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Treatment of patients with chronic granulomatous disease with recombinant human interferon-gamma does not improve neutrophil oxidative metabolism, cytochrome b558 content or levels of four anti-microbial proteins

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SUMMARY

Recombinant interferon-gamma (rIFN-γ) has been described to enhance phagocyte functions *in vitro* and *in vivo* in several patients with chronic granulomatous disease (CGD). To demonstrate the clinical usefulness of this treatment, 128 patients were treated in a randomized, double-blind multicentre study with a placebo preparation or with rIFN-γ. We analysed parameters of neutrophil oxidative and non-oxidative metabolism in 16 patients enrolled in this study. No enhanced superoxide-release was observed in patients treated with rIFN-γ compared to placebo-treated patients. Phagocyte cytochrome b558 content also remained unchanged. Levels of four non-oxidative antimicrobial proteins (cathepsine G, azurocidine, p29b, lactoferrin) rose, fell, or remained unchanged, irrespective of treatment with rIFN-γ or placebo.

Keywords chronic granulomatous disease recombinant interferon-gamma cytochrome b558 anti-microbial proteins

INTRODUCTION

Chronic granulomatous disease (CGD) is a group of rare congenital phagocyte disorders characterized by recurrent pyogenic infections of patients with catalase-positive bacteriae and fungi. Due to defects in the NADPH oxidase the phagocytic cells of these patients are unable to generate superoxide (O_2), hydrogen peroxide and other microbicidal oxygen metabolites in response to ingested microorganisms [1,2]. Recombinant interferon-gamma (rIFN-γ) is a lymphokine that has been shown to activate macrophages. *In vitro* and *in vivo* treatment of normal monocytes and neutrophils enhances the production of reactive oxygen intermediates and improves the capacity of killing of phagocytosed microorganisms [3,4].

Recent pilot studies demonstrated a partial correction of the phagocyte defect in patients with X-linked as well as autosomal recessive CGD by subcutaneous rIFN-γ [5–9]. Treatment resulted in five to 10-fold increase in superoxide production of neutrophils and monocytes; bactericidal activity rose proportionally and the phagocyte cytochrome b558 contents increased up to 50% of normal values [6,7]. Subsequently a randomized, placebo-controlled international phase III study of rIFN-γ in patients with CGD was performed showing clinical usefulness of the drug for infection prophylaxis [10]. Here we demonstrate analyses of NADPH oxidase activity, cytochrome b588 content, anti-microbial protein (AP) levels [11] in neutrophils and of serum IgG subclasses in 16 patients enrolled in this rIFN-γ study.

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PATIENTS AND METHODS

Patients

Twelve infection-free patients with complete X-linked cytochrome b558 deficiency (eight unrelated male patients, two cousins and one uncle and his nephew), two patients, one male, one female, with autosomal-recessive cytochrome B558 deficiency and two patients, one female, one male with autosomal-recessive cytochrome b558-positive CGD were enrolled in the study with local Ethical Committee approval. All 16 patients were on prophylactic antibiotics (co-trimoxazole) treatment, none received anti-mycotics or corticosteroids. Neutrophils from a healthy control were included in each analysis.

The diagnosis of CGD has been established by defective nitroblue tetrazolium (NBT) reduction in the NBT slide-test after stimulation with phorbol myristate acetate (PMA) and

opsonized zymosan particles (OPZ), deficient oxygen consumption or superoxide release from neutrophils upon maximal stimulation with PMA and fMLP, and in most cases by determination of cytochrome b558 content of neutrophils. X-linked inheritance has been established by the mosiac in the NBT test of the mothers of these patients. Autosomal-recessive inheritance was diagnosed by normal NBT test in the parents and determination of cytochrome b558 content and where necessary of soluble cytosolic factors of neutrophils.

Study design

The complete protocol of the study is described elsewhere [10]. Briefly, the influence of rIFN-γ was investigated in a multicentre, randomized, double-blind, placebo-controlled trial with two parallel groups (designated A and B). The patients received rIFN-γ (50 μg/m^2) (group A) or placebo (group B) subcutaneously three times a week for an average of 9 months in addition to co-trimoxazole prophylaxis. Neutrophil functions were assayed on day 0, 90 and 180. Subsequently an interim analysis was performed, and the study was unblinded and stopped, because clinical data were clearly in favour of rIFN-γ treatments. There has been no violation of the treatment protocol in our patient group. Nine out of the 16 patients had received the placebo preparation and seven patients received rIFN-γ. In the placebo group two episodes of serious infection necessitating hospitalization had occurred (one lymphadenitis, one aspergillus pneumonia), and in the IFN group one episode (a case of pneumonia).

