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RESEARCH ARTICLE

Effect of recombinant human IFNγ in the treatment of chronic pulmonary complications due to sulfur mustard intoxication

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Abstract

Pulmonary problems are among the most common chronic complications of sulfur mustard (SM) intoxication and adversely affect patients' quality-of-life. The present trial investigated the impact of immunotherapy with interferon (IFN)-γ on quality-of-life, respiratory symptoms, and circulating immunologic and oxidative parameters in patients suffering from chronic SM-induced complications. Patients ($n = 15$) were administered IFNγ (100 μg) every other day for a period of 6 months. Assessment of quality-of-life [using St. George respiratory Questionnaire (SGRQ) and COPD Assessment Test (CAT) indices], the severity and frequency of respiratory symptoms, and serum levels of immunologic [including interleukin (IL)-2, IL-4, IL-6, IL-10, IFNγ, calcitonin gene related peptide (CGRP), matrix metallopeptidase (MMP)-9, and tumor necrosis factor (TNF)-α], oxidative stress [malondialdehyde (MDA) as well as total and reduced glutathione, and catalase and superoxide dismutase (SOD) activity], and fibrogenic [transforming growth factor (TGF)-β] parameters were performed at baseline and at trial end. The results indicated that IFNγ therapy is associated with improvements in SGRQ ($p < 0.001$) and CAT ($p < 0.001$) scores, decreased severity of cough ($p = 0.001$), dyspnea ($p < 0.001$), and morning dyspnea ($p < 0.001$), reduced frequency of sputum production ($p < 0.001$) and hemoptysis ($p < 0.001$), and elevated FEV_1 ($p = 0.065$). Serum levels of IL-4 ($p < 0.001$), IL-6 ($p < 0.001$), IL-10 ($p < 0.001$), CGRP ($p < 0.001$), MMP-9 ($p = 0.001$), TNFα ($p < 0.001$), TGFβ ($p < 0.001$) and MDA ($p = 0.001$) were decreased while those of IL-2 ($p < 0.001$), IFNγ ($p < 0.001$), and both total ($p = 0.005$) and reduced glutathione ($p = 0.061$) increased by the end of the trial. It was concluded that IFNγ has favorable effects on the quality-of-life and alleviates respiratory symptoms in patients suffering from chronic SM-induced pulmonary complications. A modulation of cytokines and oxidative stress appears responsible for the clinical efficacy of IFNγ.

Keywords

Anti-oxidant, immunomodulation, interferon-γ, mustard gas, quality-of-life

History

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Introduction

Sulfur mustard (SM; $C_4H_8CL_2S$), also known as mustard gas, is the first and by far the most studied chemical blistering agent. Since its last deployment in the Iraq–Iran conflict (1980–1988) (Anonymous, 1986), a large and growing body of research has been focused on the mechanisms as well as clinical outcomes of SM intoxication (Ghabili et al., 2011). Apart from the early and acute effects of SM during the first several hours of exposure, SM causes numerous late complications that affect daily activities and quality-of-life of exposed patients (Panahi et al., 2008). Chronic SM-induced complications often afflict eyes, skin, and lungs. Pulmonary complications are very common and include bronchiolitis, chronic bronchitis, and pulmonary fibrosis (Emad & Rezaian, 1997; Khateri et al., 2003). These late pulmonary complications are resistant to most available treatments and their management is mainly palliative, through administration of corticosteroids and other immunosuppressants. These agents have been repeatedly reported to be associated with several adverse effects on long-term use and, on the other hand, have limited efficacy in the control of respiratory symptoms (Hengge et al., 2006). Thus, there remains a real need for identification of new medications to effectively control the severity and frequency of chronic complications due to SM intoxication (Panahi et al., 2012a–e; Sahebkar, 2012).

