RESEARCH PAPER

Genotoxicity assessment of recombinant human interferon gamma in human lymphocytes

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Abstract

Interferon (IFN)-gamma as a recombinant pharmaceutics, being launched in Iran as the third country in the world, despite its unclear genotoxic potential in *in vivo* models. In the present study, the genotoxic effects of this recombinant human IFN-gamma were evaluated in peripheral blood lymphocytes from 24 healthy volunteers. The induction of chromosomal alterations including chromosomal aberrations, and micronuclei (MN) in binucleated cells were performed by different cytogenetic assays. Heparinized whole-blood samples from volunteers after cell pretreatment were treated with 0.33-66.6 IU/µl of IFN-gamma. The treated cells were then determined for chromosomal aberrations, and micronuclei in binucleated cells. The negative and positive controls were phosphate buffered saline (PBS), and mitomycin C, respectively. Results revealed that cells treated with IFN showed no significant difference from cells treated with PBS indicating this recombinant protein under the performed treatment regimen lacked of genotoxic potential.

Introduction

After the first trials on the efficacy of the interferon (INF)-gamma and in the last 25-plus years since those, IFN-gamma has been used in a wide variety of life threatening clinical indications for its antiviral, antibac-

terial, antiproliferative, and immunomodulatory properties. IFN-gamma, also known as type II interferon. It is a cytokine which is critically involved in the innate as well as adaptive immunity (Panahi *et al*, 2012) by which has been shown to have enhancing effects in reversing hepatic fibrosis (Yao *et al*, 2010), systemic sclerosis (Vlachoyiannopoulos *et al*, 1996), improving the lung function in mustard-gas exposed patients with bronchiolitis (Panahi *et al*, 2005; Ghanei *et al*, 2006), in atopic dermatitis (Panahi *et al,* 2011) and chronic sulfur mustard-induced cutaneous complications (Panahi *et al,* 2012).

Interferon gamma is produced by Th_1 cells, cytotoxic T cells, and NK cells with the pleotropic cytokine that has the important functions on all virtually immune cells as well as innate and adaptive immune responses (Panahi *et al,* 2012). After having provided the recombinant form of IFN-gamma, the first clinical trials of this cytokine was run in 1986 to evaluate the therapeutic potentials of this newly discovered recombinant drug. All the initial studies were focused on the tolerability at first. The pharmacology of IFN-gamma with its antitumor and anti-infection studies were performed systematically after then (Miller *et al,* 2009).

The toxicity of recombinant IFN-gamma from the overall studies was shown to be similar to some preparations of IFN-alpha. According to one study, however, the recorded result showed this drug was less hepatic and hematological toxicity in comparison to other IFNs (Sriskandan *et al,* 1986). Additionally, it has been shown to lack of toxic reaction when studied in animals using rats and dogs as the studied models (Yuan *et* al, 1995) but IFN-gamma is a highly species-specific cytokine and has the most restricted host range of activity among other interferons. This factor is highly concerned.

Recombinant human IFN-gamma is one of the first species-specific recombinant proteins to be thoroughly assessed in conventional safety models used for xenobiotics. Acute single-dose intravenous toxicity studies with rHuman IFN-gamma were performed in rats, marmosets, and squirrel monkeys with no indications of toxicity. A complete series of subchronic toxicity studies and segment I and II reproductive studies in the rat revealed no evidence of toxicity at any of the doses tested (Terrell *et al*, 1993). The adverse effects seen in toxicity studies with cytokines and growth factors are often exaggerated pharmacological effects of the molecules, and therefore can only be studied in a responsive species. In situations in which a high degree of species specificity is encountered, studies employing a newly formulated recombinant protein in a homologous species may provide a useful test system for preclinical safety assessment.

