Development and Use of Enzyme-Linked Immunosorbent Assays (ELISA) for the Detection of Protein Aggregates in Interferon-Alpha (IFN-α) Formulations

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Received March 11, 1997; accepted July 7, 1997

Purpose. Protein aggregates are thought to be involved in the immunogenicity of recombinant proteins in humans. To probe human IFN-α formulations for the presence of soluble protein aggregates, enzyme-linked immunosorbent assays (ELISA) were developed.

Methods. For the detection of IFN-α-IFN-α and HSA-IFN-α aggregates, sandwich ELISAs were developed using a monoclonal anti-IFN-α antibody as a capture antibody and the same anti-IFN-α antibody and an anti-human serum albumin (HSA) antibody (HRP-labeled), respectively.

Results. Marketed freeze-dried, HSA-containing IFN-α formulations tested in the ELISAs all contained IFN-α-IFN-α and/or HSA-IFN-α protein aggregates, although in varying amounts. These aggregates were predominantly IFN-α dimers and 1:1 conjugates of HSA with IFN-α. Test formulations revealed that aggregation of IFN-α was strongly affected by the presence of pharmaceutical excipients, pH of the formulation, lyophilisation procedure, and storage temperature and time.

Conclusions. The ELISAs are rapid, highly specific for aggregates in the presence of both IFN-α and HSA monomers and allow the direct detection of both types of aggregates in formulations in the nanogram range. The new assays will assist the monitoring of the aggregate-inducing processes during IFN-α formulation and storage in an early phase and the development of aggregate-free IFN-α formulations.

KEY WORDS: interferon alpha (IFN-α); human serum albumin (HSA); enzyme-linked immunosorbent assay (ELISA); protein formulation; protein aggregates.

INTRODUCTION

Therapeutic strategies involving the administration of recombinant human proteins such as interferons (IFNs), colony-stimulating factors, hormones, and growth factors are rapidly expanding. The majority of these products have amino acid sequences almost identical to the corresponding human proteins and were expected to be well tolerated by the human immune system. However, in clinical studies most of them were immunogenic to varying degrees and induced antibodies that, in some cases, affected therapeutic efficacy.

Factors discussed to affect the immune response against human recombinant proteins fall into three major categories: (a) source of recombinant protein (production in yeast; bacteria or mamalian cells, presence of contaminating proteins, glycosylation differences; etc.), (b) clinical factors (dose/duration of treatment; underlying disease; presence of autoantibodies; route of administration; etc.), and (c) formulation parameters (excipients; chemical and physical protein modifications; etc.). In particular the latter has been discussed intensively since, for example, aggregated human growth hormone (1), insulin (2), or IgG (3) were more immunogenic than the monomer.

In this study we analysed interferon alpha (IFN-α) formulations for possible aggregate formation since all marketed IFN-αs are reported to induce antibodies to some extent (4,5). In general, protein aggregates can be induced by stress conditions, such as exposure to extremes in temperature and pH, introduction of a high air/water or solid/water interface, or addition of certain pharmaceutical additives (6,7). In marketed IFN-α formulations human serum albumin (HSA) is used as constituent because of its good solubility, its thermal stability, and its abilitiy to prevent surface adsorption of active proteins. However, HSA can also interact with other proteins (8,9). Therefore, we developed ELISAs for the detection of both IFN-α-IFN-α and HSA-IFN-α aggregates. Compared to common techniques used for the detection of protein aggregates, for example sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF), high-performance liquid chromatography (HPLC), capillary zone electrophoresis, spectroscopic methods, or immunoblotting techniques (6,10), the ELISAs are highly sensitive, need low protein concentrations, operate in the presence of excipients and require no pretreatment of the samples. By using these highly sensitive ELISAs, we detected protein aggregates in marketed IFN-α formulations and identified some of the parameters that induce protein aggregation in formulations.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all reagents were of anaytical grade quality from Fluka. Mouse monoclonal antibodies (MAbs) generated against leukocyte-IFN-α (LI) clone 1 (LI-1) and clone 9 (LI-9) were described by Staehelin et al. (11). These anti-IFN-α antibodies and Peroxidase-labelled LI-1 (LI-1-HRP) were provided by Roche Diagnostics, Kaiseraugst, Switzerland. Peroxidase-labelled polyclonal goat anti-human HSA antibody (anti-HSA-HRP) was from Nordic Immunological Laboratories (Code GAHu/Alb/PO). Bovine milk (instantized), human serum albumin (HSA), fetal calf serum (FCS), and bovine serum albumin (BSA) were from Sano Lait (Coop, Switzerland), Swiss Red Cross, AMIMED, and Sigma, respectively. The recombinant Interferon alphas, Roferon A® (r-human IFN-α2a), Intron A® (r-human IFN-α2b), and Berofor® (r-human IFN-α2c), and interferon alpha purified from cell culture supernatants, Wellferon® (human IFN-α-n1), were from Roche (Switzerland), Essex Chemie (Switzerland), Bender & Co. (Austria), and Wellcome (England), respectively. IFN-α bulk solution (266 MIU IFN-α2a/ml, 1.2 mg/ml) was from F. Hoffmann-La Roche AG (Basel) and free of any detect-

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ABBREVIATIONS: ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; LI-1 and LI-9, mouse monoclonal antibodies to human lymphocyte, interferon alpha (IFN-α); HRP, horseradish peroxidase; HSA, human serum albumin; SMM, slow moving monomer of IFN-α.

