Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 4874–4878, July 1988
Medical Sciences

Recombinant human interferon-γ reconstitutes defective phagocyte function in patients with chronic granulomatous disease of childhood

(neutrophils/monocytes/lymphokine/superoxide/bactericidal activity)

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Communicated by Seymour J. Klebanoff, February 29, 1988 (received for review December 11, 1987)

ABSTRACT **Monocytes from 19 of 30 patients with the classic phenotype of chronic granulomatous disease of childhood (CGD) responded to 3 days of treatment in culture with recombinant human interferon-γ (rHuIFN-γ) at 100 units/ml by producing superoxide after stimulation with phorbol 12-myristate 13-acetate. Cells from 15 of 16 patients with cytochrome *b*-positive CGD (15 with autosomal and 1 with X chromosome-linked inheritance) and cells from 4 of 14 patients with cytochrome *b*-negative CGD (13 with X chromosome-linked and 1 with autosomal recessive inheritance) responded. Subcutaneous rHuIFN-γ (0.01–0.05 mg/m^2) administered as a single dose, daily or every other day, for five or six doses to 3 patients whose phagocytes responded to rHuIFN-γ *in vitro* resulted in significant improvement in phagocyte bactericidal activity against *Staphylococcus aureus* and increases in superoxide production. Studies on 1 patient's cells indicated the increases in superoxide production correlated with increased membrane cytochrome *b*. The effects of rHuIFN-γ persisted for more than a week following cessation of therapy. Thus, we have demonstrated a partial correction *in vivo* of these CGD patients' phagocyte defect with rHuIFN-γ. Moreover, the data suggest that a significant proportion of patients with CGD will respond to rHuIFN-γ with augmentation of phagocyte microbicidal function.**

Chronic granulomatous disease of childhood (CGD) represents a group of patients whose phagocytes fail to produce superoxide and hydrogen peroxide when activated (1–4) and who have greatly increased susceptibility to infections. The disease can be inherited as X chromosome-linked (65%), autosomal recessive (34%), or rarely autosomal dominant patterns (1, 3, 4). X chromosome-linked CGD is usually associated with absence of a membrane cytochrome b_{558}, a component of the electron transport chain linked to NADPH oxidase (5–7). Most autosomal recessive CGD may result from the lack of a cytosolic factor required for activation of oxidative metabolism (8, 9).

Interferon-γ (IFN-γ) enhances the oxidative burst of normal phagocytes (10, 11). Furthermore, it has been reported that treatment of phagocytes from three of nine patients with CGD, *in vitro* with IFN-γ, enhances superoxide production (12). Responders in that study were considered variants of X chromosome-linked CGD in that phagocytic cells from these variants demonstrated significant superoxide production in response to phorbol 12-myristate 13-acetate (PMA) without recombinant human IFN-γ (rHuIFN-γ) treatment.

In this report we demonstrate that in 19 of 30 patients with the classic phenotype of CGD, representing all reported inheritance patterns, treatment of monocytes *in vitro* with rHuIFN-γ enhances oxidative metabolism. Most patients whose phagocytes are positive for cytochrome *b* were responders, though one-third of X chromosome-linked, cytochrome *b*-negative patients were also in the responding group. More importantly, administration of rHuIFN-γ subcutaneously to 3 CGD patients, whose cells responded *in vitro*, reconstituted defective bactericidal neutrophil and monocyte function to near normal.

MATERIALS AND METHODS

Patient Population. The diagnosis of CGD and inheritance pattern have been reported previously for 22 of the 30 patients participating in this study (7). CGD patients not previously reported were evaluated by using the same methods and criteria as in our previous reports (3, 7). As described (3), for these studies X chromosome-linked CGD patients are males whose mothers' neutrophils are 20–80% nitroblue tetrazolium (NBT) negative (CGD phenotype), and autosomal recessive patients are patients whose mothers' neutrophils are NBT positive (normal phenotype). One family with probable autosomal dominant inheritance has been described (3).

All of our patients were diagnosed in early childhood and have had one or more episodes of severe deep tissue bacterial or fungal infection. All of the patients have neutrophils that are NBT negative and that produce no detectable superoxide during 5 min of PMA stimulation. The two autosomal dominant patients' monocytes produced trace amounts of superoxide following PMA stimulation during an hour-long assay.

