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.

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suspensions were washed, submitted to hypotonic lysis by treatment with 155 mM NaCl to remove remaining erythrocytes, washed again, and counted (PMN: staining with Türk solution [Merck, Darmstadt, Germany]; monocytes: nonspecific esterase stain [Technikon, Tarrytown, New York, NY]). Mononuclear cells were suspended in culture medium and adding 100 μL of PMA. Results are expressed in nanomoles of O₂ per 10⁶ 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 suspension containing each 10⁶ monocytes/mL. One hundred microliters of the reaction mixture at 550 nm in a Dynatech Microtiter reader (MR600, Becton Dickinson, 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.

<table>
<thead>
<tr>
<th>Patient/Sex</th>
<th>Inheritance</th>
<th>NBT Test (%)</th>
<th>PMN*</th>
<th>Monocytes*</th>
<th>Cytochrome b₅₅₅ (pmol/10⁶ PMN)</th>
<th>7 D5 (p47-phox)</th>
<th>47 Kd (p47-phox)</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<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>
</tr>
<tr>
<td>3/M</td>
<td>AR</td>
<td>3-11</td>
<td>0.1</td>
<td>0.2</td>
<td>7.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4/F</td>
<td>AR</td>
<td>6</td>
<td>0.2</td>
<td>2.7</td>
<td>12.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5/M</td>
<td>AR</td>
<td>3</td>
<td>2.1</td>
<td>0.4</td>
<td>10.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6/M</td>
<td>X</td>
<td>80</td>
<td>1.7</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7/M</td>
<td>X</td>
<td>14</td>
<td>2.7</td>
<td>2.4</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>8/M</td>
<td>X</td>
<td>11</td>
<td>3.2</td>
<td>0.7</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9/M</td>
<td>X</td>
<td>0</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10/M</td>
<td>X</td>
<td>0</td>
<td>0.3</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11/M</td>
<td>?</td>
<td>0</td>
<td>0.5</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12/M</td>
<td>?</td>
<td>53</td>
<td>0.6</td>
<td>3.8</td>
<td>-</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>13/M</td>
<td>?</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>14/M</td>
<td>?</td>
<td>76</td>
<td>6.1</td>
<td>3.5</td>
<td>0.8</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>23.1</td>
<td>16.2</td>
<td>540</td>
<td>480</td>
<td>160</td>
</tr>
</tbody>
</table>

* 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>INFα</td>
<td>Bioferon</td>
<td>20</td>
<td>E.coli</td>
<td>500</td>
</tr>
<tr>
<td>INFβ</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>IL-1β</td>
<td>Boehringer M.</td>
<td>10</td>
<td>E.coli</td>
<td>100</td>
</tr>
<tr>
<td>IL-3</td>
<td>Genzyme</td>
<td>350</td>
<td>Yeast</td>
<td>100</td>
</tr>
<tr>
<td>IL-6</td>
<td>Genzyme</td>
<td>200</td>
<td>E.coli</td>
<td>100</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Genzyme</td>
<td>50/250</td>
<td>Yeast</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not determined; -, absent; +, detectable; AR, autosomal recessive; X, X chromosome recessive.

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 Baumgarten3 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, Becton Dickinson, 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 Baumgarten3 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, Becton Dickinson, Germany). The standard 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 b₅₅₅ determination in PMN. Cytochrome b₅₅₅ content of PMN was determined as sodium dithionite-induced difference spectrum, as described by Lutter et al.27

FACS analysis of the cytochrome b₅₅₅ a-chain with 7D5 MAb. PMN (1 × 10⁵) 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 IgG1 [Coulter Electronics, Krefeld, Germany]; positive control: Leu M1 [Becton

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Dickinson, Mountain View, CA)) at 4°C for 60 minutes. After washing, PMN were incubated with fluorescent isothiocyanate (FITC)-
conjugated F(ab'), goat antimouse IgG and IgM (Pansel & Lore, Frankfurt, Germany) at 4°C for 30 minutes. The cells were fixed in 1%
(w/v) paraformaldehyde and analyzed with a FACScan flow
cytometer (Becton Dickinson). Fluorescent emission of 2,7-dichlo-
rofluorescein of individual PMN was determined by computer gating (FACScan research software).18