T-helper (T_H)-1 and T_H2 cytokines are known to possess opposite effects on collagen synthesis. While T_H1 cytokines contribute to formation of normal tissue, T_H2 forms are implicated in development of fibrosis (Tzouvelekis et al., 2010). Increased production of T_H2 cytokines is a reported feature of idiopathic pulmonary fibrosis (IPF), and causes over-proliferation of fibroblasts, up-regulation of *TGF*β, and collagen accumulation in pulmonary tissue (Keane, 2008). These effects have been attributed to a deficiency in the endogenous production of IFNγ in patients with IPF. Previous findings have indicated that IFNγ inhibits fibroblast proliferation in pulmonary tissue, thereby reducing extracellular matrix and

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connective tissue formation (Li & Zhai, 2008). These findings have been corroborated by observations on the complete or partial deficiency of IFNγ in patients with pulmonary fibrosis compared to normal subjects (Gurujeyalakshmi & Giri, 1995; Prior & Haslam, 1992). Besides, IFNγ can counterbalance T_H2-associated cytokine overproduction and down-regulate gene expression of *TGF*β—one of the most potent fibrogenic factors (Du Bois, 1999; Ziesche & Block, 2000).

Owing to the above-mentioned casual link between IFNγ deficiency and development of pulmonary disorders, the present trial sought to investigate the clinical efficacy of immunotherapy with IFNγ in patients suffering from chronic SM-induced pulmonary complications, despite being treated with conventional medications.

Materials and methods

Subjects

The study was approved by the institutional Ethics Committee and written informed consent obtained from the participants. The trial was conducted among SM-intoxicated veterans suffering from chronic respiratory complications who were hospitalized in Baqiyatallah Hospital between April 2010 and January 2012. The key inclusion criterion was a presence of documented respiratory problems due to mustard gas exposure. Exclusion criteria were any history of hypersensitivity/adverse events in response to interferon (IFN) preparations, history of hepatic, renal or cardiac dysfunction, and/or psychotic disorders.

After initial examinations, eligible participants were instructed to self-inject recombinant IFNγ-1b (γ-Immunex®, Exir Pharmaceutical Company, Tehran, Iran) via subcutaneous injection, except for the first two doses which were injected by nurse at the Hospital. The dosage of IFNγ was one vial (100 μg) every other day for a period of 6 months. Fasted serum samples were collected from the antecubital vein at baseline as well as at the end of trial. Collected blood samples were centrifuged at 1500 × g for 10 min to obtain serum; all serum samples were then kept at −20 °C until used for analysis. Sera were analyzed for immunologic [including interleukins (IL) 2, 4, 6 and 10, IFNγ, calcitonin gene related peptide (CGRP), matrix metallopeptidase (MMP)-9, and tumor necrosis factor (TNF)-α], oxidative stress [e.g. malondialdehyde (MDA), catalase, total and reduced glutathione and superoxide dismutase (SOD)], and fibrogenic [transforming growth factor (TGF)-β] parameters. Serum levels of IL-2, IL-4, IL-6, IL-10, IFNγ, CGRP, MMP-9, TGFβ, and TNFα were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA). Serum activities of SOD and catalase as well as concentrations of total and reduced glutathione were determined spectrophotometrically using routine procedures (Akerboom & Sies, 1981; Chance & Maehly, 1995; Uchiyama & Mihara, 1978).

In addition to evaluation of biochemical parameters, all participants underwent spirometry (HI-801 Chest M.I. Spirometer, Tokyo, Japan) at baseline and at the end of trial. The spirometer was calibrated using a device provided by the manufacturer. To assess pulmonary function, forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and forced expiratory flow 25–75% ($FEF_{25–75\%}$) were measured. The severity of cough, dyspnea and morning dyspnea together with the frequencies of sputum production and hemoptysis were also recorded for participants at baseline and at the end of trial. Assessment of the clinical symptoms was performed through interview and based on the St. George's respiratory questionnaire for COPD patients (Meguro et al., 2007). All patients were monitored for the occurrence of adverse events during the course of the trial, and were asked to report any side-effects due to IFNγ use.