Reagents. Materials were obtained as follows: RPMI 1640 medium (GIBCO), human AB serum (Hazleton Laboratories, Denver, PA), Histopaque 1083, NBT dye, PMA, superoxide dismutase, catalase, and ferricytochrome *c* (Sigma), nonspecific esterase stain (Technikon, Tarrytown, NY). A laboratory strain of *Staphylococcus aureus* 502A was used for the bactericidal assays. The rHuIFN-γ for *in vitro* studies and for *in vivo* administration to patients was generously provided by Genentech (South San Francisco, CA).

Cell Preparation. Monocytes and neutrophils were separated from heparinized (5 units/ml) peripheral venous blood by Histopaque density gradient and dextran sedimentation with subsequent hypotonic lysis of erythrocytes (13).

Mononuclear cells to be cultured were washed and suspended in RPMI 1640 medium with 5% heat-inactivated human AB serum. For most studies of *in vitro* effects of rHuIFN-γ on monocytes, a million monocytes (by nonspecific esterase stain) (14) were placed in each well of a 24-well

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Abbreviations: CGD, chronic granulomatous disease of childhood; IFN-γ, interferon-γ; rHuIFN-γ, recombinant human IFN-γ; PMA, phorbol 12-myristate 13-acetate; NBT, nitroblue tetrazolium.

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culture plate and allowed to adhere for 2 hr before rinsing and adding medium with and without rHuIFN-γ. The remaining cells were >90% monocytes. When very small numbers of cells were available, mononuclear cells were cultured in 1 ml of medium in small round-bottomed tubes without depleting lymphocytes. For a few assays mononuclear cells were cultured in small Teflon-coated beakers. For time courses and dose–response studies, monocytes were cultured at 10^5 cells per well in 96-well microtiter plates. Neither the presence nor the absence of lymphocytes prevented a response of monocytes to rHuIFN-γ.

For studies of the *in vitro* effect of rHuIFN-γ, monocytes were cultured for 3 days with or without rHuIFN-γ at 100 units/ml added to the medium at the time of initiation of the culture. On the third day monocytes were assayed for PMA-stimulated NBT reduction and superoxide production. About a third of the patients were studied on more than one occasion.

NBT Reduction Assay. PMA-stimulated NBT studies were carried out for 20 min at 37°C with PMA at 100 ng/ml as the stimulus. The cells were then fixed and stained with safranin (15).

Superoxide Assays. During the course of the study two closely related methods were used for evaluation of superoxide production. For both methods a solution of 160 μM ferricytochrome *c* and catalase at 1000 units/ml in Hanks' balanced salts solution with and without superoxide dismutase was used. Superoxide production was measured with and without PMA at 100 ng/ml as the stimulus.

For most studies the cumulative superoxide production at 1 hr was determined by spinning out the cells and scanning the solution from 560 nm to 540 nm on a Perkin–Elmer lambda 3 spectrophotometer. The peak absorbance was measured from the baseline of each scan and the amount of superoxide was calculated from the difference in optical density between the solutions with and without superoxide dismutase, using a millimolar extinction coefficient of 21.1 per cm light path (oxidized minus reduced). Resting values were subtracted from stimulated values (16, 17).

If mononuclear cells were grown in 96-well tissue culture plates, the superoxide assay was run on the adherent cells and read at multiple time points on a Dynatech Elisa reader at 550 nm using absorbance at 490 nm as the reference standard (18).

Bactericidal Assay. Bactericidal assays were run on freshly isolated neutrophils and monocytes. Briefly, assay tubes were set up with 5×10^6 phagocytic cells per tube in Hanks' balanced salts solution with 0.1 ml of serum and 0.1 ml of bacteria at 2.5×10^8 bacteria per ml in a final volume of 1 ml. The tubes were rotated at 37°C and sampled at 20, 45, and 90 min by diluting 10 μl of the mixtures into 10 ml of distilled water and plating 75 μl of the dilution with trypticase soy agar. The plates were incubated at 37°C overnight and the number of bacterial colonies was quantified as described (13). Samples of neutrophils were run in triplicate. Because of the need to minimize the total blood volume obtained from the patients, monocytes were run in only one assay tube at 5×10^6 monocytes per tube. However, this monocyte assay tube was sampled in triplicate at each time point.