*p7-phox analysis in PMN by immunoblot.* A polyclonal rabbit antiserum was generated against a synthetic peptide (PDDLKL-
PTDNQTKPETYLM) corresponding to amino acid residues 122
to 141 of the 47-Kd neutrophil cytosolic factor (p47-phox).28,29 The
topeptide was kindly prepared by Dr B. Schmidt (Institute for Bio-
chemistry II, University of Göttingen, Germany). The peptide was
coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde as
described elsewhere11 and injected in rabbits. Antiserum (NCF-
122) was assayed for reactivity with the peptide by Western blot anal-
ysis and by enzyme-linked immunosorbent assay (ELISA). Purified
PMN were lysed for 5 minutes on ice at a concentration of 5 × 107
cells/ml in solubilization buffer (3 mmol/L MgCl2, 15 mmol/L
HEPES buffer, pH 7.4, 2 mmol/L phenylmethylsulfonylfluoride
(PMSF), 1 mmol/L Leupeptin, and 10% [vol/vol] Triton X-100) and
then centrifuged for 5 minutes at 13,000g. Supernatants representing
the cytosol enriched fraction were frozen in aliquots at −70°C. Protein
determination was performed by the method of Bradford,32 using bovine serum
albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE) was performed according to Laemmli with 2 × 106 cell
equivalents/lane. Electrophoretic transfer of proteins from SDS-
polyacrylamide gels onto nitrocellulose (Schleicher & Schüll, Dassel,
Germany) was performed as described elsewhere.33 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, München,
Germany) and 5-bromo-4-chloro-indolylphosphate (SIGMA).

*Assay of endoxin.* Lipopolysaccharide (LPS) content of cyto-
kines and cell culture reagents was determined by the Limulus amoe-
bocyte 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 Ficol; PBS contained less than 1 pg/ml and AB-medium
less than 10 pg/ml. The cytokine preparations were LPS-free (IL-3,
TNFa, 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 manifes-
tation 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, TNFa, IL-1β, IL-3, IL-6,
and GM-CSF for enhanced stimulated superoxide production
after 72 hours of monocyte culture (Table 3).

**Table 3. Influence of Cytokines on Superoxide Production
of Monocytes From Healthy Donors**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>FMLP (0.1 μmol/L)</th>
<th>medium (2 ng/mL)</th>
</tr>
</thead>
<tbody>
<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 produc-
tion in AR-CGD was IFNγ, followed by IL-3, TNFa,
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 TNFa produced during infections
could stimulate phagocytes to become more efficient in de-
stroying 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 pro-
duction 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 TNFa, 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 PMA-stimulated superoxide produc-
tion was observed only in two of four patients and after
FMLP stimulation in one of four patients. Cultivation of

Monocytes from healthy donors have been cultured for 72
hours without a cytokine (medium) or with the cytokines described
under Materials and Methods. After stimulation with PMA or FMLP, the
production of superoxide has been determined. Means ± SD are given
(number of experiments: medium, n = 17; medium with cytokines, n = 3
to 6).

* p < .05 compared with monocytes cultured in medium without a
cytokine.

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Fig 1. Superoxide production of monocytes (nmol O2/10^6 monocytes/2 h) from patients with CGD cultured for 72 hours without and with cytokines (500 U/mL for IFNγ and TNF-α, 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., who 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 a 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

We thank M. Nakamura (Tokyo, Japan) for kindly providing us the monoclonal antibody 7D5, and J. Roessler (Hannover, Germany) for helpful advice.

REFERENCES


18. Kharazmi A, Nielsen H, Bentzen K: Recombinant interleukin 1α and β prime human monocyte superoxide production but have no effect on chemotaxis and oxidative burst response of neutrophils. Immunobiology 177:32, 1988


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