Increased production of nitric oxide by neutrophils from patients with chronic granulomatous disease on interferon-gamma treatment

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ABSTRACT

Chronic granulomatous disease (CGD) is a rare immunodeficiency disorder in which phagocytic leukocytes fail to generate superoxide (O2−) and antimicrobial oxidants. The therapeutic validity of interferon-gamma (IFN-γ) has been well established in CGD patients but its underlying mechanisms remain poorly understood. One probable mechanism has been suggested to be modulation of nitric oxide (NO) release from phagocytic cells. Herein, we investigated NO production from neutrophil cells in CGD patients on treatment with IFN-γ in vivo and in vitro.

We measured NO levels in sera from 19 CGD patients (group I: 7 patients treated with TMP-SMX, group II: 12 patients treated with TMP-SMX and IFN-γ simultaneously) and healthy control individuals (8 cases). We also measured NO production from neutrophils in both patients groups as well as in control group after adding 100 U IFN-γ in vitro.

Our results showed that there was a significant difference between the groups in the NO levels of serum; patients who received IFN-γ had significantly higher amount of NO than the other groups. Besides, NO levels increased significantly after adding 100 U IFN-γ in vitro in three studied groups, considerably in the patients on treatment with IFN-γ.

As a brief conclusion, the effect of IFN-γ in increasing NO production is obvious. This could be an explanation for the therapeutic effect of IFN-γ in patients with CGD as NO acts as a bactericidal agent and plays a role in host defense mechanism instead of O2−.

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1. Introduction

Chronic granulomatous disease (CGD) is an uncommon primary immunodeficiency affecting the innate immune system, characterized by recurrent, often life-threatening bacterial and fungal infections and by granuloma formation in vital organs. The disease is caused by mutations in any one of the genes encoding subunits of the superoxide generating phagocyte NADPH oxidase, resulting in an absence or very low levels of enzyme activity [1]. Conventional treatment consists of lifelong anti-infectious prophylaxis with antibiotics such as trimethoprim-sulfamethoxazole (TMP-SMX) [2], antimycotics such as itraconazole [3], and/or interferon-gamma (IFN-γ); a proinflammatory cytokine [4]. Despite these strategies, the annual mortality is still between 2% (autosomal recessive CGD) and 5% (X-linked CGD) according to the U.S. CGD registry [5].

It was shown that long-term oral TMP-SMX prophylaxis has markedly reduced the rate of infection among patients with CGD [6]. Further reduction of the infection rate among persons with CGD has been achieved with IFN-γ. Injected subcutaneously 3 times weekly of IFN-γ has reduced the rate of serious infection in persons with CGD by 67%. This therapy was effective for all genetic types of CGD, in which children less than 10 years of age appeared to get the most benefit. Moreover, side effects related to IFN-γ treatment were reported to be moderate [4]. The effectiveness of IFN-γ in reducing infections has also been obtained in a trial on mice with CGD [7].

Despite several decades of work on IFN-γ and its many effects on phagocytes, it is not known yet which mechanism(s) is/are important for preventing infection in CGD [8].

Initial reports on oxidative functions in CGD patients have suggested augmented superoxide production and bacterial killing in neutrophils...
and increased gene expression for the oxidase components, i.e., cytochrome b558 after IFN-γ treatment [9,10]. Polymorphonuclear neutrophils (PMNs) from patients with CGD have a defect in the NADPH oxidase-superoxide-generating system and are thus unable to generate superoxide anion (O$_2^-$), hydrogen peroxide, and other reactive oxygen radicals during phagocytosis [11]. Nitric oxide (NO) was mentioned as a critical target of superoxide [12]. Human PMNs produce NO spontaneously [13] or after activation [14]. Both inducible and constitutive isoforms of NO synthase (NOS) have been purified from human PMNs [15,16]. Several lines of evidence imply that this function is of importance for host defense as it provides an additional microbial killing pathway in PMN [15,17]. It has been mentioned that NO possesses cytotoxic and bactericidal actions, particularly against intracellular pathogens [15]. NO released from PMNs, in healthy population, is known to rapidly react with O$_2^-$ to form the stable peroxynitrite anion [12]. Since this reaction is not occurred in CGD, NO by itself may be of a relatively larger importance [15]. Moreover, it has been shown that PMNs from CGD patients produce NO in vitro [18].