***In Vivo* Administration of rHuIFN-γ.** Patients chosen for initial studies with subcutaneous rHuIFN-γ were three adults with the classic phenotype of CGD, whose monocytes responded to rHuIFN-γ in the *in vitro* studies. Two individuals have autosomal recessive, cytochrome *b*-positive CGD (L.O. and J.H., Table 1), whereas the third individual has autosomal dominant, cytochrome *b*-positive CGD (C.H., Table 1). All were free of active infection, with normal erythrocyte sedimentation rate, complete blood count, and blood chemistry, which included renal and liver function studies. The rHuIFN-γ was freshly prepared from lyophilisate and stored for no more than a few hours at 4°C before administration. The rHuIFN-γ was given as a single subcutaneous dose of 0.01 mg/m² (L.O., C.H.) or 0.05 mg/m² (J.H.). The specific activity of rHuIFN-γ provided by Genentech is $\approx 2 \times 10^7$ units/mg, referenced to the NIH IFN-γ standard. Blood counts, chemistry, vital signs, and any symptoms were monitored during and after the study period. Administration of rHuIFN-γ to patients with CGD was done within the context of NIH-approved Clinical Protocol 87-I-136.

Electrophoretic Transfer Blot Detection of Cytochrome *b*. One million neutrophils were extracted in 50 μl of 18 mM Tris glycine buffer (pH 8.0) containing 1% Triton X-100 and protease inhibitors, solubilized in $NaDodSO_4$ sample buffer, electrophoresed into 10% polyacrylamide gels (19), and electroblotted onto nitrocellulose (20). A synthetic peptide corresponding to the 19 C-terminal amino acids of the large subunit of cytochrome *b* (21) was conjugated to keyhole limpet hemocyanin and used to immunize rabbits. The resulting antiserum had characteristics similar to previously described antiserum to the large subunit of cytochrome *b* (22). This antiserum detected a 91-kDa peptide in normal neutrophils that was missing from most X chromosome-linked CGD neutrophils (23). The antiserum was used with peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in an electrophoretic transfer blot assay to detect changes in the amount of cytochrome *b* large subunit. Preliminary experiments demonstrated that Triton X-100 extraction solubilized all detectable neutrophil cytochrome *b* large subunit and that the development of the peroxidase reaction was linear in the range of 0.5×10^6 to 5×10^6 neutrophils.

Statistical Analysis. Except where specifically indicated, Student's *t* test was used to determine statistical significance of the difference between two sets of data.

RESULTS

Dose–Response and Time Course for rHuIFN-γ Effect on CGD Monocytes *in Vitro*. Based on preliminary screening, several patients with the classic phenotype of CGD were identified whose peripheral blood monocytes were found to produce superoxide when cultured in the presence of rHuIFN-γ. Monocytes from four of these patients were cultured in the presence of different concentrations of rHuIFN-γ for 3 days in 96-well microtiter plates and superoxide production was measured (Fig. 1 *Upper*). A response to rHuIFN-γ could be seen with as little as 10 units/ml, was highly significant at 100 units/ml ($P < 0.01$), and increased through 10,000 units/ml.

Monocytes from one of the responding patients were then used to establish the effect of different incubation times with rHuIFN-γ. Fig. 1 *Lower* shows that the effect of a single addition of rHuIFN-γ to a culture of CGD monocytes was maximum at 48–72 hr. The effect of rHuIFN-γ on monocytes could be inhibited by the addition of cycloheximide at 10 μg/ml to the culture simultaneously with rHuIFN-γ (data not shown).

Based on these results, a concentration of rHuIFN-γ of 100 units/ml was used as the standard dose and 3 days was used for the standard time in culture for subsequent studies of *in vitro* responses of CGD monocytes to rHuIFN-γ. With all of the doses of rHuIFN-γ used and with PMA at 100 ng/ml as the stimulus for activation of oxidative metabolism, the kinetics of superoxide production was linear through 1 hr with normal monocytes and responding CGD monocytes (not shown). In subsequent studies cumulative superoxide production by monocytes was measured at 1 hr.