Evaluation of quality-of-life was performed using St. George respiratory Questionnaire (SGRQ) and COPD Assessment Test (CAT) indices. SGRQ is a life quality index specifically designed for patients with asthma, COPD and bronchiectasis. This questionnaire contains 50 items that are categorized into two parts. The first part (symptoms) pertains to the frequency and severity of clinical symptoms and the second part (activity and impacts) asks about the impact of disease on patient's daily physical activities and psychosocial functioning. SGRQ can also be classified into three sub-scales of 'symptoms', 'activities', and 'impacts'. Each sub-scale as well as total score ranges between 0–100, with higher scores indicative of more severe impairments to the quality-of-ife due to disease (Meguro et al., 2007).

CAT is another index designed for the assessment of quality-of-life in COPD patients and contains eight questions, each with a score range of 0–5. Thus, the overall CAT score could vary between 0–40, with higher scores indicative of more severe impairments to the quality-of-life due to disease (Jones et al., 2009).

All patients were under treatment with salmeterol (two puffs q12h; 50 μg/puff), *N*-acetylcysteine (600 mg q12h), and omeprazole (20 mg/day). These medications remained unaltered through to the end of the trial.

Statistical analysis

Statistical analyses were performed using SPSS software version 15.0 (Chicago, IL). Data were expressed as mean ± SD or as number (%). A *per protocol* statistical approach was used for data analysis. Categorical variables were compared using McNemar's test. Pre- vs post-trial comparisons were performed using paired samples *t*-tests (in case of normal distribution of data) and Wilcoxon signed-ranks test (in case of non-normal distribution of data). A two-sided p value of < 0.05 was considered statistically significant.

Results

Out of the 24 participants who were initially recruited into the trial, nine failed to complete the trial. The major reason for dropping out was living in another city and being unable to return to the hospital for the final visit. Therefore, information from just 15 individuals [age (years): 47.67 ± 8.77; height (cm): 171.43 ± 5.51; weight (kg): 75.93 ± 11.91; and BMI (kg/m^2): 25.76 ± 3.52] who completed the trial with full baseline and post-trial data on the efficacy measures and biochemical parameters was included for the final analyses and interpretation.

Effect of IFNγ on respiratory symptoms

Treatment with IFNγ, was associated with significant reductions in the frequencies of all evaluated respiratory symptoms including cough ($p = 0.001$), dyspnea ($p < 0.001$), morning dyspnea ($p < 0.001$), sputum production ($p < 0.001$), and hemoptysis ($p < 0.001$) (Table 1).

Effect of IFNγ on quality-of-life indices

All assessed indices of quality-of-life were significantly improved by IFNγ therapy. The sub-scale [symptoms ($p = 0.001$), activities ($p < 0.001$) and impacts ($p < 0.001$)] and total ($p < 0.001$) SGRQ scores were significantly reduced by the end of trial. In the same manner, there were significant reductions in the CAT score ($p < 0.001$) as well as patients' need to oxygen therapy ($p = 0.004$) and 6-month hospitalization ($p = 0.001$) (Table 2).

Table 1. Effect of IFNγ on severity or frequency of respiratory symptoms.

Symptom	Pre-trial	Post-trial	*p* value
Cough	3.53 ± 0.52	2.73 ± 0.59	< 0.001
Dyspnea	3.80 ± 0.41	0.60 ± 0.63	< 0.001
Morning Dyspnea	3.60 ± 0.74	2.80 ± 0.68	< 0.001
Sputum production (%)	100	73.3	< 0.001
Hemoptysis (%)	86.66	6.67	< 0.001

Values shown are mean ± SD; $n = 15$.

Table 2. Effect of IFNγ on quality-of-life indices.

Parameter	Pre-trial	Post-trial	*p* value
St. George (symptoms)	87.43 ± 5.94	75.56 ± 9.91	0.001
St. George (activity)	72.58 ± 8.33	45.33 ± 4.89	< 0.001
St. George (impacts)	69.88 ± 8.64	37.98 ± 9.35	< 0.001
St. George (total)	73.62 ± 6.35	46.51 ± 5.71	< 0.001
CAT	35.06 ± 1.83	26.80 ± 2.90	< 0.001
Need for oxygen therapy	2.46 ± 0.83	1.46 ± 0.51	0.004
6-months hospitalization	3.50 ± 0.75	1.64 ± 0.49	0.001

CAT, COPD Assessment Test.
Values shown are mean ± SD; $n = 15$.

