukin-1β (IL-1β) (100 U/mL), and IL-3 (100 U/mL). After 3 days of culture, the phorbolmyristate acetate (2 ng/mL) and the formyl-methionyl-leucyl-phenylalanine (0.1 μmol/L)-stimulated superoxide-production was determined in a microtiter system. In nearly all of the 14 patients examined (5 autosomal, 5 X-chromosomal, and 4 of unknown inheritance), an improvement of superoxide production could be demonstrated. The most impressive effect with the cytokines newly tested was seen with monocytes from autosomal CGD patients treated with IL-3 and stimulated by phorbolmyristate acetate. In single patients cultivation of monocytes with IL-6 and granulocyte-macrophage colony-stimulating factor resulted in only slight improvement of superoxide production. Our findings indicate that cytokines other than IFNγ can positively modulate the defective respiratory burst in CGD and that each patient reacts with an individual pattern to different cytokines.

**MATERIALS AND METHODS**

**Characterization of patients with CGD.** The CGD patients were characterized by inheritance pattern, nitroblue tetrazolium (NBT) test and stimulated superoxide production of PMN and monocytes, cytochrome b558 content in membrane fractions of PMN as determined by sodium dithionite-induced difference spectrum, or by fluorescence-activated cell sorter (FACS) analysis with the monoclonal antibody (MoAb) TDS against the cytochrome b558 α-chain, and in some patients by determination of the p47-phox protein in cytosols of PMN using Western blot analysis (Table 1). Residual NADPH-oxidase activity was supposed if the patient’s PMN showed more than 3% NBT-positive cells and/or measurable cytochrome c reducing activity. CGD patients with X-chromosomal recessive inheritance pattern (X-CGD) are males whose mothers showed a mosaicism of cells with normal and deficient formazan formation in the NBT test and intermediate superoxide production. CGD patients with autosomal inheritance (AR-CGD) are males or females whose mothers’ phagocytes had normal NBT reduction and superoxide formation. All AR-CGD patients tested had cytochrome b558 in their phagocyte membranes and residual superoxide production, except for one with presumably deficiency of the α-chain of cytochrome b558. In some patients of this group, p47-phox protein was not detectable in Western blots of PMN lysates. The five X-CGD patients lacked cytochrome b558; two were “classical” X-CGD patients without residual superoxide-producing capacity and the three other X-CGD patients showed residual NADPH-oxidase activity (Table 1).

**Reagents and chemicals.** Tissue culture reagents (Dulbecco’s phosphate-buffered saline [PBS], RPMI 1640, Hanks Balanced Salt Solution [HBSS], HEPES-buffer, penicillin G, and streptomycin) were obtained from Biochrom (Berlin, Germany). All other chemicals were from Sigma (Deisenhofen, Germany). Human AB-serum consisted of a serum pool from 20 healthy donors. Human recombinant cytokines were obtained as shown in Table 2.

**Isolation and superoxide assay of PMN and freshly isolated monocytes.** Peripheral blood PMN and monocytes were isolated from peripheral blood anticoagulated with heparin after dextran sedimentation by density gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden).24 For determinations of superoxide production of freshly isolated phagocytes, PMN and mononuclear cells were obtained from Biochrom (Berlin, Germany). All other chemicals were from Sigma (Deisenhofen, Germany). Human AB-serum consisted of a serum pool from 20 healthy donors. Human recombinant cytokines were obtained as shown in Table 2.

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**The aim of our study was to determine if cytokines other than IFNγ were able to prime cultured human peripheral blood monocytes also from patients with CGD for enhanced production of reactive oxygen species.**

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suspensions were washed, submitted to hypotonic lysis by treatment with 155 mmol/L NH₄Cl to remove remaining erythrocytes, washed again, counted (PMN: staining with Türk's solution [Merck, Darmstadt, Germany]; monocytes: nonspecific esterase stain [Technikon, Tarrytown, New York, NY]), and suspended in HBSS without phenol red. Supernatide production was determined as superoxide dismutase inhibitable cytochrome c reduction after stimulation of the cells with 1 μg/mL phorbol-12-myristate-13-acetate (PMA). Results are expressed in nanomoles of O₂ per 2.5 × 10⁵ cells per 60 minutes.

