Influence of albumin content and freeze-drying on the immunogenicity of human recombinant Interferon alpha-2b in mice

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ABSTRACT

Antibodies against interferon (IFN) could explain the lack of response to treatment or the development of autoimmune processes. The albumin content and the freeze-drying process of IFN alpha formulations could be involved in the generation of such antibodies in treated patients. To study this, the immunogenicity of four formulations of human recombinant IFN alfa-2b was compared in mice. One-hundred sixty Balb/c mice were randomly and evenly distributed among the following four formulations: Heberon Alpha R, lyophilized containing 1.5 mg albumin per 3×10^6 IU of IFN, lyophilized albumin-free, or liquid albumin-free, and Reaferon-ES, lyophilized with 5 mg albumin per 3×10^6 IU of IFN. They were immunized subcutaneously with 100 000 IU of IFN alpha-2b three times a week for four weeks. Mice immunized with Reaferon-ES produced the highest serum titers of IFN alpha-2b antibodies by ELISA in the last 2 weeks, while the Heberon Alpha R liquid albumin-free formulation was the least immunogenic. Intermediate titers were obtained with the Heberon Alpha R lyophilized formulations, although they were larger for the formulation with albumin. We can conclude that albumin content and the freeze-drying process seem to have an influence on the formation of antibodies in mice.

Keywords: IFNα-2b, immunogenicity, anti-IFN antibodies, albumin, lyophilization

RESUMEN

La formación de anticuerpos contra el interferón (IFN) puede ser causa de no respuesta al tratamiento o el desencadenamiento de procesos autoinmunes. El contenido de albúmina y el proceso de liofilización de las formulaciones de IFN alfa pueden ser factores involucrados en la generación de tales anticuerpos. Para investigar lo anterior se comparó la inmunogenicidad de cuatro formulaciones de IFN alfa-2b humano recombinante en ratones. Un total de 160 ratones Balb/c se distribuyeron al azar y uniformemente para recibir una de las siguientes cuatro formulaciones: Heberon Alfa R liofilizado conteniendo 1.5 mg de albúmina por 3×10^6 UI de IFN, liofilizado sin albúmina o líquido sin albúmina, y Reaferon-ES, liofilizado que contiene 5 mg de albúmina por 3×10^6 UI de IFN. Ellos se inmunizaron subcutáneamente con 100 000 UI de IFN alfa-2b tres veces por semana durante cuatro semanas. Los ratones inmunizados con Reaferon-ES produjeron los títulos de anticuerpos contra IFN alfa-2b más elevados por ELISA en las últimas 2 semanas, mientras que el Heberon Alfa R líquido sin albúmina resultó ser la formulación menos inmunogénica. Con las formulaciones liofilizadas de Heberon Alfa R se obtuvieron títulos intermedios, aunque superiores para la que contiene albúmina. Podemos concluir que el contenido de albúmina y el proceso de liofilización parecen tener influencia sobre la formación de anticuerpos en ratones.

Palabras clave: IFNα-2b, inmunogenicidad, anticuerpos anti-IFN, albúmina, liofilización

Introduction

Every protein has the potential to induce an antibody response. These antibodies can be generated by a few injections as the in case of vaccines and therapeutic antibodies, or during prolonged treatments (e.g. months, years) with some homologous recombinant proteins such as insulin, the growth hormone and cytokines [1].

Interferons (IFNs) are cytokines with antiviral, antiproliferative and immunomodulatory properties, among others [2]. IFN alpha has been produced by recombinant DNA techniques since the end of the 1970's [3]. The amino acid sequences of human recombinant IFNs are similar to those of their natural homologous species, but they can differ in glycosylation patterns, other post-transcriptional changes and small differences in their three-dimensional structures. Vallbracht et al. were the first to report the presence of antibodies against IFN in patients [4]. Antibody induction has been thereafter reported in clinical trials with commercial preparations of different human recombinant IFNs in patients with several diseases, although with varying incidence [5, 6]. The determination of these antibodies (when they are neutralizing antibodies) is of a high practical importance since they could be a cause of the lack of response to the treatment with this molecule and they could also trigger autoimmune processes.

The analysis of IFN immunogenicity and its formulations is complex and requires continuous research since many questions on the causes and factors involved in this event have not been well clarified. Controlled studies in humans for this purpose are not easily carried out. It is therefore necessary to perform experiments in animal models in the search for information that may lead to possible predictions within the clinical setting.

