Protein Aggregates Seem to Play a Key Role Among the Parameters Influencing the Antigenicity of Interferon Alpha (IFN-α) in Normal and Transgenic Mice

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Received March 11, 1997; accepted July 7, 1997

Purpose. During long-term treatment of various malignant or viral diseases with IFN-α up to 20% of patients develop anti-IFN-α antibodies for as yet unknown reasons.
Methods. To address this issue, a mouse model using Balb/C mice was established and the relevance of several potentially anti-IFN-α antibodies inducing factors was studied.
Results. The model revealed that both a higher frequency of injections and a higher dosage of IFN-α were more immunogenic and that the route of administration affected the antibody response to IFN-α. The intrinsic immunostimulatory activity of IFN-α itself also enhanced the immune response. IFN-α protein aggregates (IFN-α-IFN-α and human serum albumin (HSA)-IFN-α aggregates), which were recently identified in all marketed IFN-α products, were significantly more immunogenic than IFN-α monomers. These aggregates broke the tolerance against human IFN-α monomers in human IFN-α transgenic mice.
Conclusions. Based on these animal studies it is proposed that the immune response to IFN-α in humans is most probably elicited by a combination of several factors among which IFN-α protein aggregates seem to play a key role.

KEY WORDS: interferon alpha (IFN-α); animal model (mouse); antigenicity; protein aggregates; route of administration; dosage regimen; immunomodulation.

INTRODUCTION

Originally, immunogenicity problems with protein therapeutics were mainly attributed to contaminating proteins (1,2), to modifications of proteins during purification (3), or to the use of animal-derived proteins. With the introduction of recombinant DNA technology and the possibility to produce 'human identical' counterparts of animal proteins, most of the originally encountered immunogenicity problems with animal proteins have been solved. However, most of the recombinant therapeutic human proteins are still immunogenic, in particular during long-term treatment. Current efforts to explain their immunogenicity focus predominantly on formulation, clinical or physiological parameters, such as modifications during storage of formulations (4), route of administration (5), underlying disease, cumulative dosage, duration of treatment, dosage regimen, or the presence of autoantibodies (6). However, systematic studies analysing the significance of individual factors are missing. Because of a particular interest in human interferon alpha (IFN-α), it was decided to study the significance of some of these factors on the immunogenicity by using IFN-α as a model protein.

IFN-α is administered for treatment of malignant and viral diseases and induces anti-IFN-α antibodies in a significant number of patients. From the clinical literature it is very difficult to identify those factors potentially affecting the immunogenicity of IFN-α due to major differences in study design and assay methodologies. Nonetheless, it has been claimed that some recombinant IFN-α products are more immunogenic than others or than nonrecombinant ones (7). The latter are purified from cell culture supernatants and contain about 22 different subtypes of IFN-α (8). In contrast, E-coli-produced, recombinant products contain only one subtype of IFN-α and differ in only one or two amino acids of their primary sequence (7,9). These minor variations are not very likely to explain the proposed differences in the immunogenicity of recombinant IFN-αs, since (a) natural IFN-α subtypes differ in up to 30% of their primary sequence (9), (b) antibodies generated against one subtype can cross-react with others (10), and seem to recognise conformational epitopes rather than linear ones (11), and (c) peptides representing different recombinant IFN-α show identical reactions in human T cell proliferation assays or in binding to MHC class II molecules (12). Thus other, yet unidentified factors, must exist for potential differences in their immunogenicity.

In principle, there is no substitute for clinical trials to identify with absolute certainty those factors that are responsible for the immunogenicity of therapeutic proteins. However, since comparative studies in humans designed to identify antibody-inducing factors are unethical it was hoped that comparative studies in animals would give valuable basic information about the relative importance of the tested parameters for the antigenicity of IFN-α. Therefore, two mouse models (Balb/C and IFN-α transgenic FVB/NTac) were established using human IFN-α as an antigen. The factors tested in normal mice were: dosage regimen, route of administration, immunomodulating effect of murine interferon, and the effect of IFN-α aggregates. The latter two parameters were additionally tested in a human IFN-α transgenic mouse model.