We speculated that the effect of IFN-γ on NO production would be one of the probable underlying mechanisms explaining the therapeutic effect of IFN-γ in patients with CGD. This study was therefore designed to investigate the effect of IFN-γ on NO levels in patients with CGD both in serum and in vitro from their neutrophils.

2. Materials and methods

2.1. Study population

Nineteen patients with CGD who had been diagnosed on the basis of abnormal neutrophil function test defined by nitroblue tetrazolium reduction (NBT) test and DHR (dihydrorhodamine) were enrolled in this study. They were evaluated in Immunology, Asthma and Allergy Research Institute (IAARI); a main referral center for immunodeficiency disorders in Iran from May 2008 to February 2009. All of the patients received prophylactic antibiotic therapy with TMP-SMX (usually 5 mg/kg b.i.d.). These patients had neither infection clinically nor hospitalization during the study time. In addition to TMP-SMX, patients received prophylactic antibiotic therapy with 100 μg/mL penicillin, and 100 μg/mL streptomycin. To avoid accidental neutrophil activation and clumping, neutrophil preparations were carefully washed by centrifugation at 200 × g, the supernatant was aspirated, and cells were gently resuspended with a pipet. The sudden changes in temperature were avoided. The purity of neutrophil populations was greater than 95% on Giemsa stain, and neutrophil viability was greater than 98% as determined by trypan blue dye exclusion. Less than 5% of neutrophil preparations showed a polarized shape; a sensitive marker for neutrophil activation [20].

After cells counting, the suspensions were adjusted to a final concentration of 2 × 10$^6$ cells/ml. Then suspended cells were allocated into 2-well culture plates, at a density of 2 × 10$^6$ cells/well. Cell cultures were incubated at 37 °C and 5% CO$_2$ with 100 U/mL penicillin, and 100 μg/mL streptomycin. To avoid accidental neutrophil activation and clumping, neutrophil preparations were carefully washed by centrifugation at 200 × g, the supernatant was aspirated, and cells were gently resuspended with a pipet. The sudden changes in temperature were avoided. The purity of neutrophil populations was greater than 95% on Giemsa stain, and neutrophil viability was greater than 98% as determined by trypan blue dye exclusion. Less than 5% of neutrophil preparations showed a polarized shape; a sensitive marker for neutrophil activation [20].

We divided the patients into 2 groups: patients under treating with IFN-γ simultaneously (group I) and patients under treating with IFN-γ after 7 days (group II). We also studied a control group containing healthy nonsmoking individuals (control group).

At first, 24 patients with CGD had been entered to this study but 5 of them were excluded; one patient was diagnosed recent to the study start point so he did not receive any drug. One patient was withdrawn from the study because of discontinuing of IFN-γ due to its adverse effects, and 3 patients discontinued IFN-γ therapy for the reasons such as loss of health insurance and nonadherence to the treatment so they were excluded from this study. Our report is therefore based on findings from 19 patients with CGD.

Control individuals (age range: 2–35 years old) did not receive any drug for at least 7 days before their enrollment to the study. They had not been also affected with any infection during the last 3 days. Informed consent was obtained from each healthy volunteer and all patients with CGD or their guardians before their enrollment to the study.

3. Sampling, neutrophils isolation and NO measurement

3.1. Blood sampling

Blood samples (1–2 ml) were obtained from all patients and control individuals. The samples were centrifuged within 30–45 min of collection and sera were stored at −20 °C until the time of NO measurement (within 10 days). Three ml of blood was additional obtained from the patients and collected into sterile heparinized syringes for PMN isolation.