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It has been described that a formation of complexes between IFN alpha molecules and the human serum albumin (HSA) that is regularly used as a stabilizer for the preparations could increase IFN immunogenicity [7, 8]. Furthermore, during lyophilization a group of molecular aggregates can be formed, which could amplify the induction of antibodies [7, 9].

The purpose of this study was to compare the immunogenicity of four formulations of human recombinant IFNα-2b in mice. The formulations differ in albumin content and in a liquid or lyophilized presentation. A simple enzyme immunoassay was developed to detect anti-IFN antibodies in mice sera.

Material and methods

Animals

A total of 160 male BALB/c mice of 16-18 g and 6-7 weeks old were used. The mice came from the National Center for the Production of Laboratory Animals (CENPALAB, Havana). They were kept under controlled temperature (19 ± 2 ºC) and humidity (55 - 65%) conditions. Food and water intake were *ad libitum*. Before the experiment, mice were kept for 15 days under acclimatization. The protocol was approved by the Ethics Committee for Biological Assays in Laboratory Animals of the Center for Genetic Engineering and Biotechnology [10], where the animals were kept during the experiment.

Interferons

Four different human recombinant IFNα-2b formulations were applied: 1) Reaferon-ES® (NPO Vecktor, Koltsovo, Novosibirsk Region, Russia), obtained in a bacterium (*Pseudomona putida)*. It is presented in ampoules, as a lyophilized powder for injection, containing: 3×10^6 IU of human recombinant IFNα-2b and 5 mg HSA. 2) Heberon Alpha R® (Heber Biotec S.A., Havana), produced in a bacterium (*Escherichia Coli*), in their traditional formulation. They are presented as a lyophilized powder for injection, in vials, containing 3×10^6 IU IFNα-2b, 1.5 mg HSA, 10 mg dextran, 5 mg mannitol, 7 mg NaCl, 11.4 mg $Na_2HPO_4 \cdot 2 \ H_2O$ and 6 mg $NaH_2PO_4 \cdot 2 \ H_2O$ per vial; 3) Heberon Alpha R® lyophilized without albumin, containing 3×10^6 IU IFNα-2b, 20 mg sucrose, 7 mg polyethylenglycol 8000, 9.2 mg $Na_2HPO_4 \cdot 2 \ H_2O$ and 6.3 mg $NaH_2PO_4 \cdot 2 \ H_2O$ and 4) Heberon Alpha R® liquid, without albumin, with 10×10^6 IU IFNα-2b, 10 mg benzyl alcohol, 0.2 mg polysorbate 80, 4.67 mg NaCl, 14.41 mg $Na_2HPO_4 \cdot 2 \ H_2O$, 2.96 mg $NaH_2PO_4 \cdot 2 \ H_2O$ and water for injection to complete 1 mL. For the ELISA, the buffered human recombinant IFNα-2b produced in *E. coli* was used. The authors received all the formulations of IFNα-2b from Heber Biotec Havana Cuba, free of charge.

Design

The immunization schedule lasted 4 weeks. One hundred and sixty BALB/c mice were randomly distributed in 4 groups (40 mice per group), corresponding to each formulation tested. In turn each group of animals was divided into four parts. The mice under study were slaughtered and bled at the end of each week to verify the development of anti-IFN antibody titers.

For all groups a dose of 100 000 IU of IFNα-2b was given subcutaneously, three times a week. Lyophilized formulations were used immediately after being reconstituted, where each 3×10^6 IU vial or ampoule was diluted with 3 mL of water for injection, and 100 µL were then taken for injection. Each vial of liquid IFN (10×10^6 IU) was diluted with 9 mL of water for injection and 100 µL were injected.

Mice were slaughtered at least 48 hours after the last IFN administration to allow complete plasmatic clearance so that anti-IFN antibodies are not masked in the ELISA determinations by their link to circulating IFN molecules. The ocular dissection method was used for bleeding and blood samples were collected into labeled Eppendorf tubes. Samples were centrifuged for 5 minutes at 250 x *g* and the sera obtained were stored at - 20 ºC for later analyses.

