Prolonged Recombinant Interferon-γ Therapy in Chronic Granulomatous Disease: Evidence Against Enhanced Neutrophil Oxidase Activity

By Richard C. Woodman, Richard W. Erickson, Julie Rae, Howard S. Jaffe, and John T. Curnutte

Recombinant interferon-γ (rIFN-γ) therapy has become an effective form of prophylaxis for patients with chronic granulomatous disease (CGD). Preliminary studies with CGD suggested that rIFN-γ treatment enhanced phagocyte oxidase activity and increased superoxide (O$_2^-$) production. We evaluated various aspects of neutrophil NADPH oxidase activity in 19 CGD patients (representing all four known types of CGD) receiving prolonged rIFN-γ therapy (6 to 27 months). In contrast to earlier studies, we failed to detect any improvement in neutrophil NADPH oxidase activity in 18 of the 19 CGD patients as determined by (1) intact cell O$_2^-$ production (continuous assay), (2) nitroblue tetrazolium (NBT) staining, (3) cytochrome b$_{558}$ spectroscopy, and (4) activity levels of cytosol and membrane oxidase components using a cell-free activation system. One patient with a variant form of X-linked CGD had a transient increase in neutrophil O$_2^-$ production following 3 months of rIFN-γ therapy. However, this was not sustained, and was not associated with any change in cytochrome b levels. In some patients, rIFN-γ therapy was associated with the appearance of a small subset of circulating monocytes (1% to 20%) that were NBT-positive. Although the functional significance of this monocyte subpopulation needs to be determined, these results suggest that one possible mechanism by which rIFN-γ may benefit CGD patients is by partially correcting the respiratory burst defect in a subset of monocytes. We conclude that the clinical benefit of prolonged rIFN-γ therapy in the vast majority of CGD patients is not due to enhanced neutrophil NADPH oxidase activity. The mechanism of action of rIFN-γ in most CGD patients remains unknown.

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CHRONIC GRANULOMATOUS disease (CGD) is a rare inherited disorder characterized by recurrent pyogenic infections. Bacterial killing is impaired because of an inability of phagocytic cells to convert molecular oxygen into superoxide (O$_2^-$) by an enzyme known as NADPH oxidase. Recently, it has become apparent that the oxidase is a complex multicomponent enzyme system that includes several cytosolic components and a unique membrane-bound heterodimeric cytochrome b (termed cytochrome b$_{558}$). It is therefore not surprising that CGD, like many other inherited diseases, is actually a heterogeneous group of disorders, each with a different genetic defect affecting a separate component of the oxidase. The majority of patients (55% of cases) have the classic X-linked form of CGD due to a complete deficiency of the 91-Kd glycoprotein subunit (gp91-phox) of cytochrome b$^{13}$ (CGD type X91$^{-}$). The gene encoding for this protein is located at the Xp21.1 locus; point mutations and small deletions in this gene will probably be most commonly responsible for this form of CGD.$^{3,7}$ Occasionally, patients have a variant form of X-linked CGD with greatly diminished, but detectable, levels of cytochrome b and O$_2^-$ production (X91$^{-}$). In 5% of cases, CGD is due to an inherited abnormality in the 22-Kd subunit (p22-phox) of cytochrome b, which is encoded by a gene on chromosome 16 (CGD type A22$^a$). Autosomal recessive CGD can also occur due to deficiencies of a 47-Kd cytosolic phosphoprotein (p47-phox) (CGD type A47$^b$, 35% of cases)$^{7}$ or a 67-Kd cytosolic polypeptide (p67-phox) (CGD type A67$^a$, 5% of cases)$^9$, which are encoded by genes on chromosomes 7 and 1, respectively.$^{19}$ CGD patients with defects in proteins other than the cytochrome b subunits or p47-phox and p67-phox have not been reported.$^3$

The ability of interferon-γ (IFN-γ) to enhance or prime the oxidase of normal human phagocytic cells in vitro and thereby augment O$_2^-$ production in response to a variety of stimuli has been extensively studied, but the precise mechanism of action remains uncertain.$^{1,3,13}$ Preliminary studies in selected patients with X-linked variant CGD$^{13,14}$ and autosomal recessive/cytochrome b-positive CGD$^9$ demonstrated that brief in vitro or in vivo administration of recombinant IFN-γ (rIFN-γ) significantly enhanced phagocyte O$_2^-$ production and Staphylococcus aureus bacterial killing. In two patients with variant X-linked CGD, rIFN-γ treatment in vivo was also associated with increased spectral levels of neutrophil cytochrome b, probably due to an increase in the level of the gp91-phox cytochrome subunit as measured by

*The nomenclature for CGD refers to the mode of inheritance (X for X-linked, A for autosomal recessive), the affected protein (91-, 22-, 47-, or 67-Kd oxidase subunit), and the measured level of the affected protein (undetectable ('), diminished ('), or normal (')).
Western blot analysis.14 Earlier studies with monocytes from these two patients had demonstrated increased gp91-
phox mRNA expression following in vitro administration of rIFN-γ.15 More recent studies involving these same two CGD patients suggest that the respiratory burst defect was partially corrected by IFN-γ at the level of the myeloid progenitor cell.16 Although these preliminary studies sug-
gested a possible mechanism of action for rIFN-γ in CGD, they were limited by the brief duration of rIFN-γ adminis-
tration and the number of patients studied.