Cell culture of monocytes and superoxide assay of cultured monocytes. For culturing monocytes, mononuclear cell suspensions were washed three times with PBS, pH 7.4, without calcium and magnesium at 4°C. Cell count of monocytes in the mononuclear cell suspensions was performed by nonspecific esterase stain (Technikon; Tarrytown, NY), and in HBSS without phenol red. Supernatide production was determined as superoxide dismutase inhibitable cytochrome c reduction after stimulation of the cells with 1 μg/mL phorbol-12-myristate-13-acetate (PMA). Results are expressed in nanomoles of O₂ per 2.5 × 10⁵ cells per 60 minutes.

<table>
<thead>
<tr>
<th>Patient/Sex</th>
<th>Inheritance</th>
<th>NBT Test (%)</th>
<th>PMN</th>
<th>Monocytes</th>
<th>Cytochrome b₅₅₈</th>
<th>7 DS (p22-phox)</th>
<th>47 Kd (p47-phox)</th>
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<tr>
<td>1/M</td>
<td>AR</td>
<td>2</td>
<td>0.13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>2/M</td>
<td>AR</td>
<td>2-4</td>
<td>1.14</td>
<td>2.0</td>
<td>10.2</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>AR</td>
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<td>0.1</td>
<td>0.2</td>
<td>7.6</td>
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<td>ND</td>
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<tr>
<td>4/F</td>
<td>AR</td>
<td>6</td>
<td>0.2</td>
<td>2.7</td>
<td>10.2</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>5/M</td>
<td>AR</td>
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<td>2.1</td>
<td>0.4</td>
<td>10.4</td>
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<td>−</td>
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<tr>
<td>6/M</td>
<td>X</td>
<td>80</td>
<td>1.7</td>
<td>0.8</td>
<td>0</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7/M</td>
<td>X</td>
<td>14</td>
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<td>+</td>
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<tr>
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<td>X</td>
<td>11</td>
<td>3.2</td>
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<td>0</td>
<td>0.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>10/M</td>
<td>X</td>
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<td>−</td>
<td>+</td>
</tr>
<tr>
<td>11/M</td>
<td>?</td>
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<td>0.5</td>
<td>0.2</td>
<td>0</td>
<td>NC</td>
<td>ND</td>
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<tr>
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<td>0.6</td>
<td>3.8</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>13/M</td>
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<td>14/M</td>
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<tr>
<td>Controls</td>
<td></td>
<td>99-100</td>
<td>23.1 ± 4</td>
<td>16.2 ± 4</td>
<td>9 ± 3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not determined; −, absent; +, detectable; AR, autosomal recessive; X, X chromosomal recessive.

* Freshly isolated from peripheral blood.

Table 2. Source and Properties of Cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Manufacturer</th>
<th>Specific Activity (10⁶ U/mg)</th>
<th>Source</th>
<th>Test Concentration (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Bioferon</td>
<td>20</td>
<td>E coli</td>
<td>500</td>
</tr>
<tr>
<td>IL-3</td>
<td>Bioferon</td>
<td>200</td>
<td>CHO cells</td>
<td>500</td>
</tr>
<tr>
<td>TNFα</td>
<td>Boehringer M.</td>
<td>20</td>
<td>Yeast</td>
<td>500</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Genzyme</td>
<td>50</td>
<td>Yeast</td>
<td>100</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Bioferon</td>
<td>20</td>
<td>E coli</td>
<td>100</td>
</tr>
<tr>
<td>IFNβ</td>
<td>Boehringer M.</td>
<td>200</td>
<td>CHO cells</td>
<td>100</td>
</tr>
<tr>
<td>IL-6</td>
<td>Genzyme</td>
<td>200</td>
<td>E coli</td>
<td>100</td>
</tr>
</tbody>
</table>

The remaining cells were greater than 90% to 95% monocytes and greater than 99% viable (as determined by trypan blue exclusion test). Cells were cultured in 5% CO₂ at 37°C for 72 hours. Standard culturing conditions and optimal cytokine concentrations were established from preliminary experiments with blood from healthy donors. The superoxide production of cultured monocytes was measured as superoxide dismutase inhibitable cytochrome c reduction by a modified Pick and Mizel method.25 Stimulation was performed with PMA (2 ng/mL) or formyl-methionyl-leucyl-phenylalanine (FMLP; 0.1 μmol/L). The cumulative superoxide production was measured 120 minutes after the addition of the reaction mixture at 550 nm in a Dynatech Microtiter reader MR600 (Dynatech, Denkendorf, Germany) using absorbance at 490 nm as reference. The superoxide production was calculated from the difference of the optical densities between the wells with and without superoxide dismutase in nanomoles of superoxide per 10⁵ monocytes per 120 minutes.