Laboratory evaluations

Neutrophils were isolated from citrated blood sampled before the next injection of the study drug [12]. NBT reduction was performed as described by Bohler *et al.* [1] after stimulation with PMA (200 ng/ml) as well as with OPZ (150 μg/ml). The percentage of formazan-positive cells was determined by two persons counting three hundred on each slide and averaging the results. Superoxide formation was measured at 37°C as the superoxide dismutase (SOD)-sensitive redution of cytochrome c with PMA (100 μg/ml) and PMA + fMLP (1 μM) as stimuli. Cytochrome b558 was measured by determination of the reduced minus oxidized spectrum in a Triton X-100 extract. For all calculations of cytochrome b558 content an extinction coefficient of 106 mM^{-1}cm^{-1} for the Soret band was used [13]. Western blot analysis of cytochrome b was performed according to Verhoeven *et al.* [14]. Antibodies used were MoAb 48 against the heavy chain and MoAb 449 against the light chain of cytochrome b558 (Dirk Roos, Amsterdam, The Netherlands); alternatively rabbit antisera were used from C. Parkos, La Jolla, CA, against the light chain and from S. Orkin, Boston, MA, against the heavy chain of cytochrome b558. The Killing Assay with *Staphylococcus aureus* 502A was performed according to the standardized protocol [10]. AP levels were determined as follows: neutrophil pellets from healthy donors or patients at day 0 and day 90 of treatment were resuspended in Krebs–Ringer phosphate buffer with glucose in the presence of aprotinin (5 μg/ml), pepstatin (5 μg/ml), and PMSF (5 mM) and extracted with 50 mM Tris, pH 6·8, 1% SDS, 5% glycerol, 2% β-mercaptoethanol at 10^7 cells/ml. Samples were heated at 100°C for 3 min, centrifuged at 10 000 ***g*** for 10 min, and 10-μl aliquots (equivalent to 10^5 neutrophils) were used for SDS–PAGE and Western blot analysis using monospecific, polyclonal rabbit antibodies as described [15]. Levels of cathepsin G, azurocidine, p29b (all from azurophilic granules) and lactoferrin (from specific granules) were determined by densitometry as related to standard curves run with 0·1–1 μg of each of the purified proteins. Total IgG was determined nephelometrically and IgG subclasses were determined by conventional radial immunodiffusion using subclass-specific polyclonal antisera from Janssen Biochimica.

RESULTS

Six of the X-linked cytochrome b558-deficient patients and one patient with autosomal 67-kD protein deficiency were treated with rIFN-γ. The other six X-linked patients received placebo. The two patients with autosomal cytochrome b558 deficiency and one patient with 47-kD protein deficiency also received placebo. The results are summarized in Figs 1, 2 and 3. Briefly, none of the parameters observed changed in the rIFN-γ-treated patients compared with the placebo-treated controls. In the cytochemical NBT test (Fig 1a) all the cells remained formazan negative in all patients after stimulation with PMA (not shown) as well as with OPZ. The deficient superoxide production (Fig. 1b) was not improved in patients under rIFN-γ treatment. The cytochrome b558 content (Fig. 1c) remained below detection limit in all patients with cytochrome b558 deficiency, and was equally unchanged in one rIFN-γ-treated patient with 67-kD protein deficiency. In Western blots prepared from Triton X-100 cell extracts from day 0 and day 180 no change in either of the two subunits of cytochrome b558 was observed (not shown). The killing of *S. aureus* 502A (Fig. 1d) was not significantly increased in rIFN-γ-treated patients after 1 h (not shown) and 2 h of incubation. The total IgG and the IgG subclass levels remained unaffected by rIFN-γ treatment (Fig. 2). A marked increase or decrease in the level of one or more of the AP occurred over the 90-day interval in 10 out of the 16 patients. These changes did not appear to be correlated with treatment status (Fig. 3).