Effect of IFNγ on spirometric findings

IFNγ treatment was associated with a borderline elevation in FEV_1 ($p = 0.065$). FVC and FEV_1/FVC values were also increased by the end of trial, but these increases did not reach statistical significance ($p > 0.05$) (Table 3).

Effect of IFNγ on immunologic parameters

The 6 months of immunotherapy with IFNγ was associated with a decreasing trend in circulating levels of all evaluated immunologic mediators, i.e. IL-4 ($p < 0.001$), IL-6 ($p < 0.001$), IL-10 ($p < 0.001$), CGRP ($p < 0.001$), MMP-9 ($p = 0.001$), TNFα ($p < 0.001$) and TGFβ ($p < 0.001$). The only exception was IL-2 ($p < 0.001$) for which a significant elevation was found after IFNγ therapy. Mean serum concentrations of IFNγ, were elevated by ≈ 145% at the end of the trial ($p < 0.001$) (Table 4).

Effect of IFNγ on oxidative stress biomarkers

Comparison of baseline vs post-trial levels of total and reduced glutathione revealed, respectively, significant ($p = 0.005$) and borderline significant ($p = 0.061$) increases. In contrast, lipid peroxidation was attenuated by IFNγ therapy, as assessed by serum concentrations of MDA ($p = 0.001$). Serum levels of enzymatic anti-oxidants, SOD and catalase, remained statistically unaltered by the end of trial ($p > 0.05$) (Table 5).

Adverse events

Any adverse reactions that were reported due to IFNγ, were not severe in nature and occurred at a very low frequency in the study population. These adverse events included flu-like symptoms in seven patients, nausea and vomiting in one patient and headaches in one patient over the study entirety.

Discussion

This study set out to assess the efficacy of IFNγ in improvement of quality-of-life and alleviation of clinical symptoms in patients suffering from chronic pulmonary complications due to SM exposure. The findings revealed a favorable effect for IFNγ therapy in improving quality-of-life indices, clinical symptoms and immunologic and oxidative stress parameters. Although spirometric parameters showed an increasing trend from baseline to the end of trial, these changes were not of statistical significance, apart from FEV_1 that had a borderline significant elevation. Findings from a previous investigation implied that treatment with IFNγ improved spirometric function (Ghanei et al., 2006). In the study by Ghanei et al. (2006), treatment with 200 μg IFNγ (three times per week) plus 7.5 mg of prednisolone (once a day) for 6 months was associated with significant elevation of both FEV_1 and FVC compared to the control group. Lack of significant changes in spirometric parameters in the present study could be due to the smaller population size and not administering an oral corticosteroid as adjunctive therapy.

According to a previous clinical report, the CD4:CD8 lymphocyte ratio is increased in the bronchoalveolar lavage fluid (BALF) of patients with SM-induced bronchiectasis compared to healthy controls (Emad & Emad, 2007). It has been shown that alveolar macrophages release IL-1 and TNFα in response to pulmonary stimulation that is the same as what occurs following SM intoxication. These cytokines, on their own, can evoke the release of IL-6 and IL-8 via autocrine or paracrine

Table 3. Effect of IFNγ on spirometric parameters.

Parameter	Pre-trial	Post-trial	*p* value
FEV_1 (%)	48.43 ± 14.27	55.32 ± 18.37	0.065
FVC (%)	53.85 ± 12.21	61.55 ± 13.35	0.092
FEV_1/FVC (%)	92.28 ± 17.59	95.42 ± 18.55	0.433

FEV_1, Forced expiratory volume in the first second; FVC, forced vital capacity; $FEF_{25–75\%}$, forced expiratory flow 25–75%.
Values shown are mean ± SD; n = 15.

Table 4. Effect of IFNγ on immunologic parameters.