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able IFN-α aggregates. The IFN-α (SMM, 0.96 mg IFN-α/ml) used to develop the IFN-α-IFN-α aggregate ELISA contained 5% IFN-α dimers and was kindly provided by Mr. Ettlin (F. Hoffmann-La Roche Ltd, Basel).

Enzyme-linked Immunosorbent Assays (ELISA)

NUNC 96F MAXISORP polystyrene microtiter plates were used for all experiments. Unless otherwise stated, experiments were performed at room temperature. Between the individual incubation steps, wells were washed 3 times with 400 μl deionized water and twice with 400 μl 0.05% Tween 20/deionized water (washing buffer) to remove unbound material. If necessary, samples were prediluted in plastic tubes or Nunclon® MicroWell Plates U96 in 100 mM sodium phosphate/10% FCS, pH 6.5 buffer (saturation buffer). During the incubation steps, microtiter plates were sealed with an adhesive sealing tape (Gibco BRL). The buffers used for the ELISAs were stable for 3 months at 4°C.

The following general procedure was used for all types of ELISA: Microtiter plates were coated with 200 μl of antibody dilution in 100 mM sodium hydrogen carbonate, pH 7.9–8.1 (coating buffer) for 2 h at room temperature (RT) or overnight at 4°C. The wells were washed and remaining non-specific protein binding sites on the plastic's adsorptive surface were saturated with 250 μl of saturation buffer for 2 h at RT or overnight at 4°C (plates can now be stored at 4°C for a maximum of 3 months). The saturation buffer was removed and the wells were washed. For the IFN-α-IFN-α aggregate ELISA, 200 μl of serially diluted sample was mixed with 50 μl of Peroxidase (HRP)-labelled second monoclonal antibody and was added to the wells overnight at RT. For the HSA-IFN-α ELISA, 200 μl of a serial dilution of the sample was first added to the wells overnight at 4°C, wells were washed and 50 μl of the HRP-labelled anti-HSA antibody was added for 4 h at RT. The wells were washed and 200 μl of a 1:20 dilution of substrate (480 mg 3,3′,5,5′-Tetramethylbenzidin in 10 ml acetone/90 ml ethanol/0.6 ml 30% H_2O_2) in substrate buffer (30 mM citric acid/0.015% Kathon (Christ AG), pH 4.1) was added. The reaction was stopped by adding 50 μl of 1 M H_2SO_4. The absorbancies were read at 450 nm in an Dynatech MR7000 automated plate reader. IFN-α was determined as described (12).

Data Analysis

The relative titers of IFN-α-IFN-α and HSA-IFN-α aggregates in samples were calculated from IFN-α-IFN-α or HSA-IFN-α aggregate standards used in each experiment. Standard and samples were adjusted to the same IFN-α protein concentration, serial dilutions were prepared and the assay was performed. The concentrations of IFN-α (x-axis) and the corresponding extinction readings (y-axis) were plotted on linear scales. Linear regression analysis was performed within the linear part of the sigmoidal curve of the standards (the linear part was in the range of about 5–50 ng IFN-α/ml) and the half-maximal extinction within this range was determined. Relative aggregate titers in samples were then expressed as the reciprocal of the dilution at which the samples reach this half maximal extinction (e.g., sample dilution at 50% of maximal extinction = 1:250, aggregate titer = 250).

Generation of Artificial IFN-α-IFN-α and of HSA-IFN-α Aggregates

IFN-α-IFN-α aggregates were generated by incubating 350 μl of IFN-α2a (150 μg IFN-α2a/ml PBS) with 5.6 μl of glutardialdehyde (2.5% solution in PBS) for 2 min at room temperature. The reaction was terminated by adding 4.2 μl of 0.1M $NaBH_4$ in 0.1N NaOH for 20 h at room temperature. The resulting IFN-α2a sample contained approx. 30% dimers and higher oligomers as shown by HPLC size exclusion chromatography.