A recently completed multicenter, double-blind, placebo-
controlled randomized phase III study of 128 CGD patients demonstrated that prolonged prophylactic rIFN-γ therapy led to a 70% reduction in the relative risk of serious infections for both X-linked and autosomal recessive pa-
tients.17 A critical question that remains unanswered by this study is the mechanism of action of rIFN-γ in CGD. In contrast to earlier studies, there was no correlation in the phase III study between clinical benefit from rIFN-γ administration and either enhanced neutrophil (or mono-
cyte) O2− production (discontinuous assay) or in vitro S.
aureus bacterial killing.

To investigate further the effects of rIFN-γ in CGD, we
prospectively examined several aspects of neutrophil respira-
tory burst activity (including cell-free NADPH oxidase activity) in 19 CGD patients after prolonged rIFN-γ ther-
apy (6 to 27 months). All four known phenotypes of CGD
were represented in this study, as well as two patients with a
variant form of X-linked CGD.

MATERIALS AND METHODS

Patient population. All CGD patients were studied at the
General Clinical Research Center of The Scripps Research Insti-
tute. Sixteen participants participated in the double-blind, placebo-
controlled randomized phase III study,18 while four patients received rIFN-γ under a compassionate protocol. One of the patients enrolled in the phase III study could not be reevaluated after 3 months of therapy because of recurrent infections and a
resulting inability to travel to the study center. Clinical data from
this patient are included, but follow-up neutrophil studies were not
performed. Informed consent was obtained from all patients
according to protocols approved by the Scripps Human Subjects
Committee. Fourteen males and five females with a mean age of
11.8 years were studied. The diagnosis of CGD was established
before rIFN-γ therapy according to the entry criteria of the randomized study (O− production ≤ 20% of normal and an
abnormal nitroblue tetrazolium [NBT] test).17 Each patient was
classified according to pattern of inheritance (X-linked or autoso-
nal recessive) and deficiency of one of the known oxidase compo-
nents (gp91-, p22-, p47-, or p67-phox) as determined by cytochrome
b spectroscopy.18 Northern blot analysis of p22- and gp91-phox
mRNA,18 cytosol complementation studies,19 and (in some cases)
immunoblot analysis20 All patients received rIFN-γ subcutaneously
three times per week (0.05 mg/m2/dose) for periods of 6 to 27
months. Seventeen of the 19 patients were on daily antibiotic
administration and either enhanced neutrophil (or mono-
cyte) O2− production (discontinuous assay) or in vitro S.
aureus bacterial killing.

Superoxide and cytochrome b55 levels. Intact cell O2−
assays and cytochrome b measurements were performed on neutro-
phils (> 95% purity) isolated from whole blood by a previously
published method.26 Cytosol and plasma membranes, used for
cell-free oxidase activation, were prepared from unstimulated
neutrophils collected by leukapheresis and fractionated as de-
scribed elsewhere.28 The rates of O2− production by intact neutro-
phils in suspension and by neutrophil membranes and cytosol in
the cell-free activation system were determined by measuring the
kinetics of superoxide dismutase (SOD)-inhibitable reduction of
ferricytochrome c. For intact neutrophils, cells were stimulated
with phorbol myristate acetate (PMA; final concentration, 200
ng/mL) as previously described.23 NADPH oxidase activity in a
cell-free system was determined with 40 μmol/L sodium dodecyl
sulfate (SDS) using a previously reported method.29

Neutrophil cytochrome b55 levels were determined by dithionite
difference spectroscopy as previously described.22 In patient M.P.,
with a variant form of X-linked CGD (X91−), cytochrome b
spectroscopy was performed on solubilized neutrophil membranes
using the same method.

NBT slide test. The reduction of NBT dye by neutrophils and
monocytes after stimulation with PMA (final concentration, 200
ng/mL) was performed using a modification of a previously
published method.30 The percentage of NBT-positive cells was
determined by light microscopy after counting 200 or more
neutrophils (or ≥ 50 monocytes). In patients with variant X-linked
CGD, the staining intensity of the patient's NBT-positive cells
compared with control cells was also determined.