Determination of adherent cell protein. To exclude the possibility that variable numbers of monocytes were eliminated by washing the culture wells, the amount of cell protein adherent in culture wells was determined after 72 hours of monocyte culture. The method of Bradford2 modified by Baumgarten26 with bovine serum albumin as a standard was used. The test was evaluated photometrically within 1 hour after the addition of the dye with a Dynatech Microplate reader MR600 at 630 nm. The calibration was performed with bovine serum albumin solutions in 120 μL PBS by adding 30 μL of the dye solution. There were no statistically significant variations in the content of adherent cell protein between different culture wells in different compartments of the microtiter plates.

NBT test. The NBT reduction of isolated PMN was determined by using the method of Preisig and Hitzig.26

Cytochrome b₅₅₈ determination in PMN. Cytochrome b₅₅₈ content of PMN was determined as sodium dithionite-induced difference spectrum, as described by Lutter et al.27

FACS analysis of the cytochrome b₅₅₈ α-chain with 7D5 Monb. PMN (1 × 10⁶) in 20 μL PBS without calcium and magnesium with 0.02% [wt/vol] sodium azide were incubated with 7D5 antibody or control antibodies (negative control: mouse IgG [Cooper Electronics, Krefeld, Germany]; positive control: Leu M1 [Becton
Dickinson, Mountain View, CA) at 4°C for 60 minutes. After washing, PMN were incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab)2, goat antimouse IgG and IgM (Pascal & Lorei, Frankfurt, Germany) at 4°C for 30 minutes. The cells were fixed in 1% (wt/vol) paraformaldehyde and analyzed with a FACSscan flow cytometer (Becton Dickinson). Fluorescent emission of 27-dichlorofluorescein of individual PMN was determined by computer gating (FACSscan research software).

**p47-phox analysis in PMN by immunoblot.** A polyclonal rabbit antiserum was generated against a synthetic peptide (PDDKLKPTDNGQTTPKETLM) corresponding to amino acid residues 122 to 141 of the 47-kDa neutrophil cytosolic factor (p47-phox). The peptide was kindly prepared by Dr. B. Schmidt (Institute for Biochemistry II, University of Göttingen, Germany). The peptide was coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde as described elsewhere and injected in rabbits. Antiserum (NCF-122) was assayed for reactivity with the peptide by Western blot analysis and by enzyme-linked immunosorbent assay (ELISA). Purified PMN were lysed for 5 minutes on ice at a concentration of 5 × 10^6 cells/ml in solubilization buffer (3 mmol/L MgCl_2, 15 mmol/L HEPES buffer, pH 7.4, 2 mmol/L phenylmethylsulfonylfluoride [PMSF], 1 mmol/L Leupeptin, and 1% [vol/vol] Triton-X 100) and then centrifuged for 5 minutes at 13,000 g. Supernatants representing the cytosol enriched fraction were frozen in aliquots at −70°C. Protein was determined by the method of Bradford using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli with 2 × 10^6 cell equivalents/lane. Electrophoretic transfer of proteins from SDS-polyacrylamide gels onto nitrocellulose (Schleicher & Schüll, Dassel, Germany) was performed as described elsewhere. After incubation with the NCF-122 antiserum, the blots were incubated with alkaline phosphatase-conjugated goat antirabbit IgG (Dianova, Hamburg, Germany) and developed with NBT chloride (BioRad, Munich, Germany) was performed as described elsewhere.