Evaluation of *in Vitro* rHuIFN-γ on Cultured Monocytes from 30 Patients with CGD. Table 1 shows the results of PMA-stimulated superoxide production by monocytes from CGD patients and normal controls after 3 days in culture with or without rHuIFN-γ. Cultured monocytes from all of the CGD

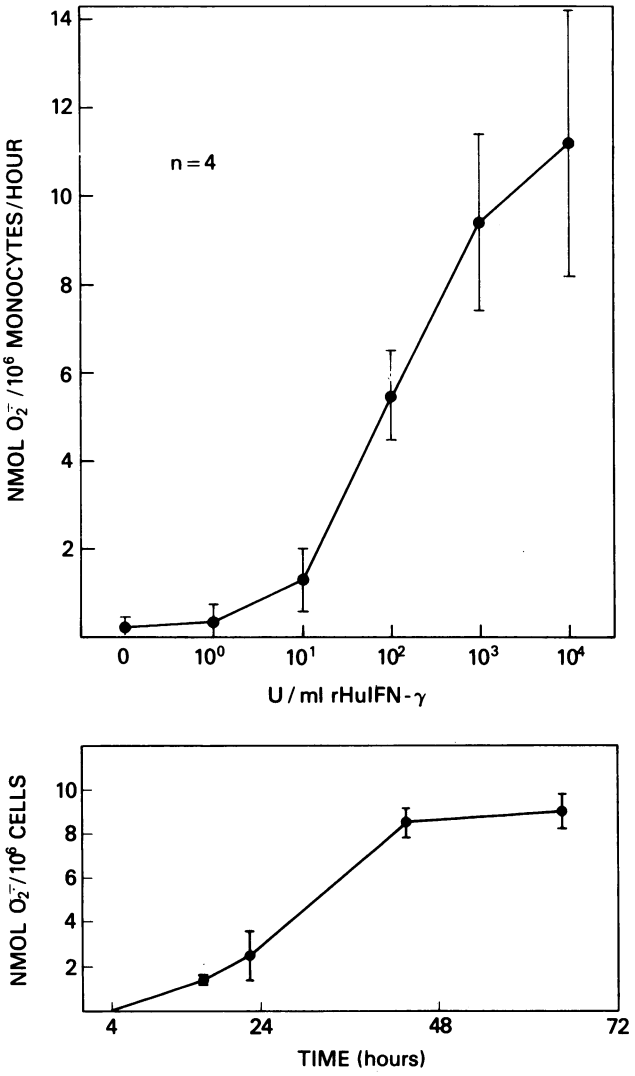

FIG. 1. Dose–response and time course for the rHuIFN-γ effect on CGD monocytes *in vitro*. After culture with or without rHuIFN-γ, monocytes were stimulated with PMA at 100 ng/ml and the cumulative amount of superoxide produced over 1 hr was determined in a ferricytochrome *c* reduction assay. (*Upper*) Superoxide production measured after 3 days of culture in the presence of various concentrations of rHuIFN-γ (average of four different cytochrome *b*-positive CGD patients). (*Lower*) Superoxide production measured after various times in culture with rHuIFN-γ at 100 units/ml.

patients in this study produced little or no superoxide without rHuIFN-γ. Monocytes from 15 of 16 patients whose phagocytes contain cytochrome *b* and 4 of 14 patients whose phagocytes are deficient in cytochrome *b* showed enhancement of PMA-stimulatable superoxide production following rHuIFN-γ treatment. The difference in response rate of monocytes from cytochrome *b*-positive and cytochrome *b*-negative CGD patients was significant ($P < 0.01$, Fisher exact test).

The results of superoxide measurement were supported by NBT reduction assays, though not all monocytes demonstrated equal NBT reduction. Following incubation with rHuIFN-γ and stimulation with PMA, an average of 36% ± 3% of monocytes from nine responding CGD patients showed moderate reduction of NBT, although the amount of NBT reduction seen was less than that of normal monocytes.

Subcutaneous Administration of rHuIFN-γ to CGD Patients. Based on the above *in vitro* studies, rHuIFN-γ was administered subcutaneously to a patient with autosomal recessive, cytochrome *b*-positive CGD whose cells had responded to rHuIFN-γ *in vitro* (patient L.O., Table 1). The rHuIFN-γ at 0.01 mg/m^2 was given first as a single dose, to evaluate safety and effect, and then given 6 days later for five daily doses. The patient had no symptoms and no changes in blood chemistry or cell counts following the injections of rHuIFN-γ or for several weeks after the last dose. One day after the single injection there was an increase in neutrophil and monocyte PMA-stimulated superoxide production (Fig. 2 *Upper*). However, maximum responses were noted in neutrophils and monocytes after 3 days of daily injections (day 9). The rate of superoxide production by monocytes following *in vivo* rHuIFN-γ was about 10% of normal monocytes, whereas