Parameter	Pre-trial	Post-trial	*p* value
IL-2 (pg/ml)	37.19 ± 3.65	51.95 ± 5.82	< 0.001
IL-4 (pg/ml)	43.80 ± 13.65	23.95 ± 10.06	< 0.001
IL-6 (pg/ml)	1.88 ± 0.49	0.95 ± 0.25	< 0.001
IL-10 (pg/ml)	33.77 ± 22.23	22.23 ± 6.27	< 0.001
IFNγ (pg/ml)	10.03 ± 5.42	24.62 ± 8.05	< 0.001
CGRP (pg/ml)	49.67 ± 7.55	36.25 ± 5.57	< 0.001
MMP9 (ng/ml)	22.55 ± 14.56	6.36 ± 0.02	0.001
TNFα (pg/ml)	29.38 ± 3.94	15.94 ± 3.78	< 0.001
TGFβ (pg/ml)	41.36 ± 7.06	19.80 ± 3.69	< 0.001

IL, interleukin; IFN, interferon; CGRP, calcitonin gene related peptide; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; TGF, transforming growth factor.
Values shown are mean ± SD; $n = 15$.

Table 5. Effect of IFNγ on oxidative stress markers.

Parameter	Pre-trial	Post-trial	*p* value
Catalase (U/ml)	26.45 ± 5.20	25.37 ± 2.99	0.180
SOD (U/ml)	1.48 ± 0.39	1.33 ± 0.38	0.100
Total glutathione (μg/ml)	3.90 ± 4.09	8.80 ± 9.00	0.005
Reduced glutathione (μg/ml)	9.04 ± 10.99	11.02 ± 10.56	0.061
MDA (nmole/ml)	21.73 ± 12.62	9.49 ± 3.89	0.001

SOD, superoxide dismutase; MDA, malonedialdehyde.
Values shown are mean ± SD; $n = 15$.

routes and thereby trigger an inflammatory response (Losa Garcia et al., 1999). There are proof-of-concept studies that clearly indicate a heightened state of inflammation in SM-intoxicated veterans, accompanied by increased pulmonary and systemic levels of IL-6 and IL-8, even 20 years post-exposure (Pourfarzam et al., 2009; Yaraee et al., 2009). Overall, it appears that counterbalancing the T_H1/T_H2 system in favor of T_H2 cytokines is an important underlying mechanism in the development of SM-induced inflammation.

As a T_H1 cytokine, IFNγ may correct the above-mentioned immunologic imbalances and attenuate the production of T_H2 cytokines such as IL-6 and TNFα. Evidence from the current trial supported this notion and further confirmed the efficacy of IFNγ in the attenuation of SM-induced inflammation. Aside from pro-inflammatory cytokines, treatment with IFNγ caused a decline in serum levels of IL-4, IL-10, CGRP, and TGFβ. IL-4 activates eosinophils and leads to IgE over-production (Gruner et al., 1991; Sato et al., 1998), the latter playing a key role in the development of asthma in SM-injured individuals. Reduction of serum IL-10 that was observed in the present study is a contrasting finding as IL-10 is a cytokine with documented anti-inflammatory properties (de Vries, 1995). Circulating IL-10 levels rise during the recovery period and might serve as a biomarker for the subsidence of inflammation. Hence, it might be speculated that reduced levels of IL-10 are due to the lack of complete recovery of patients and, therefore, immunotherapy should be continued until complete subsidence of inflammation is achieved. CGRP has been reported to contribute to the exacerbation of airway inflammation. Blockage of this neuropeptide (via immunization) has been shown to suppress SM-induced inflammation by $\approx$ 50% (Louis et al., 1989). Thus, reducing systemic concentrations of CGRP is a plausible mechanism for the anti-inflammatory effects of IFNγ. Among the biochemical mediators of pulmonary fibrosis, TGFβ has the most pivotal effect. Several lines of evidence have shown that T_H2 cytokines and fibroblast count are elevated in patients with pulmonary fibrosis, which enhance TGFβ status and accumulation of collagen in the airways (Raghu et al., 1989; Rahman, 2005). There is also evidence indicating impaired IFNγ production in patients with fibrosing lung diseases (Prior & Haslam, 1992) that results in the over-proliferation of fibroblasts and excessive collagen production.