Determination of anti-human IFNα antibodies in mice sera

A simple enzyme immunoassay was used to detect anti-IFN antibodies in mice sera. The ninety-six well polystyrene plates (Costar, Cambridge, MA, USA) were coated with 100 µL/well of IFNα-2b (20 µg/mL) in 0.1 mol/L Na_2CO_3 - $NaHCO_3$ pH 9.6 for 3 h at 37 ºC. They were washed once with 0.05% polysorbate 20 (Sigma, USA) and incubated with the samples or controls (100 µL/well) for 2 h 37 ºC. The samples were diluted in 2% skim milk (Oxoid, Hampshire, England) in PBS, pH 7.2-7.4 (assay buffer). After 4 washings, an anti- mouse IgG - peroxidase conjugate (Sigma, USA) was added (100 µL/well) and incubated for 30 min at 37 ºC. After 6 washings the reaction was developed for 15 min at room temperature with 5.5 mg of *o* - phenylendiamine (Merck, Germany) and 5.5 mL of 0.015% H_2O_2 (Caledon, Canada) in 11 mL of citrate-phosphate pH 5.0 (100 µL/well). Finally, the reaction was stopped with 50 µL/well of 2 M H_2SO_4. The absorption was read at 492 nm in a plate reader (PR 521, Tecnosuma, Havana). The positive control was a pool of mice sera with high anti-IFN titer. The sera of non-immunized BALB/c mice were used as negative controls.

Sample evaluations for formulation and week of treatment were performed blindly. Nine two-fold dilutions were tested: from 1:20 to 1:5120. The sera with extinction values higher than 3 standard deviations above the average of the negative controls in each plate (cut-off) were considered to be positive antibody responses. The antibody titer was the inverse of the highest positive dilution. Simultaneously, to test specificity, the samples were pre-incubated at a 1:20 dilution with 100 µg/mL of IFNα-2b for 1 h at 37 ºC. Samples were confirmed to be positive when the fall of the absorption value after this pre-incubation was more than the absolute cut-off value.

Statistical analyses

For each evaluation time, the logarithms of the titers obtained were tested for normal distribution by the Shapiro-Wilk test and for variance homogeneity by the Levene test. Since assumption of normality was not valid for all groups, a non-parametric Kruskal-Wallis test was applied to compare them. When the differences were statistically significant, the Dunn

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multiple comparison test was carried out. A non para-
metric Mann-Whitney U test was applied to compare
groups for the presence of HSA as an ingredient in the
formulation. The significance level chosen was 0.05.

Results

The differences in the generation of anti-IFN antibody
titers with the formulations administered were analy-
zed. Antibodies were not detected after the first week
of immunization. In general, the order according to ti-
ters was: Reaferon-ES® > Heberon Alpha R® classic >
Heberon Alpha R® lyophilized without albumin >
Heberon Alpha R® liquid without albumin (Figure 1).
Titers rose after the second week but formulations did
not differ significantly at that time. During the third
week significant differences ($p < 0.05$) were detected
between Heberon Alpha R® liquid and the rest of the
formulations. During the fourth week, differences bet-
ween Reaferon-ES® and Heberon Alpha R® formula-
tions without HSA, in the liquid and lyophilized form,
were significant ($p < 0.05$). A prominent increase of
Reaferon-ES® titers was observed. Interestingly, at the
fourth week, only one sample was positive, from a mou-
se receiving Reaferon- ES® at the highest dilution by
ELISA (1:5120), while only one sample with Heberon
Alpha R liquid® without albumin was still negative.

The comparative analysis of the influence of cer-
tain preparation-derived factors on the generation of
anti-IFNα 2b antibodies in BALB/c mice is shown in
table 1. When the formulations were grouped by the
albumin factor, titers in the third and fourth weeks
were significantly higher ($p = 0.007$ and $p = 0.001$,
respectively) in the groups of mice receiving the for-
mulations containing HSA. Formulations using the
freeze-drying process developed titers on the third
week that were markedly higher ($p = 0.000$) than the
liquid formulations. According to the source of the
preparations, Reaferon-ES® was significantly more
immunogenic than Heberon Alpha R® formulations at
week 3 ($p = 0.007$) and week 4 ($p = 0.000$). Howe-
ver, for factors lyophilization and the source of the
preparations, the number of mice for the comparisons
was unbalanced, which assumes that a statistical bias
is introduced.

The specificity of the immunoassay was confir-
med in all cases since the absorbance values decrea-
sed completely after pre-incubation with IFNα-2b
(100 µg/mL) when the values were compared with
those for the positive samples at the same dilution
(data not shown).

Discussion

Human recombinant IFNα-2b is expressed in bacteria
(*E. coli*) with a high purity. This product has shown
its efficacy in the treatment of viral [11-13], oncolo-
gic [14] and fibrotic conditions [15] in several clinical
trials. Nevertheless, neutralizing antibodies for IFNα-
2b have been detected in 2.5% of all the patients trea-
ted with Heberon Alpha R® (Cervantes-Llano M, un-
published results).