MATERIALS AND METHODS

Unless stated otherwise, all reagents were of analytical grade quality from Fluka or Merck.

Animals

Eight week old, female Balb/C mice or 8–12 week old FVB/NTac mice of both genders (IFN-α2b transgenic or littermates) were from Biological Research Laboratories Ltd., Füllinsdorf, Switzerland and were kept in a conventional mouse facility under specific pathogen-free conditions. Test groups generally consisted of 5 mice.

Ethics

All animal experiments were approved by the institutional animal experimentation and ethics committee and adhered to

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the guidelines on animal experimentation of the National Health and Medical Research Council ("Principles of Laboratory Animal Care"; NIH publication 85-23, revised 1985).

Human IFN-α Transgenic Mice

A 4.5 kb EcoRI fragment encompassing the human IFN-α2b gene was obtained from a plasmid subclone of lamda 91 (13). The plasmid was a gift of Dr. Sidney Pestka, UMDNJ, Piscataway, NJ. The fragment was isolated using geneclean beads (Bio101 Inc.). DNA was used at a concentration of 1 ng/μl in 0.1 mM EDTA, 5 mM Tris-HCl, pH 7.4. Prior to microinjection, it was passed through a 0.2 micron nylon filter, followed by centrifugation in an Eppendorf microcentrifuge for 30 minutes.

FVB/N mice (=littermates) from Taconic Inc. were used (14). Two founders containing the intact interferon transgene were identified and one, line 45, was used for all experiments. Prior to the experiments, transgene carrying mice were identified by PCR analysis.

Preparation of Samples

Protein Samples

IFN-α2a bulk solution (F. Hoffmann-La Roche Ltd., Switzerland), IFN-α2a-IFN-α2a aggregates, and albumin-IFN-α2a aggregates were diluted to 3 μg IFN-α-2a/ml in sterile 10 mM sodium phosphate/150 mM NaCl buffer, pH 7.4 (PBS), containing 0.5% glycocholic acid (Calbiochem, 360512) for the normal mouse model. For the transgenic mouse model, IFN-α2a was diluted to 200 μg/ml with sterile PBS pH 7.4. Glycocholic acid was added to prevent adsorption of proteins to surfaces. Samples were used within 2 hours after preparation or were stored at −80°C and thawed directly prior to use.

Recombinant Murine-IFN-α (r-mur-IFN-α) and Polyinosinic-polycytidylic Acid (poly IC) Samples

R-mur-IFN-α (Holland biotechnology, B040a) and poly IC (Sigma) were diluted to a final concentration of 10.000 IU/ml and of 1 mg/ml in PBS, pH 7.4 containing 0.1% mouse serum albumin (MSA, Sigma), respectively.

Aggregate Samples

Aggregates of IFN-α2a and of IFN-α2a with HSA (Swiss Red Cross) or with MSA were prepared by cross-linking with glutaraldehyde as described (15). For the transgenic mouse model, glutaraldehyde was added to a final concentration of 0.12% to either 200 μg IFN-α2a/ml or to a mixture of 200 μg/ml IFN-α2a and 600 μg/ml albumin. After 2 min at room temperature, cross-linking was stopped by addition of 12 μl of 0.1 M sodium borohydride in 1 N sodium hydroxide solution for 20 hours at room temperature. Protein mixtures without glutaraldehyde and sodium borohydride solution were used as controls in some experiments.

Sample Administration

Samples of 100 μl were administered once a week per mouse by intraperitoneal (ip.) injection into the left side of the peritoneum for 5 weeks. For the determination of the immunomodulating activity, murine IFN-α was injected at the other side of the peritoneum to prevent interactions of the samples at the site of injection. Intravenous (iv.) injections (100 μl once a week per mouse) were administered via the tail vein. For subcutaneous (sc.) and intramuscular (im.) injections, 50 μl of a double concentrated sample was injected under the skin of the neck or into the muscles above the knee of the right hind leg, respectively.