In 1996, it was suggested in literatures that different cytokines such as human IFN-alpha and gamma, tumor necrosis factor alpha, epidermal growth factor and interleukin-2 might exhibit genotoxic effects in human peripheral blood lymphocytes. The suggestion was from the investigation of these cytokines in such human blood lymphocyte cultures that showed the parabolic-like relationship between the dose and the frequency of sister chromatid exchanges (SCE) (Lazutkal, 1996). From the evidence above, the objective of this study was to examine the genotoxicity potentials of recombinant IFN-gamma (gamma-Immunex®) which was provided by

Exir Pharmaceutical Company in human peripheral blood lymphocytes using two major cytogenetic endpoints: chromosome aberrations and micronuclei (MN), for genotoxicity studies and biomonitoring purposes.

Materials and Methods

Chemicals: Gamma-Immunex® was obtained from Exir Pharmaceutical Company (Brojerd-I.R., Iran). The stock solution was 2x106 IU (international unit) / 500 µl, that is equal to 4000 IU/µl. This chemical was tested at 0.33, 0.66, 1.32, 3.33, 6.66, 13.2, 26.4 and 66.6 IU/µl. Phosphate buffered saline (PBS) was served as a negative control, and Mitomycin C (MMC, CAS: 50-07-7), was served as a positive control. All chemicals used except IFN-gamma were purchased from Sigma-Aldrich.

Cells and culture conditions: Human peripheral blood lymphocytes from 24 healthy donors were collected in lithium heparinized tubes. Blood were grown in cell suspension in RPMI-1640 medium (Gibco), supplemented with 15% heat inactivated foetal bovine serum (Gibco), glutamine, heparin and antibiotics [penicillin (100 U/ml) and streptomycin (0.1 mg/ml)]. Phyto-haemagglutinin (PHA, HA 15) was added immediately to all cultures to stimulate blood growth, and the cultures were incubated at 37°C and 5% CO_2 atmosphere.

Chromosome aberrations in metaphases: For chromosome aberration analysis in metaphases, lymphocytes were stimulated to grow for 24 h. Chemicals were then added for 6 h. Afterwards, cells were collected, washed twice with PBS, and allowed to grow in complete medium supplemented with PHA for an additional 24 h. Colcemid (at final concentration of 1.0 µg/ml) was added for the last three hours before fixing cells. For fixing lymphocytes at 54 h, cultures were removed from an incubator. Medium from each culture bottle was transferred into a centrifuge tube and tubes were centrifuged at 800 rpm for 8 min. After then, the supernatant was decanted until 0.5 cm above the pellet. The pellet was gently broken and cell suspension was resuspended in 5 ml prewarmed (37°C) hypotonic solution (KCl 5.6 g/l = 0.075M) and incubate for 10 min in a water bath at 37°C. Cells were again centrifuged, supernatant was discarded and pellet was fixed in a fixation solution (5 ml) that was composed of 3:1 methanol: acetic acid and further centrifuged at 800 rpm for 8 min. The fixative was used two more times and finally supernatant was decanted and pellet was resuspended in 0.4 ml of fixative. Slides were prepared by dropping the cell suspension on precleaned object glasses with a micropipette. When drops were maximally expanded and Newton rings began to appear, slides were blown gently to accomplish a flattening of the cells. For analyzing, slides were stained with 2% aqueous Giemsa solution (Gurr R66) for 5 min, rinsed a few seconds in distilled water, air-dried and mounted with a cover-glass using Depex (Miller *et al*, 1997).

Slides scoring and evaluation: For each dose chromosomal alterations namely chromatid-type aberrations in first division metaphases were analyzed at two intervals each on 12 donors (24 donors in total). In the first part of the study, 12 healthy donors of Tehran among the different classes of chromatid-type aberrations, namely chromatid breaks, gaps, tri- and quad-radials were observed more prevalently in healthy donors even in negative controls. From this observation, it gave the conclusion that these volunteers would have had

exposed different chemicals from environmental resources according to air pollution in Tehran or other possible lifestyle factors and this made the experiment had to start by choosing 24 healthy young age donors (12 males and 12 females aged 20-30) who were born and lived in unpolluted areas outside of Tehran**.**