The direct relationship between the response to
treatment and antibody formation has not been clearly
verified. There are studies where relapses in some pa-
tients have concurred with the development of neu-
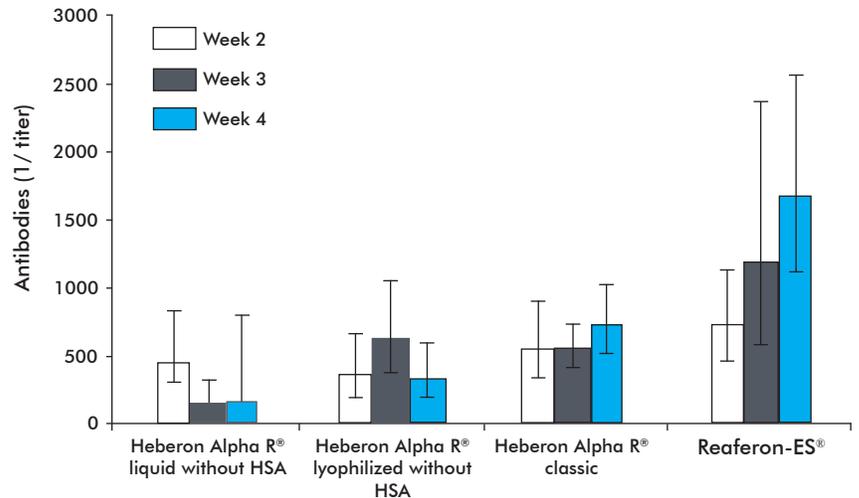tralizing antibodies to IFNα-2 [16-18]. The response

Figure 1. Serum anti-IFN α2b antibody titers in BALB/c mice immunized subcutaneously, 3 times a
week, with 100 000 IU of IFNα-2b for a maximum of 4 weeks. Each bar is the geometric mean and
the 95% confidence intervals of titers from 10 mice. Antibodies were not detected after the first week
of immunization. Differences were statically significant ($p < 0.05$, Dunn's test) at week 3: Heberon
Alpha R® liquid without HSA vs the other formulations; week 4: Reaferon-ES® vs Heberon Alpha R®
liquid without HSA and Reaferon-ES® vs Heberon Alpha R® lyophilized without HSA.

could be recovered if the therapy is switched to natu-
ral IFN [19]. On the other hand, certain authors have
found no clinical implications due to the generation of
these antibodies [20]. It is possible that, in these cases,
the affinity of IFN for its receptors was higher than
for the antibodies present in the serum. Alternatively,
patients could have responded before developing the
neutralizing antibodies. The formation of antibodies
is a potential problem when the treatment is prolon-
ged or the doses are high [21]. These antibodies could
also trigger autoimmune processes [22, 23].

This immune response depends on a great variety
of protein-, formulation-, and subject- derived factors.
Differences in manufacturing and purification proce-
dures can affect the antigenicity reported for the com-
mercial IFN formulations [24]. Some contaminating
molecular species within the preparations have been
found to have immunogenic properties [25]. On the
other hand, for many years, all IFN formulations have
contained HSA as a stabilizer because of its solubili-
ty, thermal stability and ability to avoid the surface
adsorption of active proteins. Nevertheless, immu-
nogenic reactions could be expected with that blood
derivative as well [26]. Since the albumin can cova-
lently interact with other proteins [27, 28], IFN-HSA
aggregates can be formed [7, 8], which increase its
molecular weight, and therefore its immunogenicity.
These aggregates are favored when the storage condi-
tions and the pH of the formulation are inadequate [7,
29]. At certain pH ranges the lyophilization process
can notably affect HSA-IFN and IFN-IFN aggregate
formation [7]. This has also been reported for the hu-
man growth hormone [9].

The characteristics of the disease under treatment
also affects the generation of antibodies. A high inci-
dence has been reported in oncological [30] and viral
[31] diseases. Individual characteristics may also be
involved as shown by results in several mouse strains
[32, 33] and by the existence of natural autoantibod-

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ies in certain subjects [34]. The study of administration frequency has revealed that the more frequent the administration of IFN, the higher the possibility of developing antibodies [35], which has also been observed in mice [32, 33]. Some systemic administration routes have been shown to be more immunogenic than others [32, 33], and the higher the dose and the duration of the treatment with recombinant IFN, the greater the possibility of developing antibodies [35].