3.2. Neutrophils isolation and culture

The neutrophils from heparinized blood were isolated by a dextran/ficoll method [19]. Erythrocytes were removed using dextran sedimentation (1:1 mixture of blood: 6% dextran/HBSS). Contaminating erythrocytes were removed by hypotonic water lysis. Neutrophils were isolated from the resulting cell suspension using Ficoll-Histopaque density centrifugation. The entire isolation was performed at 4 °C in Ca$^{2+}$-free PBS to prevent cell activation. The isolated neutrophils were washed twice with PBS and resuspended in RPMI 1640, 100 U/mL penicillin, and 100 μg/mL streptomycin. To avoid accidental neutrophil activation and clumping, neutrophil preparations were carefully washed by centrifugation at 200 × g, the supernatant was aspirated, and cells were gently resuspended with a pipet. The sudden changes in temperature were avoided. The purity of neutrophil populations was greater than 95% on Giemsa stain, and neutrophil viability was greater than 98% as determined by trypan blue dye exclusion. Less than 5% of neutrophil preparations showed a polarized shape; a sensitive marker for neutrophil activation [20].

After cells counting, the suspensions were adjusted to a final concentration of 2 × 10$^6$ cells/ml. Then suspended cells were allocated into 2-well culture plates, at a density of 2 × 10$^6$ cells/well. Cell cultures were incubated at 37 °C and 5% CO$_2$ with 100 U/mL human recombinant IFN-γ for 24 h. Control wells were supplemented by PBS in the equal volume.

3.3. Determination nitrite and nitrate (NOx) by Griess method

Serum NOx was measured by the Griess reaction. As biological samples such as serum and plasma have a high content of protein and high turbidity, deproteinization step is necessary in the Griess reaction assay for such samples [21,22]. So the serum samples were deproteinized by adding trichloroacetic acid (15%) to the serum (sample: TCA, 1:3, v/v) followed by centrifugation at 10,000 × g for 10 min [21]. One hundred μL of the supernatant was applied to a microplate well and then 100 μL vanadium (III) chloride (8 mg/ml) was added to each well to reduce nitrate to nitrite. Griess reagents were added and after 30 min incubation at 37 °C, absorbance was read at 540 nm using the ELISA reader (Sunrise, Tecan, Austria). Concentration of NOx in serum samples was determined from the linear standard curve established by 0–100 μM sodium nitrate [21,23].

Moreover, cell-free culture fluids were obtained by centrifugation and assayed for the stable end product of NO, nitrite, using the Griess method described above. NaN$_3$O$_2$ in RPMI used to construct a standard curve for each plate reading.

3.4. Statistical analysis

SPSS program (SPSS Inc., Chicago, IL, USA; Version 13) was used for data analysis. Data are expressed as median and/or mean±s.d. Data were analyzed with the Mann Whitney test for comparing the results between independent groups and Wilcoxon signed rank test for comparing the results between before and after experiment with IFN-γ in vitro. Differences were considered significant at p<0.05.

4. Results

We studied 9 male and 10 female patients with CGD who had been referred to IAARI for following up their disease. The median age of the patients was 6 years (range: 1.5 to 27 years). The median age of the patients in group I was 19 years...
(range: 2 to 27 years) while it was 6 years in group II (range: 1.5 to 19 years) and 8.5 years in the control group (range: 2 to 35 years).

Twelve patients received IFN-γ at least 3 months during the study. They came to our center every 3 months for following up their disease. Two patients received IFN-γ for 6 months. Three patients received IFN-γ for 9 months, and the rest (6 patients) received IFN-γ for 12 months.

4.1. NOx levels in vivo

The serum NOx levels were 24.9 ± 8.4 μmol/l in the control group, 24.3 ± 5.7 μmol/l in group I, and 32.2 ± 5.6 μmol/l in group II. There was a significant difference between the groups in the serum NOx levels; patients who received IFN-γ had significantly higher amount of NOx compared to other groups. They had significantly higher levels of the serum NOx compared to that in group I (p = 0.006) as well as that in control group (p = 0.04). Fig. 1 showed the difference between the groups.