Micronuclei in binucleated cells: For analyzing micronuclei in binucleated cells, lymphocytes were stimulated to grow using PHA for 24 h and then treated with the test chemicals for 6 h. The treated lymphocytes were finally collected, washed twice with PBS, and allowed to grow in complete medium supplemented with PHA for an additional 42 h. Cells were further fixed at 72 h after starting culturing. Cytochalasin B (at final concentration of 6 g/ml) was added at 44 h after PHA stimulation. For fixing lymphocytes, cell suspension was transferred into a centrifuge tube, and centrifuged at 800 rpm for 8 min. The supernatant was discarded, the pellet was gently broken and resuspended in 5 ml cold KCl (0.075M, 4-8^{o}C), and immediately centrifuged. Afterwards, pellet was fixed in a mixture of acetic-acid: methanol, 1: 3, while dispersing slowly with a vortex stirrer and cells were centrifuged at 600 rpm for 8 min. The last step was repeated twice, and finally the supernatant was decanted close to the pellet and pellet was resuspended in 0.4 ml fixative before preparing slides. Slides were prepared by dropping the cell suspension on pre-cleaned object glasses with a micropipette. When drops had maximally expanded and Newton rings begun to appear, slides were blown gently to accomplish a flattening of the cells. For analyzing, slides were stained with 4% aqueous Giemsa solution (Rurr R66) for 5 min, slides were rinsed a few seconds in distilled water, air dried and mounted with a cover-glass using Depex (Albertini et al, 1997; Miller et al, 1997).

Slides scoring and evaluation: There were 24 donors involved in this study. For each donor and each dose, 1000 cells were analyzed in duplicate experiments. The induced frequency of MN for each dose was determined as follows: induced frequency of MN = MN frequency in the treated population, and MN frequency in the negative control (treated with PBS) samples (Rudd *et al*, 1998).

Results

The spontaneous frequency of chromosomal aberrations in all 24 donors is described in Table 1. Number of gaps and breaks were counted per 100 metaphases of each volunteer for each concentration. The experiments were duplicated for each concentration. The aberrations caused by the highest concentration of Gamma Immunex® at 66.6 IU/µl was shown and the results of lower concentrations was not showed in this report (Table 1).

In lymphocytes treated with Gamma Immunex®, the observed frequency of chromatid-type aberrations was not significantly higher than samples treated with PBS. The genotoxic agent MMC tested at concentration of 1.5 x 10^{-6}M could significantly induce both chromatid-breaks as well as gaps and exchanges (Table 2).

In samples treated with different doses (0.33-66.6 IU/µl) of Gamma Immunex®**,** the induced frequency of MN was in the range of 0-4 in 1000 binucleated cells, and statistically, there was no significant difference from PBS treated control (student t-test) (Table 3). In contrast, MMC tested at 3 x10^{-7} M) significantly increased frequency of MN when compared to negative control as well as to Gamma Immunex® treated one.

Table 1 Structural chromosome aberrations in lymphocyte cultures of 24 healthy donors (12 males and 12 females)

No.	Sex/age	Mitomycin C (1.5 µM)			Gamma Immunex® (66.6 IU/µl)			PBS (1%)		
		Gaps and exchange	Breaks	Total	Gaps and exchange	Breaks	Total	Gaps and exchange	Breaks	Total
1	M/27	8	2	10	2	1	1	2	0	2
2	F/24	2	0	2	1	0	0	0	0	0
3	F/25	6	2	8	1	1	1	1	0	1
4	F/26	4	2	6	0	1	1	0	0	0
5	M/24	2	1	3	1	0	0	0	0	0
6	M/25	5	2	7	1	0	1	1	0	1
7	M/27	7	2	9	1	0	1	1	0	1
8	F/27	6	2	8	0	1	0	0	0	0
9	F/25	3	1	4	0	0	1	0	0	0
10	F/24	9	3	12	0	1	1	1	0	1
11	M/29	6	2	8	1	0	0	0	0	0
12	F/23	4	1	5	0	0	1	0	1	1
13	F/28	5	1	6	1	0	0	1	0	1
14	M/26	3	1	4	1	0	0	0	0	0
15	M/23	3	2	5	2	1	0	0	0	0
16	M22	2	0	2	1	0	1	0	0	0
17	F/26	1	0	1	1	0	1	1	0	1
18	M/28	9	3	12	1	0	2	1	1	2
19	M/24	7	4	11	1	0	1	0	1	1
20	F/28	6	2	8	1	0	1	0	0	0
21	F/25	4	1	5	1	0	1	1	0	1
22	F/24	5	3	8	1	1	0	0	0	0
23	M/23	6	1	7	0	0	1	0	1	1
24	M/26	6	2	8	0	1	0	0	1	1