The results obtained demonstrate the effect of albumin content and lyophilization on the immunogenicity of IFNα-2b formulations. Studies with these factors are not reported in humans or animals. Other factors, regardless of the characteristics of the formulations, remained well controlled throughout the study. Moreover, storage conditions and quality requirements of the formulations were regularly checked and found to be appropriates.

The Reaferon-ES® formulation was the most immunogenic. This product is presented as a lyophilized powder and has the highest albumin content. Nevertheless, other factors such as differences in the production strain and species and the characteristics of the manufacturing and purification processes cannot be discarded.

On the other hand, Heberon Alpha R® liquid without albumin produced the lowest anti-IFN antibody titers in immunized mice. When formulations were grouped according to the presence of HSA, titers were significantly higher in those with HSA. Likewise, an evident influence of the lyophilization process on IFN antigenicity was found.

The ELISA test used for the determination of antibodies against recombinant IFNα in mice is appropriate in terms of sensitivity, precision and high specificity, agreeing with reports [33]. Absorbance values of the positive samples were substantially reduced by pre-incubation with soluble IFNα-2b. Similar sandwich-type ELISA's in the literature refer only to determinations in human serum [36, 37].

Although the results can not be extrapolated to patients receiving recombinant IFNα-2b, they can be taken as indicators. Due to ethical and practical reasons it is difficult to carry out such studies in humans under controlled conditions. Therefore studies in human beings refer only to observations on some immunogenic factors, mainly those that are subject-dependent [29, 35]. Furthermore, antibody titers should be much lower in human beings than in rodents due to the poor molecular homology between IFNs from both sources [38]. In

Table 1. Influence of several preparation-derived factors on the generation of anti-IFNα 2b antibodies in BALB/c micea

	Human serum albumin (HSA) content		
Week[b]	Formulations with HSA (N = 20) Heberon Alpha R® classic + Reaferon-ES®	Formulations without HSA (N = 20) Heberon Alpha R® classic + Reaferon-ES®	p[c]
2	2.8 ± 0.6 (2.5; 3.4)[d]	2.5 ± 0.3 (1.6; 3.1)	0.145
3	2.8 ± 0.8 (2.2; 3.7)	2.5 ± 1.1 (1.3; 3.4)	0.007
4	3.1 ± 0.5 (2.5; 3.7)	2.5 ± 0.6 (0.0; 3.4)	0.001
	Presentation		
Week	Lyophilized formulations (N = 30) Heberon Alpha R® classic + lyophilized without HSA + Reaferon-ES®	Liquid formulation (N = 10) Heberon Alpha R® liquid	p
2	2.8 ± 0.6 (2.5; 3.4)[d]	2.5 ± 0.6 (1.9; 3.1)	0.659
3	2.8 ± 0.8 (2.2; 3.7)	2.1 ± 0.6 (1.3; 3.1)	0.000
4	3.1 ± 0.5 (2.5; 3.7)	2.7 ± 1.9 (0.0; 3.1)	0.115
	Source of the preparations		
Week	*Escherichia coli* (N = 30) Heberon Alpha R®	*Pseudomona putida* (N = 10) Reaferon-ES®	p
2	2.5 ± 0.4 (1.6; 3.4)	2.8 ± 0.6 (2.5; 3.4)	0.109
3	2.5 ± 0.6 (1.3; 3.4)	3.3 ± 0.7 (2.2; 3.7)	0.007
4	2.8 ± 0.7 (0.0; 3.4)	3.3 ± 0.4 (2.8; 3.7)	0.000

[a]Mice were immunized subcutaneously, 3 times a week, with 100 000 IU of IFNα-2b for a maximum of 4 weeks. Formulations are grouped according to whether HSA is included or not as ingredient, whether it is a liquid or lyophilized presentation, and the source of preparations. each time the median ± interquartile range (minimum; maximum) of the logarithms of the antibody titers is showed.
[b]Antibodies were not detected after the first week of immunization.
[c]Statistical analysis was carried out using the Mann-Whitney U test.

this study, IFNα-2b was administered by the subcutaneous route because it enhanced immunogenicity and constitutes, together with the intramuscular route, the two most frequent administration routes used for IFNs.

Although an extrapolation to the clinical setting is limited, as explained above, this study contributes to the characterization of the pharmacological properties of the formulations evaluated. The identification of the factors affecting its immunogenicity will help design different therapeutic regimes and eventually confirm them in human beings by thorough observations in treated patients.

We can thereby conclude that the origin of the IFNα-2b preparations could be a crucial factor in their immunogenicity. The albumin content and the freeze-drying process seem affect the formation of antibodies in mice.

Acknowledgments

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