RESULTS

The results of rIFN-γ therapy in CGD have recently been
reported.17 The 16 patients from Scripps Clinic entered in the
phase III study are included in that report. Among the
eight patients eventually found to be on rIFN-γ, there were
a total of five serious infections† (primary and recurrent)
compared with 10 infections in the eight patients receiving
placebo. In terms of the number of infections, the Scripps
patients were similar to the larger group of CGD patients
reported in the phase III study.

Neutrophil superoxide production and cytochrome b levels. The effect of prolonged rIFN-γ therapy on intact neutro-
ophil O2− production and cytochrome b levels is shown in
Table 1. In contrast to earlier studies,13–15,21 O2− production
was measured using a sensitive continuous assay. Despite
prolonged rIFN-γ therapy varying from 6 to 27 months, only
one of the 19 CGD patients (D.H.) demonstrated any
appreciable enhancement of neutrophil O2− production.
This patient has a variant form of X-linked CGD (X91−)
and showed a transient fourfold increase in O2− production
after 3 months of therapy. However, this increase was not
sustained based on reevaluation at 9 months of therapy.
None of the CGD patients, regardless of whether they had
absent, diminished, or normal levels of neutrophil cyto-
chrome b, showed any significant change in neutrophil
cytochrome b levels following prolonged rIFN-γ therapy.

Neutrophil cell-free oxidase activity. The effects of pro-
longed rIFN-γ therapy (3 to 16 months) on the activity of
the cytosol and membrane components of NADPH oxidase
are shown in Table 2. Cytosol activity in neutrophils from
five CGD patients studied (two with cytochrome b deficiency
[X91− and A22−], two with p47-phox deficiency [A47−], and

†Defined as a clinical event requiring hospitalization and the
institution of parenteral antibiotics.17
one with p67-phaX deficiency [A67"]) did not change with rIFN-γ therapy varying from 3 to 16 months. Similarly, the one with p67-phaX deficiency [A67"] did not change with studied (two with cytochrome b deficiency [X91" and A22"], two with p47-phaX deficiency [A47"], and one with p67-phaX deficiency [A67"] was not influenced by rIFN-γ administration of 3 to 16 months.

NBT slide tests. Serial NBT slide tests were performed prospectively to assess respiratory burst activity in individual neutrophils and monocytes. Results from 15 patients entered in the randomized phase III study are shown in Table 3. In six of eight patients eventually found to be on rIFN-γ therapy, 1% to 20% of peripheral blood monocytes remained NBT-negative during rIFN-γ therapy. The acquisition of peripheral blood NBT-positive monocytes during rIFN-γ therapy did not correlate with a particular type of CGD. None of the seven patients on placebo developed NBT-positive monocytes; two of these patients with A67" CGD were tested 5 months after switching to IFN-γ therapy at the end of the phase III study and developed 2% to 3% NBT-positive monocytes. Examples of NBT-positive monocytes are illustrated in Fig 1. One of the A67" CGD patients described above also developed a few weakly NBT-positive neutrophils (2%) with an atypical perinuclear

<table>
<thead>
<tr>
<th>Type of CGD*</th>
<th>Duration of Therapy</th>
<th>O₂⁻ Production</th>
<th>Cytochrome b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mo)</td>
<td>(nmol/min/10⁷ cells)</td>
<td>Spectroscopy (μmol/10⁷ cells)</td>
</tr>
<tr>
<td>X91&quot; (7)</td>
<td>6-26</td>
<td>0.0-1.1</td>
<td>6-13</td>
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<tr>
<td>A22&quot; (1)</td>
<td>26</td>
<td>ND</td>
<td>21</td>
</tr>
<tr>
<td>A47&quot; (3)</td>
<td>13-24</td>
<td>0.0-0.3-0.3</td>
<td>5</td>
</tr>
<tr>
<td>A67&quot; (2)</td>
<td>13</td>
<td>0.0</td>
<td>53.0-59.4</td>
</tr>
<tr>
<td>X91&quot; (2)</td>
<td>3,9</td>
<td>3.1</td>
<td>11.2</td>
</tr>
<tr>
<td>M.P.</td>
<td>5,6, 19, 27</td>
<td>16.8 ± 7.3</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*See footnote (*) in text for definition of CGD subtypes.
†Number of months of IFN-γ therapy in which O₂⁻ or cytochrome b measurements were made.
‡Range of measurements for patients. Control (mean ± 1 SD) is 149.2 ± 33.8 (n = 14).
§Range of measurements for patients. Control (mean ± 1 SD) is 73.0 ± 30.9 (n = 42).
*Only six of the seven patients had cytochrome b measurements determined.
†Only one patient had O₂⁻ measurements determined.