DISCUSSION

In contrast to earlier reports [5–9] we could not observe any improvement of neutrophil microbicidal functions under therapy with rIFN-γ in six CGD patients with X-linked cytochrome b558 deficiency (the most common genetic form of the disease) and one patient with 67-kD protein deficiency. The patients described in the earlier reports could have been special responders, e.g. variants of CGD with a defect that might be partially corrected by increased expression of the relevant proteins. Increased producton of mRNA of a defective component may in most cases however not lead to expression of a functional protein.

The improved clinical state of patients treated with rIFN-γ is thus unexplained, but might be related to the ability of the cytokine to induce increased levels of respiratory burst independent antimicrobial activity, either under basal conditions, or after additional stimuli generated during the course of infection. Attempts to measure AP during infection might be complicated by partial activation and degranulation of neutrophils in the circulation. Indeed, responses to inapparent infection may have contributed to the fluctuations observed in the levels of AP in most CGD patients over a period of 90 days. The pattern of

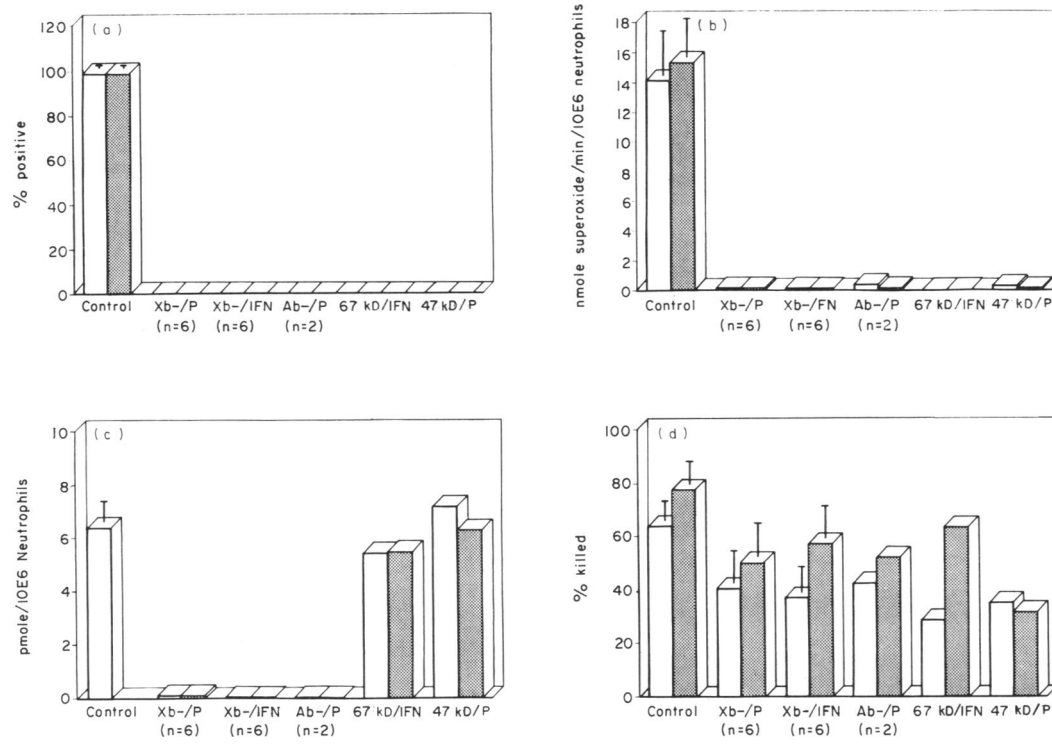

Fig. 1. Neutrophil functions and cytochrome b558 content of study patients on days 0 (□) and 180 (▦). (a): NBT test after stimulation with OPZ. Results are expressed as percentage of formazan-positive cells; (b) superoxide production after stimulation with PMA + fMLP; (c) cytochrome b558 content of neutrophils in pmole/10^6 cells; (d) killing of *S. aureus* after 2 h incubation (percentage of inoculum killed). Xb-/P: patients with X-linked cytochrome b558 deficiency; placebo treated. Ab/P: patients with a/r cytochrome b588 deficiency; placebo treated. 67-kD/IFN: patient with 67-kD protein deficiency; rIFN-γ-treated; 47 kD/P: patient with 47-kD protein deficiency; placebo treated.