Table 2 Mean of chromosome aberrations produced by different concentrations of Gamma Immunex® in human lymphocytes of 24 healthy donors *in vitro*

Substances	Dose	Structural aberrations*				Numerical aberrations	Aberrant cells (frequency±SEM)
		ctb	csb	cse	cte		
PBS (%)	1%	1	0	0	0	0	0.2 ± 0.2
MMC (M)	$1.5x10^{-6}$	19	2	3	7	1	6.2 ± 3.3**
Gamma Immunex® IU/µl	0.33	1	0	0	0	0	0.2±0.2
	0.66	1	0	0	0	0	0.2±0.2
	1.32	1	0	0	0	0	0.2±0.2
	3.33	1	0	0	0	0	0.2±0.2
	6.66	1	0	0	0	0	0.2±0.2
	13.2	1	1	0	0	0	0.2±0.2
	26.4	1	1	0	0	0	0.2±0.3
	66.6	1	1	0	0	0	0.2±0.3

* Ctb = chromatid break; Csb = chromosome break; Cse = chromosome exchange; Cte = chromatid exchange

** p-value < 0.05

Table 3 The micronucleus frequency (%± SEM) in human lymphocytes treated with Gamma Immunex®

Mitomycin C	Gamma Immunex® (IU/ml)									PBS
0.3 µM	0	0.33	0.66	1.32	3.33	6.66	13.2	26.4	66.6	1%
10.5*	3	3	4	3	5	3	4	2	4	4
± 1.2	± 1.2	±1.4	±1.1	±1.5	±1.3	±1.3	±1.2	±1.5	±1.2	±1.1

** p-value < 0.05*

Discussion

Registration of all pharmaceutical compounds requires an assessment of their genotoxic effects. Extensive reviews have shown that many compounds that are mutagenic in the bacterial reverse mutation (Ames) test are rodent carcinogens. The addition of *in vitro* mammalian tests to these compounds not only helps support the increased sensitivity for detection of rodent carcinogens and broadens the spectrum of detected genetic events, but also helps minimize the specificity of prediction (i.e., increases the incidence of positive results that do not correlate with rodent carcinogenicity). Nevertheless, a battery approach is still reasonable because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis (Albertini *et al,* 2000; Kim *et al*, 2007). On the basis of this concept, a battery of tests for assessing the genotoxic potential of Gamma Immunex® which has been formulated by Exir Pharmaceutical Company in Iran were performed.

Before running any study on the genotoxicity effects of recombinant proteins, Hoffmann-La Roche and his team from Pharma Division, Department of Toxicology in Basel, Switzerland, had assessed the genotoxicity of different recombinant proteins in 1999. They discussed various aspects of genotoxicity testing of biotechnology-derived products based on information gathered from a questionnaire which was sent to about 30 predominantly European companies. Feedback was received from 13 companies on 78 compounds, mostly recombinant proteins but also on a number of non-recombinant proteins, which had been assessed for genotoxicity in a total of 177 tests. Four of the 78 compounds appeared to elicit reproducible genotoxic effects. For one of these compounds, the activity could be related to a non-peptidic linker molecule. No scientifically convincing rationale for the other three compounds could be established although, at least for two compounds, their activity may be connected with the enzymatic/ hormonal activity (Gocke *et al*, 1999). In addition to the survey, published reports on genotoxicity testing of biotechnology products were reviewed. The data were discussed relative to whether genotoxicity testing was a valuable exercise when assessing potentially toxic liabilities of biotechnology-derived compounds. It was concluded that genotoxicity testing could be necessary for some proteins which have a position in accordance with the available guidelines addressing this area. For the 'average' protein, electrophilic reactions are difficult to envision. Indirect reactions via DNA metabolism and growth regulation seem possible for only very specific proteins such as nucleases, growth factors and cytokines. IFN-gamma is a dimerized soluble cytokine that is the only member of the type II class of interferons. This interferon was originally called macrophage-