**Assay of endotoxin.** Lipopolysaccharide (LPS) content of cyto- kines and cell culture reagents was determined by the Limulus amoebocyte lysate test (E-toxate; Sigma) as described by the manufacturer’s protocol. Only cell culture reagents and media with LPS levels lower than 10 pg/mL were used in the experiments. No LPS was detected in Ficoll; PBS contained less than 1 pg/mL and AB-medium less than 10 pg/mL. The cytokine preparations were LPS-free (IL-3, TNFα, and GM-CSF) or contained less than 1 pg/mL (IFNγ, IL-1β, and IL-6) in the highest concentration used in the experiments.

**Evaluation of data.** Significance of data from healthy volunteers was analyzed by nonparametric Mann-Whitney test.

### RESULTS AND DISCUSSION

In our patients, an unusual high number (9 of 14) with residual activity of the NADPH-oxidase (“variant CGD”) was identified. Eleven of the 14 patients under investigation were older than 10 years; in five patients, the first manifestation of the disease occurred after 10 years.

A comparison of superoxide production between freshly isolated PMN and monocytes from CGD patients showed in most patients a higher superoxide production with monocytes based on the cell number (Table 1). In experiments with monocytes from healthy volunteers, we established standard culturing conditions and showed that monocytes were primed by IFNγ, TNFα, IL-1β, IL-3, IL-6, and GM-CSF for enhanced stimulated superoxide production after 72 hours of monocyte culture (Table 3).

<p>| Table 3. Influence of Cytokines on Superoxide Production of Monocytes From Healthy Donors |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>PMA (2 ng/mL)</th>
<th>FMLP (0.1 μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.17 ± 0.36</td>
<td>0.54 ± 0.13</td>
</tr>
<tr>
<td>IFN (500 U/mL)</td>
<td>2.99 ± 0.62*</td>
<td>2.02 ± 0.6*</td>
</tr>
<tr>
<td>TNF (500 U/mL)</td>
<td>2.55 ± 0.51*</td>
<td>2.60 ± 0.73*</td>
</tr>
<tr>
<td>IL-1β (100 U/mL)</td>
<td>3.59 ± 1.06*</td>
<td>0.98 ± 0.34</td>
</tr>
<tr>
<td>IL-3 (100 U/mL)</td>
<td>3.4 ± 0.8*</td>
<td>1.42 ± 0.47*</td>
</tr>
<tr>
<td>IL-6 (100 U/mL)</td>
<td>3.17 ± 0.6*</td>
<td>2.86 ± 0.57*</td>
</tr>
<tr>
<td>GM-CSF (100 U/mL)</td>
<td>2.00 ± 0.41</td>
<td>2.00 ± 0.53*</td>
</tr>
</tbody>
</table>

Monocytes from nearly all CGD patients cultured for 72 hours in the absence of a cytokine produced more O2 than when measured immediately after isolation from peripheral blood (Table 1 and Fig 1). If cultured in the presence of cytokines, monocytes from patients with CGD were able to improve their defective oxidative metabolism. Most effective in enhancing PMA (2 ng/mL)-stimulated superoxide production in AR-CGD was IFNγ, followed by IL-3, TNFα, and IL-1β (Fig 1A). In X-CGD and in patients with unknown inheritance, other cytokines than IFNγ (IL-3 and IL-1β) were more potent in inducing an increased O2 production. Only in one patient did the addition of cytokines into the culture medium result in reduced O2 production compared with untreated cells (patient 10).

In general, monocytes from patients with AR-CGD could be primed to higher O2 production after PMA stimulation than cells from patients with X-chromosomal disease. This could explain the milder clinical course of AR-CGD patients by speculating that IL-1β or TNFα produced during infections could stimulate phagocytes to become more efficient in destroying bacteria and other microorganisms. This speculation may be supported by looking at the absolute amounts of O2 produced; in some patients, nearly 80% of the O2 production of monocytes from healthy controls was observed after culture with a cytokine.

If stimulated by FMLP (0.1 μmol/L), IFNγ was again the most potent priming agent in AR-CGD, followed by IL-3, IL-1β, and TNFα, whereas in X-CGD and patients with unknown inheritance, IL-3 and IL-1β showed a more potent priming effect than IFNγ.