Table 1. Effect of *in vitro* rHuIFN-γ on superoxide production by CGD monocytes

Subject	Superoxide, nmol produced per hr per 10^6 monocytes: Control	rHuIFN-γ*
Normal†	19.3 ± 3.9	43.3 ± 3.2
Patient		
Cytochrome *b*-negative		
X chromosome-linked		
S.H. } Siblings	0	0
E.H. } Siblings	0	0
P.K. } Siblings	0	0
R.K. } Siblings	0	0
D.H.	0	0 (2)
T.G.	0	0 (2)
E.W.	0	0
J.K.	0	0
T.B.	0	0
S.S.	0	2.3 (2)
S.P.	0	2.8 (2)
W.M.	0	3.6
M.J.	0	41.0
Autosomal recessive		
A.V.	0	0 (2)
Cytochrome *b*-positive		
X chromosome-linked		
R.D.	0	12.7
Autosomal recessive		
N.S.	0	0
S.J. } Siblings	0	4.5
B.J. } Siblings	0	4.7
D.J. } Siblings	0	6.6
J.H.	0	1.4
H.L.P.	0	1.6 (2)
D.C.	0.3	2.6
R.G.	0	2.7
T.A.	0	3.6 (3)
P.R.	0	7.5 (2)
M.B.	0	7.6
L.O.	0	9.0
K.L.	0	21.4
Autosomal dominant		
L.H. } Same kindred	2.0	9.0
C.H. } Same kindred	1.1	14.2 (2)

Monocytes were stimulated with PMA at 100 ng/ml and the cumulative superoxide produced over 1 hr was measured by using a ferricytochrome *c* reduction assay. The number in parentheses indicates the number of times a patient was studied and the results are averaged. Unless indicated, a patient was studied on one occasion.

*Monocytes were cultured for 3 days with or without rHuIFN-γ at 100 units/ml.

†Monocytes from 10 normal subjects were cultured and assayed during the course of patient studies.

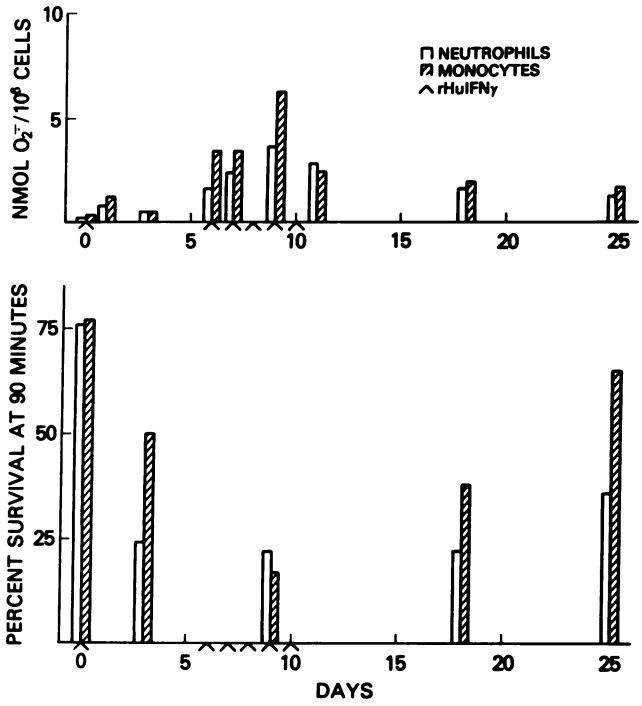

FIG. 2. Superoxide production and bactericidal activity of CGD neutrophils and monocytes from patient L.O. before (day 0), during, and after subcutaneous administration of rHuIFN-γ (0.01 mg/m^2) as indicated. (*Upper*) Cumulative superoxide production over 1 hr. (*Lower*) Percent survival of bacteria after 90 min.

that of neutrophils was about 2% of normal neutrophils. Enhanced production of superoxide was evident but was near baseline a week after the last dose of rHuIFN-γ.

To determine if the rHuIFN-γ-mediated enhancement of phagocyte superoxide production in this patient might be functionally significant, the ability of the patient's phagocytes to kill *S. aureus* was determined. The results of bactericidal assays are shown in Fig. 2 *Lower* and the kinetics of bacterial killing are shown in Fig. 3. Neutrophil and monocyte bactericidal activity for *S. aureus* was improved significantly following an initial, single injection of rHuIFN-γ. The bactericidal capacity of phagocytes was greatest during the sustained daily injections. This was coincident with the sustained increase of superoxide production shown in Fig. 2 *Upper*. The effect of *in vivo* rHuIFN-γ on monocyte bactericidal capacity persisted for at least a week after the final injection and the effect on neutrophils was still evident after >2 weeks.