activating factor, a term now used to describe a larger family of proteins to which IFN-gamma belongs. Discussion of its potential to cause genotoxic changes was based on the literature reports. Even for those products for which concerns of genotoxic/ tumourigenic potential cannot be completely ruled out because of their lack of interaction with DNA metabolism or proliferation control. the performance of standard genotoxicity assays generally appears to be of value for their safety confirmation. All information, including information on the occurrence of genotoxic impurities, has been utilized to formulate a decision approach for the genotoxicity testing of gamma interferon.

The objective of this study was to examine the genotoxicity of Gamma-Immunex® which is provided by Exir Pharmaceutical Company in human peripheral blood lymphocytes using two major cytogenetic endpoints, chromosome aberrations and MN, for genotoxicity studies and biomonitoring purposes. Gamma-Immunex® tested at different concentrations (in the range of 0.33 up to 66.6 IU/µl) for 6 h in human peripheral blood lymphocytes using chromosome aberration and MN assays. These results revealed no genotoxic potential of this chemical under investigated treatment.

Although the underlying mechanisms of genotoxic actions of cytokines remain largely unknown, generation of free radicals or interaction with enzymes such as DNA topoisomerase II may be suspected. Human INF-alpha also may be considered as an antimutagenic compound in human cells. Human tumor necrosis factor alpha has been reported to enhance cytotoxicity and DNA fragmentation produced by DNA topoisomerase II-targeted to be anticancer drugs. At the same time, it has some radio- and chemo-protective properties *in vitro* and *in vivo*. Despite these facts, the question about genotoxicity of cytokines has not yet been answered. Some problems must be solved before receiving the final answer. Firstly, much more cytokines must be tested for their genotoxic activity. Secondly, appropriate test-systems must be designed. Thirdly, genotoxicity studies of cytokines must account for cytokine interaction in the cytokine network as well as for such cytokine-induced effects as cytotoxicity and apoptosis. Fourthly, in each case, it is necessary to have experimental evidence that observed genotoxic effects are caused by cytokine under investigation and not by other factors (Lazutkal, 1996).

Therapeutic effects of gamma-Immunex® on several diseases have been demonstrated in several clinical trials (Panahi *et al*, 2011; Panahi *et al*, 2012). Experimental data suggested that some cytokines, such as IFN-gamma, exhibit cytogenetic properties in human normal lymphocytes from peripheral blood (Baka et al, 2009) and herein, it showed this IFN lackrf of genotoxic effects clearly. Baka *et al* (2009) tried to clarify the cytogenetic activity of IFN-gamma, SCE and proliferation rate index (PRI) in lymphocyte cultures from peripheral blood samples of patients immediately after diagnosis (baseline), 30 days after radiotherapy, and after the fifth instillation of IFN-gamma. They showed a decrease in SCE frequency and PRI values in lymphocytes after treatment with IFN-gamma, suggesting that IFN-gamma does not have cytotoxic activity but on the contrary, may induce repair mechanisms, as shown in earlier studies by other biologic models. The same response could be suggested for Gamma-Immunex® which should be tested by further studies.

Conclusion

Gamma Immunex® was registered as the pharmaceutical compound and is the recombinant IFN-gamma. It was shown in this study to lack of genotoxic potential under the performed treatment regimen tested in human peripheral blood lymphocyte cultures by observing of chromosomal aberrations and MN compared to the negative control.

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