The differences between autosomal and X-chromosomal patients were not so striking when monocytes were stimulated by FMLP compared with PMA stimulation (Fig 1B). The influence of IL-6 (100 U/mL) and GM-CSF (100 U/mL) could be tested only in single patients. After monocyte culture with IL-6, an increase of FMLP-stimulated superoxide production was observed only in two of four patients and after FMLP stimulation in one of four patients. Cultivation of
Fig 1. Superoxide production of monocytes (nmol O₂/10⁶ monocytes/2 h) from patients with CGD cultured for 72 hours without and with cytokines (500 U/mL for IFNγ and TNFα, 100 U/mL for IL-1β and IL-3) after stimulation with PMA (2 ng/mL) (A) or FMLP (0.1 μmol/L) (B). The patient numbers relate to Table 1.
monocytes with GM-CSF was performed in five patients for PMA-induced stimulation (four without effect and one with an increase) and in three for FMLP-induced stimulation (slight increase in all, including the patient whose monocytes showed an increase after PMA stimulation).

The superoxide assay of cultured monocytes could be repeated in seven patients. The constancy of superoxide production in individual patients could be demonstrated by repeating assays on cells from seven patients (in two of these patients even three determinations were performed). Setting the results of the first assay as 100%, the repeated assays varied between 82% and 134%.

Ezekowitz and Mühlebach et al. examined the effects of in vivo administered IFNγ on phagocyte function. In vitro, did not observe an IFNγ-induced improvement of the phagocyte respiratory burst activity. In contrast to our investigation, in only 25% of the patients was the monocyte oxidative metabolism determined or only PMN had been examined. Thus, the negative results of both examinations relate mostly to PMN. In addition, the IFNγ effect on superoxide production was tested after stimulation with very high concentrations of PMA (660 ng/mL or 200 ng/mL). In our investigation, we used a lower PMA concentration (2 ng/mL) because in preliminary experiments with monocytes from healthy donors we found that the cytokine effects on superoxide production of cultured monocytes can be shown better after stimulation with suboptimal doses of PMA. The diverging results could also be explained by the fact that in the IFNγ study the major part of the patients were classical X-CGD patients, who in our study also showed only little responsiveness to cytokines, whereas the variant AR-CGD patients did respond more.

Recently, an evaluation of long-term IFNγ treatment confirmed the nonresponsiveness of PMN oxidative metabolism to in vivo administered IFNγ. In contrast, in some AR-CGD patients with deficiency of p47-phox, IFNγ improved monocyte oxidative metabolism as determined by PMA-stimulated NBT reduction. Thus, the positive effect of IFNγ in CGD patients in vivo could be caused at least partially by improved oxidative metabolism of monocytes.

In our study, the variant CGD patients were more responsive to cytokines than the classical X-CGD patients. It is possible that in the variant CGD patients with residual superoxide production the deficient NADPH-oxidase components are produced at a low rate that is enhanced by monocyte culture and/or cytokine treatment. This would explain the improved superoxide production of cultured monocytes from these patients. The influence of the cytokines tested on the expression of cytosolic factors (supposed to be deficient in some AR patients) especially has to be discussed. In the classical CGD patients examined elsewhere the complete lack of expression of one essential NADPH-oxidase component would impair residual superoxide production and cytokine-induced enhancement of the superoxide-producing capacity of phagocytes. On the other hand, the cytokines tested could generally influence the step of the activation cascade that is linked to protein kinase C, the receptor for PMA, and thus ameliorate the response of the cells to low-dose stimulation with PMA.

We have shown that not only IFNγ but also other cytokines increase the stimulated superoxide production in cultured CGD monocytes from patients with variant CGD. Each patient showed an individual pattern of reactivity to cytokines. Testing various cytokines in monocytes of individual patients in vitro could give information about the possible therapeutic use of cytokines other than IFNγ. Identification of the mechanisms responsible for priming the respiratory burst in phagocytes of CGD patients and normal controls will be important in understanding the regulation and modulation of the NADPH-oxidase system.

ACKNOWLEDGMENT

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Improvement of superoxide production in monocytes from patients with chronic granulomatous disease by recombinant cytokines

V Jendrossek, AM Peters, S Buth, J Liese, U Wintergerst, BH Belohradsky and M Gahr