An *in vitro* study showed that TGFβ could induce the transition of alveolar epithelial cells to mesenchymal cells in a dose- and time-dependent manner. Resulting cells have fibroblast-like phenotype and express fibrogenic markers such as Type I and III collagen, connective tissue growth factor and MMP2. In addition, TGFβ has an important role in the activation and differentiation of myofibroblasts, a cell type that is involved in the fibrogenesis process (Tomasek et al., 2002; Thannickal et al., 2003). Finally, TGFβ1 has been reported to induce the mRNA transcription of collagen Type I and IV genes (Grande et al., 1997); this leads to the over-production and accumulation of the extracellular matrix (Geirsson et al., 2012). In light of the above-mentioned fibrogenic properties of TGFβ, reducing serum levels of this mediator by IFNγ could account for the observed alleviation of clinical pulmonary symptoms. Our findings are also consistent with previous reports of down-regulation of TGFβ gene by IFNγ (Gurujeyalakshmi & Giri, 1995; Wen et al., 2004).

MMP9 (gelatinase B) is another protein that has been proposed to play a prominent role in the late pulmonary complications of SM. Through degeneration of extracellular matrix proteins, MMPs are implicated in the development of tissue fibrosis, damage, and inflammation (Chakrabarti & Patel, 2005; Corbel et al., 2002; Lagente et al., 2005). MMP9 appears to serve as a key target for SM. Over-production of peroxynitrite radical ($ONOO^-$) by SM leads to an imbalance in the protease/anti-protease system that, in turn, causes MMP activation (Korkmaz et al., 2005). MMP9 is the most important type of MMP involved in SM toxicity. Previous studies have indicated that MMP9 status rises following SM injury (Calvet et al. 1999; Malaviya et al. 2010). In the current research, immunotherapy with IFNγ was associated with a remarkable decline in serum MMP9; this effect may prevent deleterious consequences of protease activation by SM.

Finally, treatment with IFNγ turned out to modulate systemic oxidative stress. Several previous studies have underlined the role of oxidative stress in SM toxicity and demonstrated that SM administration, either through topical or parenteral route, leads to a diminished activity of anti-oxidant enzymes in different tissues (Husain et al., 1996; Jafari, 2007; Pohanka & Stetina, 2009). Such an altered enzymatic activity along with the increased level of oxidant species can trigger several adverse reactions such as lipid peroxidation, membrane damage, cell death and phagocytosis, all being characteristics of SM toxicity (Han et al., 2004; Naghii, 2002). Besides, oxidative stress has an established effect in the pathophysiology of COPD and pulmonary fibrosis (Cheresh et al., 2012; Rahman, 2005). The present findings were indicative of an oxidative stress counterbalancing effect for IFNγ. This is consistent with the findings of a previous study on the extracellular anti-oxidant properties of IFNγ through induction of L-tryptophan (Trp) degradation along the kynurenine pathway in human monocytes and macrophages (Thomas & Stocker, 1999).

The present study had a number of limitations that should be taken into cautious consideration. First, the study was designed as a single arm trial and lacked a blinded placebo treatment group. Such a single-arm design is likely to introduce bias into the results as no between-group comparison could be made. Nevertheless, it must be pointed out that preparation and administration of a parenteral placebo for a biopharmaceutical to be injected regularly for a period of 6 months is a very hard task and not practical for a trial. Furthermore, due to the severe respiratory complications of SM-intoxicated veterans, it is not ethical to deprive the participants from IFNγ benefits. Second, the present trial was a pilot study and the study population was small in size. Therefore, the beneficial effects observed in the current study need to be verified in larger multi-center trials.

To conclude, the present trial provided the first evidence on the clinical efficacy of IFNγ in the improvement of quality-of-life and treatment of chronic respiratory complications due to SM. These effects are, at least in part, due to the amelioration of serum levels of ILs, TNFα, TGFβ, MMP9, CGRP, MDA, and both total and reduced glutathione. It is interesting to note that IFNγ has been previously reported to exert favorable effects in the improvement of chronic SM-induced cutaneous complications (Panahi et al., 2012c). In light of these findings, immunotherapy with IFNγ could be considered as an effective medication to be added to the armamentarium for the management of chronic complications due to SM.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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