Blood Sampling

Mice were bled from the retroorbital plexus at the beginning of the experiment and subsequently once a week. Blood was collected and pooled. After clotting for 2 hours at 4°C, serum was separated in Microtainer serum separator tubes (Becton-Dickinson, 5960) by centrifugation in a Hereaus Biofuge (4 min, 10.000 × g).

Quantification of Anti-IFN-α Antibodies and of IFN-α Aggregates

Polyclonal anti-IFN-α2a antibody generated in BALB/c or FVB/NTac mice (IFN-α-2b transgenic or littermates) were quantified by an enzyme linked immunosorbent assay (ELISA). Microtiter plates (MTPs) (Nunc Immuno Plate II F96 MAXISORP) were coated with 50 μl of IFN-α2a (10 μg/ml) in coating buffer (100 mM sodium hydrogen carbonate buffer, pH 7.9–8.1) and were incubated overnight at 4°C. Wells were washed twice with deionized water, and 200 μl of blocking buffer (200 mM Tris/HCl, 1% bovine serum albumin (BSA), 0.025% Thimerosal pH 7.5) was added for 2 hours. After a washing step consisting of two washes with deionized water and one with washing buffer (deionized water containing 0.05% Tween 20), 50 μl of serially diluted samples in dilution buffer (100 mM sodium phosphate, 10% FCS, pH 6.5) were transferred to the coated MTPs and incubated for 2 hours. After a washing step, 50μl of a horseradish peroxidase (HRP)-labelled monoclonal rat anti-mouse IgG antibody (Zymed, 04-6020) (1:500 diluted in dilution buffer) was added for 2 hours. Following a final washing step, 50 μl of the substrate solution (1:20 dilution of substrate (20 mM 3, 3′, 5, 5′-tetramethylbenzidine (TMB), 10% acetone, 90% ethanol, 50 mM H_2O_2) in substrate buffer (30 mM potassium citrate buffer, pH 4.1 containing 0.015% Kathon WT 14% (Christ AG)) was added. As a measure of HRP activity, absorbencies were read at 450 nm using a Dynatech MR7000 automated plate reader. Samples were analysed in duplicate. Anti-IFN-α2a antibody titers in mouse sera were calculated according to the antibody titer of a polyclonal mouse anti-IFN-α2a standard as previously described for the determination of IFN-α-IFN-α titers in samples (15).

IFN-α2a-IFN-α-2a and albumin-IFN-α2a aggregates were determined by ELISA as described (15). For detection of the MSA-IFN-α2a aggregates a rabbit anti-MSA antibody (RAM/Alb/PO, Nordic Immunochemicals) was used.

RESULTS AND DISCUSSION

Set-up of the Mouse Model

Ideally factors that may affect the immunogenicity of IFN-α, should be identified in a homologous animal model (i.e.

mouse model with mouse IFN-α). However, limited availability and high costs for natural or recombinant animal IFN-α rendered the use of a homologous model impracticable. Consequently, it was decided to use human IFN-α in a mouse model. Human IFN-α is not functionally active in mice but immunogenic. It was expected that samples with higher or lower immunogenicity would induce higher or lower antibody titers and/or would differ in the slope of the antibody titers.

To detect differences in antigenicity, a baseline anti-IFN-α antibody titer had to be identified that would allow the determination of an increase as well as of a decrease of the immunogenicity in subsequent studies. In previous experiments, it was found that dosages below 0.1 μg and above 3 μg of IFN-α per week ip. resulted in no or very high antibody titers, respectively. Therefore, Balb/C mice received increasing dosages of IFN-α (either 0.1 μg, 0.3 μg, 0.6 μg or 0.9 μg) once a week by ip. administration for 5 weeks. Blood samples were collected every week and anti-IFN-α antibody titers were determined by ELISA. As expected, increasing amounts of IFN-α resulted in higher antibody titers and in earlier occurrence of anti-IFN-α antibodies (Table I). Differences in and titer of antibodies were most significant at a dosage of 0.3 μg IFN-α per week. Therefore, this amount of IFN-α was used as a standard dose in subsequent experiments.