Since superoxide production may relate to levels of cytochrome *b*, we monitored changes in cytochrome *b* levels in neutrophils obtained after rHuIFN-γ administration. By using a synthetic peptide antibody to the large subunit of cytochrome *b* we demonstrated increased amounts of large subunit in neutrophils obtained from patient L.O. during the period of sustained administration of rHuIFN-γ (Fig. 4, days 8 and 11).

Two additional patients with autosomally inherited, cytochrome *b*-positive CGD (C.H. and J.H., Table 1) were treated with rHuIFN-γ (C.H. at 0.01 mg/m^2, for five daily doses; J.H. at 0.05 mg/m^2, 3 days a week for 2 weeks). Enhancement of bactericidal capacity and superoxide production by phagocytic cells was observed in both patients. For C.H.'s monocytes and neutrophils, respectively, *S. aureus* survival at 1.5 hr was 90% and 40% pre-rHuIFN-γ versus 69% and 24% (3 days), 38% and 31% (6 days), and 35% and 34% (10 days). For J.H.'s monocytes and neutrophils, respectively, *S. aureus* survival at 1.5 hr was 79% and 64% pre-rHuIFN-γ versus 31% and 28% (7 days), 50% and 36% (14 days), 22% and 18% (21 days), and 65% and 20% (28 days). Detectable increases in neutrophil superoxide production were not observed with C.H. and J.H. as had been seen with L.O. However, monocyte superoxide production increased (C.H.: 0.6 nmol of superoxide per hr per 10^6 monocytes pre-rHuIFN-γ versus 1.1, 5.0, and 3.0 nmol at days 3, 6, and 10, respectively; J.H.: 0.5 nmol of superoxide per hr per 10^6 monocytes pre-rHuIFN-γ versus 2.2, 1.2, 1.1, and 0.3 nmol at days 7, 14, 21, and 28, respectively). Thus, for all three CGD patients rHuIFN-γ administration increased bactericidal activity of monocytes and neutrophils, at the same time boosting superoxide production by monocytes.

DISCUSSION

These studies clarify and extend the recent results of Ezekowitz *et al.* (12), who reported that in nine patients with CGD, rHuIFN-γ stimulated the respiratory burst only in X chromosome-linked "variants" of CGD, who had an unusually mild clinical presentation. Two of the responders in that study were brothers whose neutrophils, prior to any rHuIFN-

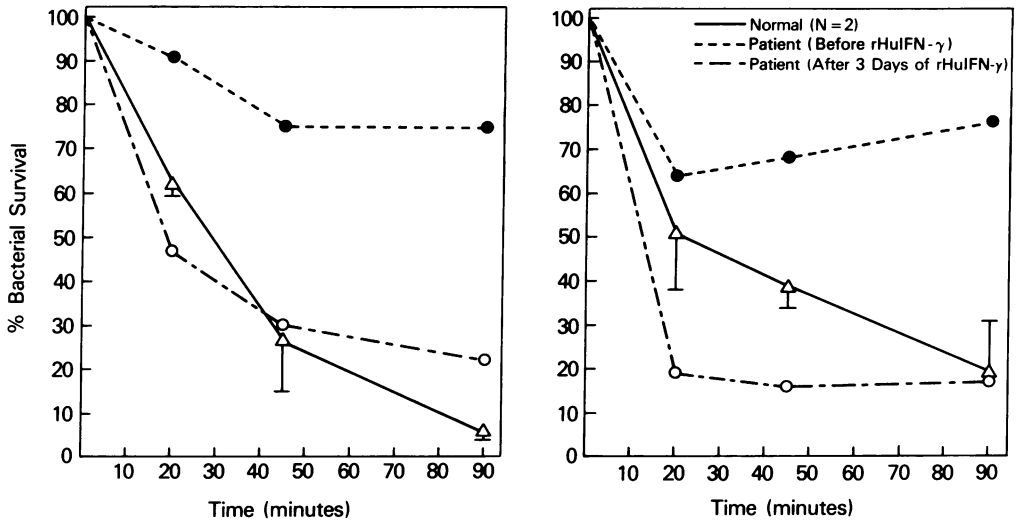

FIG. 3. Kinetics of bacterial killing by normal neutrophils and monocytes are compared to that of CGD neutrophils and monocytes, respectively, from patient L.O. before (day 0, see Fig. 3) and after (day 9, see Fig. 3) subcutaneous administration of rHuIFN-γ (0.01 mg/m^2). (*Left*) Neutrophil killing. (*Right*) Monocyte killing. The bacteria-to-cell ratios were the same for neutrophils and monocytes.