Serum NOx levels of the studied groups have been presented in Table 1 classified by gender. Comparison of serum NOx levels between males and females showed that males had significantly higher NOx values compared to females found in group II (Table 1).

Serum NOx levels of 2 patients who received IFN-γ for 3 months was 23.25 and 25 μmol/l (mean: 24.1 ± 1.2 μmol/l). The mean amount of NOx was 28.7 ± 3.2 μmol/l in 3 patients who received IFN-γ for 6 months while it was 35 μmol/l in one patient who received this drug for 9 months. The mean of NOx was 36.2 ± 3.1 μmol/l in patients who received IFN-γ for 12 months. Because of the low number of the patients in each group regarding the months of receiving IFN-γ, we could not analyse the difference in the serum NOx levels here. However the NOx levels show higher level by increasing the month number.

4.2. NOx levels in vitro

NOx production increased with adding 100 U IFN-γ on PMNs in vitro in both groups of the patients significantly; the amount of NOx in group I was 202.2 ± 60.6 (nmol/2×10^6 PMNs) and increased to 242.9 ± 79.8 (nmol/2×10^6 PMNs) at 24 h incubation with IFN-γ in 37 °C (p = 0.031, Fig. 2a). Likewise, its amount was 206.0 ± 79.9 (nmol/2×10^6 PMNs) in group II and increased to 277.5 ± 92.5 (nmol/2×10^6 PMNs) with stimulation of IFN-γ in vitro (p = 0.001, Fig. 2b).

Moreover, the amount of NOx in group control was 154.7 ± 55.7 (nmol/2×10^6 PMNs) and increased to 209.5 ± 76.9 (nmol/2×10^6 PMNs) with stimulation of IFN-γ in vitro (p = 0.023, Fig. 2c).

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male no.</th>
<th>Female no.</th>
<th>Serum NOx (μmol/l)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>4</td>
<td>7</td>
<td>21.3 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Group I</td>
<td>5</td>
<td>3</td>
<td>25.6 ± 6.5</td>
<td>0.049</td>
</tr>
<tr>
<td>Group II</td>
<td>3</td>
<td>7</td>
<td>35.9 ± 3.1</td>
<td>0.047</td>
</tr>
</tbody>
</table>

NS: Not significant.

5. Discussion

In this study, we evaluated NOx production levels in vivo and in vitro in CGD patients on treatment of TMP-SMX with/without IFN-γ. Our finding showed higher levels of serum NOx in the patients treated with IFN-γ. Likewise, the increased levels of NOx production from PMNs were found after adding IFN-γ in vitro in the both patients groups as well as in the control group. This shows the effect of IFN-γ on NO release from PMNs in the patients with CGD as well as in the normal population.

Previous report by Tsuji et al. showed the effect of TMP-SMX on NO release; they demonstrated that NO is produced in vitro by TMP-SMX with lipopolysaccharide (LPS) significantly higher compared with LPS-stimulated samples [24]. They suggested that NO production by neutrophils from the patients with CGD treated with TMP-SMX has a role in bactericidal activity instead of O2- in the host defense mechanism. We did not study the effect of TMP-SMX in patients with CGD treated with TMP-SMX, but we should say this finding is in contradiction with the finding by Condino et al. in which they demonstrated that plasma levels of NOx were not elevated after IFN-γ therapy in their CGD patients but they observed an increase in the urinary levels of NOx in their treated patients with IFN-γ [25].

In our study, male subjects in the patients group treated with IFN-γ had significantly higher NOx levels compared with female subjects. We can conclude that serum NOx levels are related to gender. This finding is partly supported by Chasemi et al. who studied 667 apparently healthy men and 1316 apparently healthy women. They showed that serum NOx levels are higher in young men compared with young women. They also showed that serum NOx concentration is more strongly related to serum triglyceride than other serum lipids [23].