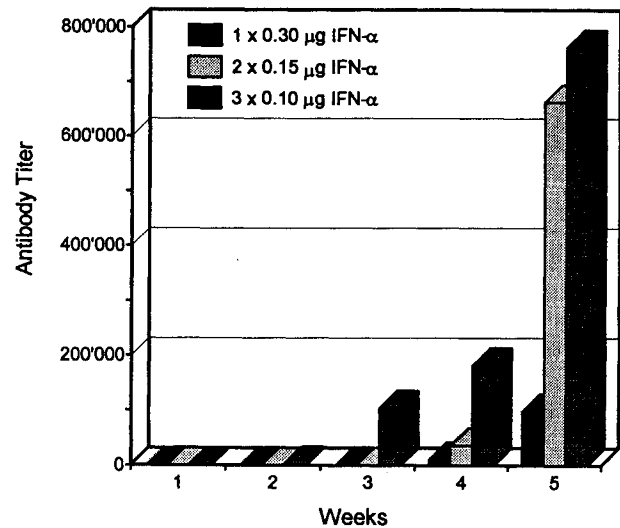

Fig. 1. Influence of the frequency of injections on the immunogenicity of IFN-α. Balb/C mice received a total cumulative dosage of 0.3 μg IFN-α per mouse per week for 5 weeks by ip. injection. Samples were divided into 1 (0.3 μg), 2 (2 × 0.15 μg), or 3 (3 × 0.1 μg) portions and were administered once, twice, or three times per week. Serum was tested for anti-IFN-α antibodies as described in the Materials and Methods section.

Frequency of Injections

In a first series of experiments, the influence of the frequency of injections on antibody formation was investigated. A total cumulative dosage of 0.3 μg of IFN-α2a per week per mouse was divided into 1 (0.3 μg), 2 (0.15 μg), or 3 (0.1 μg) portions and injected once, twice, or three times per week ip. for 5 weeks, respectively. The results in Fig. 1 show that, although all mice received the same cumulative dosage of antigen, the resulting anti-IFN-α antibody titers differed. More frequent injections resulted in higher titers and in an earlier development of antibodies than less frequent injections.

Our results indicate that the frequency of injections is an important factor for the intensity of an immune response to IFN-α. In clinical studies the frequency of injections varies from 3 to 7 injections of IFN-α per week, with dosages ranging from 3 to 36 MIU and with duration of treatment from a few weeks up to a year. This is not often taken into consideration when clinical trials are compared for IFN-α immunogenicity.

Table I. Set-up of the Mouse Model: Titration of the Baseline Titer of anti-IFN-α Antibodies

IFN-α2a administered per week	Week[a]				
	1	2	3	4	5
0.1 μg	0	0	0	0	0
0.3 μg	0	0	4,475	87,913	702,651
0.6 μg	0	0	98,241	824,406	3,403,460
0.9 μg	0	1,140	289,830	733,219	6,207,110

Note: BALB/c mice (5 animals/group) received increasing dosages of human IFN-α once a week by intraperitoneal injection. Blood was collected at the beginning of the experiment and subsequently once a week for 5 weeks. Samples were frozen at −20°C until analysis. Anti-IFN-α antibody titers in serum were determined by ELISA as described in the Material and Methods section.

[a] Mean value of anti-IFN-α2a antibody titer.

Route of Administration

There is also growing evidence that the route of administration can profoundly affect the antibody response to administered therapeutic proteins. Since IFN-α is administered clinically by intramuscular or subcutaneous injections and, in few cases, by intravenous infusion, the influence of the route of administration on the immunogenicity of IFN-α was studied. Mice received the same amount of antigen (0.3 μg) once a week for 5 weeks either ip., sc., im., or iv.. The data presented in Fig. 2 indicates that the same amount of antigen administered via different routes can induce different anti-IFN-α antibody titers; antibody titers increased in the order of iv.<<im.<ip.<sc. Both, ip. and sc. administration elicited high titers of antibodies, however, after sc. administration, the antibodies occurred earlier and antibody titers were higher. In contrast, im. administration induced hardly any antibodies against IFN-α and no anti-IFN-α antibodies were detectable in mice after iv. administration.