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*Based on intensity of NBT-staining, this patient had a transient response to rIFN-γ therapy.
†See footnote (*) in text for definition of CGD subtypes.
‡Before rIFN-γ treatment, this patient had strongly NBT-positive neutrophils (5% to 12%) and monocytes (2% to 5%), which did not change following rIFN-γ therapy.
§Based on intensity of NBT-staining, this patient had a transient response to rIFN-γ treatment.
staining pattern; a field with an unusually high percentage of these neutrophils is shown in the lower right panel of Fig 1. This was not observed in other patients and was not accompanied with any detectable release of $\mathrm{O}_2^-$ as measured by the cytochrome c assay.

Serial NBT tests from the two patients with X91- (variant) CGD were also examined before and during rIFN-γ therapy. One of these patients (M.P., on the phase III rIFN-γ arm) had a subset of strongly NBT-positive neutrophils (5% to 10%) and monocytes (2% to 5%) before treatment, which did not change despite prolonged rIFN-γ therapy (27 months). In the second patient (D.H., on compassionate rIFN-γ), 100% of the neutrophils and monocytes were weakly NBT-positive before rIFN-γ therapy. Following 3 months of IFN-γ therapy, there was a significant increase in the intensity of NBT staining. As with the intact neutrophil $\mathrm{O}_2^-$ measurements described above, these changes were transient and were not present after 9 months of IFN-γ therapy. Furthermore, they were never associated with any significant change in cytochrome b levels.

**DISCUSSION**

In this study, we evaluated several aspects of neutrophil respiratory burst activity in 19 CGD patients during prolonged rIFN-γ therapy. All four major genetic types of CGD (X91°, A22°, A47°, and A67°) were studied, including two patients with a variant form of X-linked CGD (X91°). Despite prolonged continuous rIFN-γ therapy, none of the 19 patients showed a sustained improvement in circulating neutrophil NADPH oxidase function as determined by (1) intact cell $\mathrm{O}_2^-$ production, (2) serial NBT slide tests, (3) cytochrome $b_{558}$ spectral levels, or (4) membrane and cytosol activity in the cell-free oxidase activation system. Only one patient with a variant form of X-linked CGD demonstrated a transient fourfold increase in neutrophil $\mathrm{O}_2^-$ production after 3 months of rIFN-γ therapy. However, despite continued rIFN-γ therapy, this was unsustained; it was also never associated with any change in cytochrome $b_{558}$ levels or in cell-free oxidase activity. Although some select CGD patients may have increased phagocyte $\mathrm{O}_2^-$ production following in vivo rIFN-γ administration, this does not appear to be the case in all patients with X-linked variant CGD (X91°). This discrepancy may be due to the heterogeneity of the genetic defects now known to be responsible for cytochrome b-deficient CGD. It is possible that point mutations involving the gp91-phox promoter region may be more responsive to rIFN-γ therapy than those involving the remainder of the gp91-phox gene.

Our investigations with rIFN-γ therapy are in agreement with the results of the recently reported phase III study in which there was no correlation between clinical benefit of rIFN-γ and enhanced neutrophil $\mathrm{O}_2^-$ production. The critical question that remains unanswered, therefore, is the mechanism of action of rIFN-γ in CGD. Although the functional significance of the circulating subset of NBT-positive monocytes remains to be determined, our results suggest that one mechanism in which rIFN-γ may benefit CGD patients is by partially correcting the respiratory burst defect in a subset of peripheral blood monocytes. Since monocytes spend a relatively brief time in circulation before entering tissues, the actual number of NBT-positive monocytes/macrophages may, in fact, be greater than observed in our study. Further studies to characterize these monocytes and to quantitate the percentage of NBT-positive monocytes/macrophages in tissues are required.

Other possible explanations regarding the mechanism of action of rIFN-γ in CGD include improved neutrophil microbicidal killing through a mechanism(s) other than the respiratory burst (e.g., increased levels of azurophilic granule antimicrobial proteins) or enhanced function of other cells within the immune system such as T and/or B cells. All of these could explain the reported clinical benefit of
rIFN-γ therapy in patients with both autosomal recessive and X-linked CGD. Because we only studied circulating neutrophils, we cannot exclude the possibility that rIFN-γ may augment the function of adherent neutrophils and/or tissue macrophages. It has already been established that certain cytokines may enhance the respiratory burst of neutrophils adherent to intravascular or extravascular surfaces, but have no effect on neutrophils in suspension. It is thus still possible that rIFN-γ therapy in CGD enhances the respiratory burst (and possibly other microbicidal mechanisms) of neutrophils resident within tissues.

The institution of rIFN-γ prophylaxis represents an important therapeutic advance in the treatment of CGD. Based on our studies, we plan to investigate the effects of rIFN-γ on the function of adherent phagocytic cells, as well as on the neutrophil oxygen-independent mechanisms of microbicidal killing. Determining the mechanism of action of rIFN-γ may ultimately have important therapeutic implications for other disorders of host defense.

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