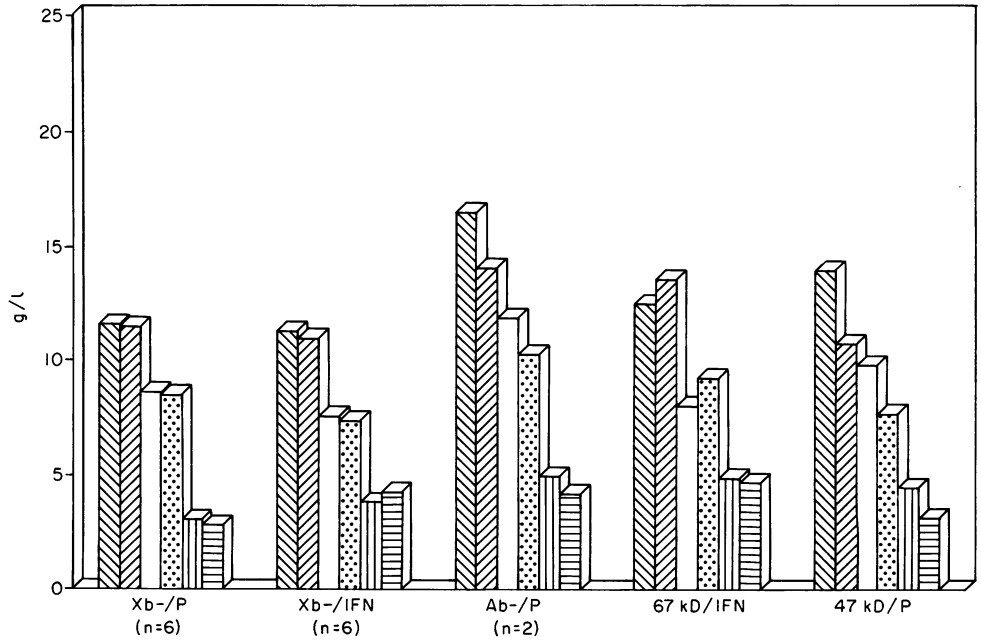

Fig. 2. Total serum IgG (day 0, ▧; day 180, ■), IgG1 (day 0, □; day 180, ▦) and IgG2 (day 0, ■; day 180, ■) levels of study patients. Abbreviations as in Fig. 1.

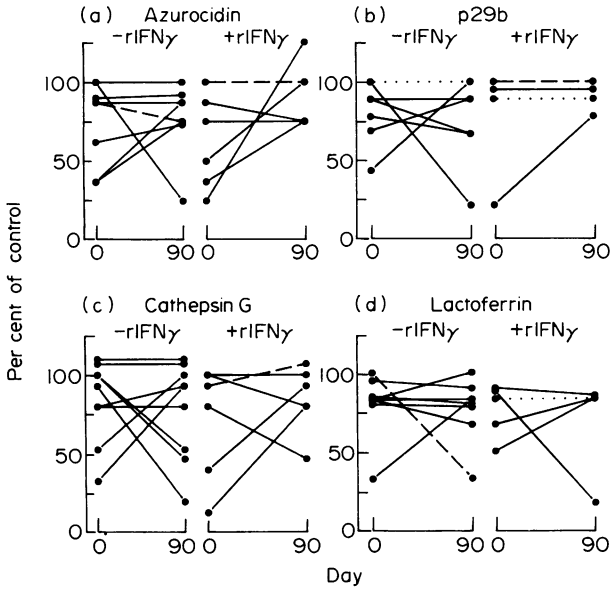

Fig. 3. AP levels measured by densitometry of Western blots are shown for each subject, with day 0 and day 90 values connected by lines. Solid lines denote results from one subject. Dashed lines are superimposable results for two different subjects. Dotted lines are superimposable results for three different subjects. Results are expressed as percentage of simultaneously determined values for four healthy donors, which varied by less than 5% and were as follows, in $\mu g/10^5$ neutrophils: azurocidin (a), 0·40; p29b (b), 0·45; cathepsin G (c), 0·75; and lactoferrin (d), 0·30.

fluctuation did not support the hypothesis that rIFN-γ induced an increase in levels of four different AP. However, we have not quantified several other AP, such as bactericidal permeability increased factor, elastase, and defensins [9] nor measured the efficency with which AP are delivered to bacteria within phagolysosomes. The apparent improvement of host response in rINF-γ-treated patients therefore remains to be explained.

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