Comparable results for the immunogenicity of different routes of administration have been reported in humans for human growth hormone (2) and human interferon beta (5). The observed differences in the immunogenicity of the different routes of administration in the mouse are likely to be a result of the microenvironment at the site of injection and/or of the amount and half-life of IFN-α in the body. Thus, after sc. injection, more immunocompetent cells will be exposed for a much longer time and in higher concentrations to IFN-α than after iv. injection. This might explain the higher immunogenicity of IFN-α in mice after sc. compared to iv. administration. Likewise, the im. route is expected to be less immunogenic than the sc. route, since IFN-α is eliminated faster from the body and fewer immunocompetent cells are present in the mus-

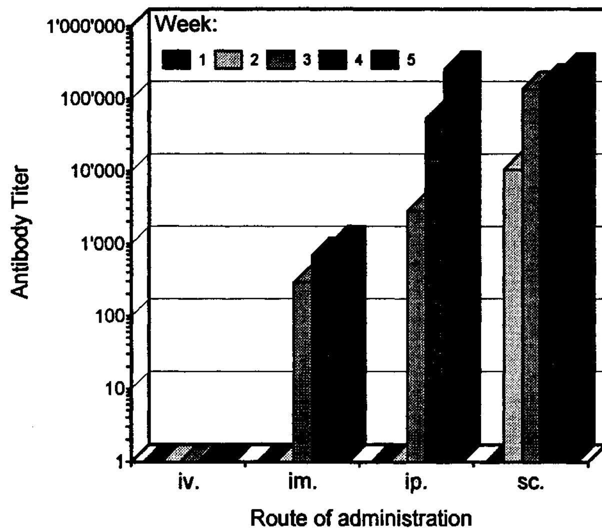

Fig. 2. Influence of the route of administration on the immunogenicity of IFN-α IFN-α (0.3 μg) was administered to Balb/C mice once a week for 5 weeks either by intravenous (iv.), intramuscular (im.), intraperitoneal (ip.), or subcutaneous (sc.) injection. Anti-IFN-α antibody titers were determined as described.

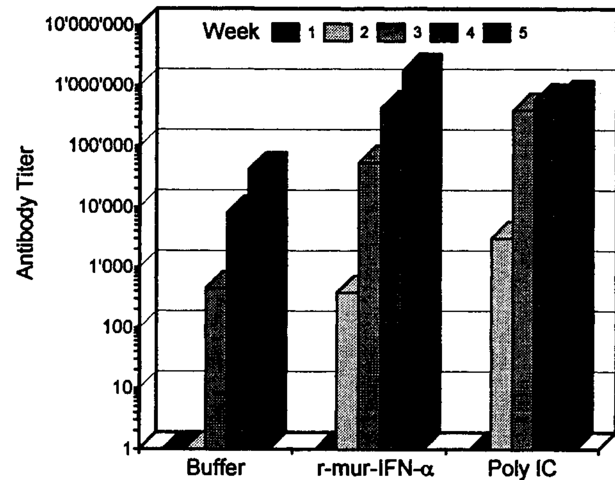

Fig. 3. Effect of concurrent immune stimulation on the induction of anti-IFN-α antibodies. Three groups of Balb/C mice received 0.3 μg of human IFN-α either with buffer, with 1000U of r-mur-IFN-α or with 100 μg of poly IC once a week by ip. administration for 5 weeks. Anti-human IFN-α antibody titers were determined as described.

cles (16). If the condition in humans were comparable, these results would suggest that in clinical studies im. or iv. administration of IFN-α should be favoured over the sc. route whenever this is compatible with the therapy.