Furthermore, we found that serum NOx levels in the patients who received IFN-γ could be related to the month number of the receiving IFN-γ. We should say this finding with the caution because of the limited number of the patients in our study. However this finding is in contrast with the results by Condino et al. [25]. They found that therapy with IFN-γ for 6 months did not enhance NO synthesis (NOS) by neutrophils or monocytes from the patients with CGD. Differences in their study design, method used for NO detection, and sample size (5 patients in their study versus 12 patients in ours) compared to ours may explain the different results.

Fig. 1. The serum NOx levels of patients with CGD treated with TMP-SMX alone or with IFN-γ simultaneously.
treatment with IFN-γ in addition to TMP-SMX. The findings related to stimulating PMNs in vitro to produce NOx are contradictory with the in vitro finding by Condino et al., in which they did not find any significant difference in NOx production from neutrophils and monocytes after adding IFN-γ in vitro [25]. But in line with our finding, Ahlin et al. reported that IFN-γ treatment in CGD is associated with an increased production of NO from PMNs when they are activated by fMLP. However, there are some differences between our study and study by Ahlin et al. They stimulated PMNs by fMLP and assessed NO production by the HbO2 (oxyhemoglobin) method (oxidation of HbO2 to methemoglobin) [15], while we used Griess method to measure NOx production from PMN after adding IFN-γ without stimulating them by fMLP or other stimulating agents like PMA. So our results in vitro reflect NO production from PMN spontaneously and not after stimulation. Moreover, they studied the bactericidal capacity of isolated PMNs from CGD patients after only two consecutive doses injection of IFN-γ, and demonstrated that enhanced bactericidal activity is associated with an increase of NO production. They concluded that this increased NO release could be instrumental in augmenting the host defense but they ended up with the question of the effect of IFN-γ treatment over a prolonged period of time on PMN NOS activity [15]. Since we compared the NOx levels between the patients with different months of IFN-γ treatment, their question can be partly answered by our finding as we explained it above. Another different point of our study with the study by Ahlin et al. is that they measured the release of NO per se [15], whereas we measured the metabolites NO2 and NO3. Corini et al. also used the measurement of NO2 and NO3 from PMN [25].

It should be mentioned that there are some difficulties in detecting NO formation in human PMNs as a consequence of its short half-life in the presence of O2 and other scavenging molecules, for example, hemoglobin [22]. Larfars and Gyllenhammar showed that NO production is dependence on Ca2+ transients. They also suggested that the small amount of NO produced by human PMNs and its easily reaction with other products of PMNs such as O2− were another possible explanations for the difficulties in detecting NO formation in human PMNs [26]. Some studies did different works and speculated about different mechanisms for beneficial effect of IFN-γ which we mention them here. Golberg et al. indicated that IFN-γ can activate a protein kinase C (PKC) mediated pathway involved in the oxidative burst and induce O2 production in phagocytes of some patients with CGD [27]. In a recent study, Fernandez-Boyanapalli et al. showed that treatment of CGD mice by IFN-γ primes macrophages via the NO-dependent pathway and enhances phagocytosis [28]. Schiff et al. also demonstrated that the protective effect of IFN-γ may be related to upregulation of Fcγ-receptor expression by phagocytes and subsequently, increased ingestion of microbes, and/or secondary to increased expression of monocyte (i2-integrins) [29]. They observed these improvements in the host defense occur in normal population as well, suggesting that IFN-γ may have broader applications in host defense disorders which is not limited to CGD [29]. We also found the increased levels of NOx production in PMNs from normal individuals besides the CGD patients groups confirming the wider application of IFN-γ. However, the definite mechanism of therapeutic effect of IFN-γ still needs further studies. Clinical studies with larger numbers of CGD patients on IFN-γ treatment during a longer follow up period are suggested to confirm our findings as well.

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