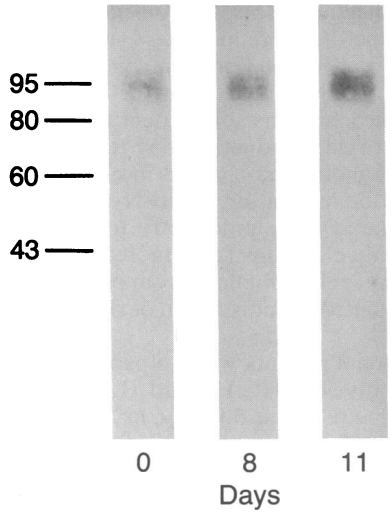

FIG. 4. Electrophoretic transfer blot of cytochrome *b* from autosomal recessive CGD neutrophils after IFN-γ *in vivo* (patient L.O., see Fig. 2). Triton X-100 extracts of 10^6 neutrophils obtained at the indicated days during the rHuIFN-γ protocol were electrophoresed into $NaDodSO_4$/polyacrylamide gels, blotted onto nitrocellulose, and probed with the antiserum against the cytochrome *b* large subunit. Molecular mass markers are indicated in kDa.

γ treatment, produced 10% and 18% of the cumulative superoxide generated by normal neutrophils 5 min after stimulation with PMA. Neutrophils from a third responder, who also had X chromosome-linked inheritance, produced about 0.5% of the superoxide generated by normal neutrophils (24). These authors used a cDNA probe (21) for the defective gene in X chromosome-linked CGD (large subunit of cytochrome *b*) to demonstrate that rHuIFN-γ greatly enhances mRNA expression of this gene in one of the two responding brothers. It was suggested that patients with the X chromosome-linked form of CGD, who retained significant capacity to produce superoxide (variant phenotype), have a defect in regulation of the synthesis of the large subunit of cytochrome *b*. The authors postulated that rHuIFN-γ enhances expression of this gene, which may partially compensate for the defect in these X chromosome-linked variant CGD patients. Monocytes from one of our X chromosome-linked CGD patients (M.J., Table 1) had a pronounced response to rHuIFN-γ to within the normal range, and this may represent a similar phenomenon.

Our studies in 30 CGD patients indicate that the enhancement of superoxide production in monocytes by rHuIFN-γ is not limited to X chromosome-linked patients with variant phenotype but can be seen in a significant subset of patients with the phenotypically classic form of CGD. In this much larger group of patients the presence of cytochrome *b* in phagocytes is correlated with enhancement of superoxide production by phagocytes after rHuIFN-γ treatment. However, one cytochrome *b*-positive CGD patient did not respond, and almost one-third of X chromosome-linked, cytochrome *b*-negative CGD patients did respond.

We show here, by using a synthetic peptide antibody directed to the large subunit of cytochrome *b*, increased level of this subunit in neutrophils obtained from a patient given rHuIFN-γ *in vivo*. It is likely that rHuIFN-γ acts to enhance expression of not only this gene but other gene products required for the oxidative burst. Nonoxidative killing mechanisms of phagocytes may also be enhanced by rHuIFN-γ, accounting for the substantial increase in bactericidal activity. It is possible that therapy with rHuIFN-γ could benefit CGD patients even where there is no enhancement of the respiratory burst.

Most exciting is our finding that with subcutaneous rHuIFN-γ, in doses easily tolerated by patients, there is reconstitution of phagocyte bactericidal activity with an associated increase of superoxide production. This provides strong evidence some patients with CGD can have their microbicidal defect corrected pharmacologically and represents an example of reconstitution of an immune function *in vivo* with rHuIFN-γ. Our *in vitro* data suggest rHuIFN-γ may benefit a significant subset of CGD patients. We look forward to more extensive clinical trials with CGD patients in the near future.

We thank Dr. Ralph Snyderman, Dr. Stephen Sherwin, and Dr. Howard Jaffe of Genentech, Inc., for their suggestions regarding the clinical protocol, for providing the IFN-γ and for their critical reading of the manuscript. We thank Patricia Runyon for her help in the preparation of the manuscript.

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