Immunomodulating Effect of IFN-α

Human IFN-α is administered to patients because of its immunostimulatory activity. As a side-effect during long-term treatment with IFN-α, a significant increase of pre-existing antitissue antibodies or their de novo synthesis in patients has been reported (17,18). It has therefore been suggested that IFN-α facilitates an antibody response against self-components and, in unphysiologically high concentrations, possibly against itself. This hypothesis was tested in the mouse model. Since human IFN-α is not functionally active in mice, the immunostimulatory effect of human IFN-α had to be simulated by simultaneous administration of recombinant mouse IFN-α (r-mur-IFN-α). Alternatively, since administered mouse IFN-α might also be immunogenic and hence increase the immune response, stimulation was additionally performed with poly IC. The latter induces various IFNs within 2 hours of injection (19,20). To compensate for this delay, poly IC was injected 2 hours before human IFN-α was administered.

In the experiment, three groups of mice received 0.3 μg of human IFN-α either with vehicle alone, with 100 μg of poly IC or with 1000 U of r-mur-IFN-α once a week by ip. injection for 5 weeks. Control groups received the same amount of either poly IC or r-murine IFN-α alone (Fig. 3). In the serum of control groups anti-IFN-α antibodies were not detectable (not shown). In contrast, in animals treated with IFN-α and r-mur-IFN-α or poly IC, the immune response occurred earlier and antibody titers were higher than with IFN-α alone.

Data suggest that IFN-α facilitates, directly or indirectly, antigen processing and presentation to the immune system. Moreover, naturally occurring anti-IFN-α antibodies have been identified in healthy blood donors (6). Hence, administration of large quantities of IFN-α may simply boost antibody secretion. Another possibility is the presence of modified IFN-α in IFN-α preparations with the same amino acid sequence but with a foreign physical structure due to denaturation or aggregation. Even small amounts of such modified IFN-α may then induce a significant immune response due to the intrinsic immunostimulatory activity of IFN-α.

Immunogenicity of Protein Aggregates of IFN-α

In the past, some immunogenicity problems related to the presence of protein aggregates have been reported, i.e. for human growth hormone, IgG and insulin (1,21,22). Recently, protein aggregates have also been identified in all marketed IFN-α products (15). All four products contained albumin-interferon (HSA-IFN-α) and additionally two contained human serum interferon-interferon (IFN-α-IFN-α) aggregates, although in variable amounts. Therefore, these types of aggregates of IFN-α were compared with IFN-α monomers for their immunogenicity in mice.

Three types of aggregates, IFN-α-IFN-α, human serum albumin (HSA)-IFN-α and mouse serum albumin (MSA)-IFN-α, were tested. The IFN-α-IFN-α aggregate sample contained approx. 50% dimers and very small amounts of larger oligomers. It represented a fraction of IFN-α that was removed from an old IFN-α bulk solution. In contrast, albumin-IFN-α aggregates were generated artificially by crosslinking of IFN-α and of HSA or MSA with glutaraldehyde at a 1:1 molar ratio. These samples contained soluble IFN-α-IFN-α and albumin-IFN-α aggregates at a ratio of about 1:4 and approx. 30% of the total amount of protein was in an aggregated form. MSA was used in some experiments to avoid the introduction of a second foreign protein into the mouse model. IFN-α alone or mixtures of IFN-α with HSA or MSA served as controls.

Mice received 0.3 μg of either IFN-α-IFN-α or albumin-IFN-α aggregates by ip. injection, once a week for 5 weeks. Control mice received 0.3 μg of IFN-α or of a mixture of

HSA or MSA and IFN-α-2a. The protein concentration was calculated based on the IFN-α concentration in the samples. Resulting anti-IFN-α antibody titers are presented in Table II. The results clearly show that both IFN-α-IFN-α and albumin-IFN-α protein aggregates were much more immunogenic than IFN-α monomers or mixtures of IFN-α with MSA or HSA. In the aggregate groups, anti-IFN-α antibodies were detected already after 2 weeks compared to 3–4 weeks in the controls and antibody titers were significantly higher. The unusually high antibody titers in the HSA/IFN-α control, compared to the MSA/IFN-α control, are presumably due to the presence of a second foreign antigen (HSA) in the mouse model.

Our studies indicate that in mice, protein aggregates are far more immunogenic than monomers. The results would thus support the few human studies that attributed immunogenicity problems with therapeutic proteins to the presence of aggregates in the formulation (1,21–23).

To address this question further, a transgenic mouse model was established. These mice carried the human IFN-α2b gene and were found to be tolerant to injections of both human IFN-α2a and IFN-α2b (12). The presence of IFN-α2b in these mice alone, however, is possibly not sufficient to imitate the situation in humans, since human IFN-α is not biologically active in mice after administration. Hence, some of the subsequent experiments were performed in the presence of mouse IFN-α to reflect the human situation more precisely.

In a first series of experiments, transgenic mice and non-transgenic littermates received 10 μg of IFN-α with or without 1000U r-mur-IFN-α twice a week for 3 weeks. Human IFN-α samples were administered sc. and r-mur-IFN-α ip. to avoid any direct protein interactions in the samples or at the site of injection. The results in Fig. 4 show that IFN-α monomers did not induce any anti-IFN-α antibodies in the IFN-α2b transgenic mice, although the injected single dose was 33 times higher than the one used in the BALB/c model and samples were injected twice a week. Concurrent immune stimulation with mouse IFN-α also did not break the tolerance towards IFN-α monomers. In contrast, the littermates responded to human IFN-α well and co-administration of r-mur-IFN-α enhanced their immune response.

In a second set of experiments, mice received 10 μg of IFN-α-IFN-α or MSA-IFN-α aggregates with or without 1000U r-mur-IFN-α twice a week for 3 weeks. Protein concentration of the samples was calculated according to the IFN-α-2a content. IFN-α alone was used as a control. Both types of aggregates, IFN-α-IFN-α and MSA-IFN-α, clearly induced anti-IFN-α antibodies, indicating that they broke the tolerance to IFN-α in the transgenic mice (Fig. 4). Concurrent immune stimulation of transgenic mice with r-mur-IFN-α augmented the antibody response to IFN-α aggregates only 2–3 times. In contrast to BALB/c mice and to littermates, transgenic mice had lower antibody titers with MSA-IFN-α than with IFN-α-IFN-α aggregates; the reasons for this are not known.

Our results with IFN-α transgenic mice indicate that both IFN-α-IFN-α and albumin IFN-α aggregates can indeed break an existing tolerance towards IFN-α monomers. If any of these two types of aggregates is more immunogenic or how the artificially created aggregates correlate to the aggregates found in marketed IFN-α formulations (15) is difficult to estimate. For example, Wellferon® has been reported to be least immunogenic but the tested formulation contained the highest total amount of IFN-α aggregates. This might be attributed to the presence of >22 IFN-α subtypes which could all form aggregates. Thus, the amount of a single IFN-α aggregate subtype might be too low to provoke an immune response, compared to recombinant IFN-α formulations with only one subtype. Consequently, not only the amount but also the composition of the IFN-α aggregates may play a role in the induction of an antibody response.

The mechanisms involved in the loss of tolerance are not clear and there are several ways in which autoreactive B cells may be activated. Alternatively, covalent IFN-α protein aggregates may contain new linear or conformational epitopes that, after processing in antigen-presenting cells, are recognized by the immune system. This may elicit antibodies that cross-react with the monomers. The relative importance of these possible mechanisms remains to be determined and further studies will be required to finally identify the level(s) at which self-tolerance is lost.

CONCLUSIONS

Several parameters were tested for their influence on the immunogenicity of IFN-α in normal and IFN-α transgenic mice

Table II. Immunogenicity of IFN-α Protein Aggregates in BALB/C Mice

Sample	Week[a]				
	1	2	3	4	5
IFN-α monomers	0	0	0	320	1,170
IFN-α-IFN-α aggregates	0	350	19,210	32,433	86,925
MSA/IFN-α mixture	0	0	0	6,647	47,205
MSA-IFN-α aggregates	0	28,706	224,698	485,967	701,791
HSA/IFN-α mixture	0	0	209,316	1,609,285	2,357,563
HSA-IFN-α aggregates	0	69,612	872,848	3,343,294	7,131,066

Note: Mice received 0.3 μg of IFN-α-IFN-α, MSA-IFN-α or HSA-IFN-α aggregates by ip. administration, once a week for 5 weeks. Equal amounts of IFN-α alone or of mixtures of IFN-α with MSA or HSA served as control. Protein concentration was calculated based on the IFN-α concentration in the samples. Anti-IFN-α-2a antibody titers were determined by ELISA as described in the Material and Methods section.

[a] Mean value of anti-IFN-α2a antibody titer.

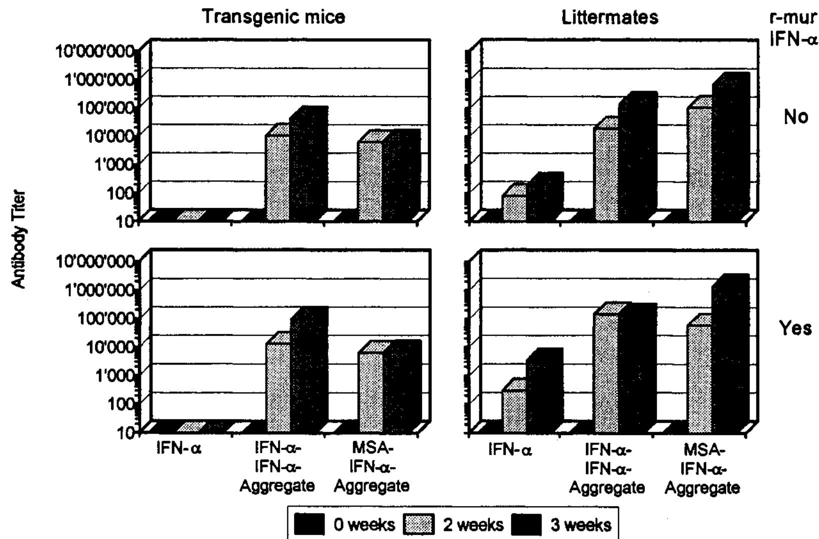

Fig. 4. Immunogenicity of IFN-α aggregates in IFN-α2b transgenic mice. The same amounts of IFN-α, IFN-α-IFN-α aggregates or MSA-IFN-α aggregates were administered twice a week to either IFN-α2b transgenic mice or to their littermates in the absence or presence of r-mur-IFN-α. Blood was collected after 0, 2 and, 3 weeks and antibody titers were determined as described in the Materials and Methods section.

and the dosage regimen, the route of administration, the intrinsic activity of IFN-α, and protein aggregates in formulations have been identified as factors that contribute to an immune response to IFN-α. Each of these factors affected the immune response alone. However, in humans it is likely that it is a net result of the tested and, presumably, as yet unidentified parameters that eventually result in an antibody response to administered IFN-α during treatment. Furthermore, if the data obtained in the mouse models can be translated into humans, our results indicate that protein aggregates in IFN-α formulations may be a major reason for an immune response to IFN-α. Thus, IFN-α protein aggregation in formulations may convert the non-immunogenic IFN-α monomers into immunogenic material. Moreover, the injection of aggregated material into a microenvironment that facilitates cooperation of the different antigen-processing and presenting cells may further increase the chance to elicit an immune response; hence sc. injection may be more immunogenic than, for example, iv. injections. Finally, other yet to be studied factors, such as underlying disease, the immune status of the patient, the presence of natural antibodies to IFN-α, or the genetic background of the patient may additionally determine whether or not a patient develops antibodies during long-term treatment with IFN-α.

The results of these experiments should have some practical implications especially for the development of new protein formulations. Since protein aggregates seem to be a major reason for an immune response, protein aggregation should be minimised or eliminated by identifying adequate formulation and/or storage conditions. Furthermore, clinical parameters, such as dosage scheme and route of administration, should be carefully reconsidered for each type of disease. Our results suggest that, whenever it is concordant with the needs of the patient and the underlying disease, proteins should be administered (i) by im. and iv. injections rather than sc., (ii) as infrequently as possible, and (iii) in amounts just sufficient to maintain effective levels.

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