Similarly, HSA-IFN-α2a aggregates were generated by incubating 350 μl of a 1:1 mixture (molar ratio) of IFN-α2a (50 μg/ml) and HSA (150 μg/ml) with 5.6 μl of glutardialdehyde (2.5% solution) for 2 min at room temperature. Cross-linking was stopped as described above.

Both IFN-α-IFN-α and HSA-IFN-α aggregates generated by cross-linking were stable for only about 1–2 weeks at 4°C (as indicated by their titers). After that time, aggregate titers decreased for unknown reasons.

High-Performance Size Exclusion Chromatography (HPSEC)

The size of aggregates present in samples was determined by HPSEC using a Bio Sil TSK 25 (30 cm × 7.5 mm, BioRad). The mobile phase (20 mM sodium phosphate/150 mM sodium chloride/0.02% sodium azid buffer, pH 6.8 containing 0.1% BSA to prevent adsorption of sample proteins to the column) was passed through a 0.45 μm steril filter just prior to use and delivered at a rate of 0.5 ml/min. Samples were injected through a 50 μl loop and collected fractions (0.5 ml) were tested for IFN-α and IFN-α protein aggregates by ELISA. The column was calibrated using a mixture of lucifer yellow (0.5KD), IFN-α (19KD), Peroxidase (44KD), BSA (65KD), and Peroxidase-labeled monoclonal antibody (200KD) dissolved in mobile phase (without BSA).

IFN-α-2a Test Formulations

Ten mM phosphate buffered saline (PBS) containing 1% benzyl alcohol as preservative was used as formulation buffer. PBS solutions containing either 0.5% human serum albumin (HSA) or 3% Saccharose were prepared. The pH of the formulations was adjusted to pH 3.5, 5.5, or 7.5 with acetic acid or ammonium acetate. IFN-α-2a bulk solution (bulk buffer: 120 mmol NaCl, 25 mmol NH_4Cl, pH 5.0) was added to the different formulations to a final IFN-α-2a concentration of 18 MIU/ml. After sterile filtration through 0.2 μm syringe filters, 1 ml test formulation was filled into sterilised 3 ml glass vials. Vials were covered with sterilised, teflonised 13 mm stoppers and were closed with aluminium caps either immediately (the liquid formulations) or after lyophilisation.

Lyophilisation was carried out in a Christ beta 2–16 lyophylisator. After cooling the vials, shelf and apparatus to 5°C, temperature was reduced to −50°C within three h. After 30 min, shelf temperature was elevated to −40°C within 30 min and left at −40°C for 16 h. Vacuum was lowered to 0.25 mbar within 15 min and was kept constant while the shelf temperature was elevated stepwise to −30°C for 15 min and then to −15°C, 0°C, 15°C, and 30°C for 2 h at each temperature. After adjusting the vacuum to 0.001 mbar within 15 min, the drying process

was continued for 4.75 h at 30°C. Finally, shelf temperature was decreased to 15°C within 5 h.

Formulations were stored at −80°C, 4°C, and 30°C (at 75% relative humidity) for one or three months.

RESULTS AND DISCUSSION

Marketed IFN-α formulations contain 10 to 5000 times more HSA than IFN-α, depending on the IFN-α dose and the manufacturer. Therefore, both IFN-α-IFN-α and, even more likely, HSA-IFN-α aggregates may form during formulation and/or storage. Standard analytical methods, such as SDS-PAGE, particle counters or HPSEC are not usually sensitive enough to directly detect small amounts of IFN-α protein aggregates in these formulations or require a pretreatment (prefiltration, addition of SDS, etc.). Therefore, assays based on the principle of an enzyme-linked immunosorbent assay (ELISA) were developed. Such ELISAs are usually rapid, sensitive, highly specific, and allow the identification of a specific compound or contaminant even in complex mixtures.

Development of an ELISA for the Detection of IFN-α-IFN-α Aggregates

The principle of the assay for the detection of IFN-α-IFN-α aggregates has been described by Pestka *et al.* (13) The assay uses the same monoclonal anti-IFN-α antibody (LI-1) as capture and as detection antibody (Fig. 1). Since the epitope recognized by LI-1 is present only once in IFN-α, monomeric IFN-α bound to surface-attached LI-1 will not be detected by the same, labelled monoclonal antibody. In contrast, dimers or oligomers of IFN-α have potential free sites available for labelled LI-1. The original radio immunoassay (RIA) used ^{125}iodine labelled antibody and worked only in the absence of monomers which rendered the assay impracticable for routine analysis of IFN-α formulations. Therefore, the RIA was converted into a more convenient ELISA that uses peroxidase-labelled LI-1 (LI-1-HRP) for detection and identifies aggregates in the presence of monomers. The ELISA system was set up using an IFN-α fraction that contained 5% IFN-α dimers (SMM).

As expected, the concentrations of both the coating and detection antibody strongly affected the detection limits of the assay. Sensitivity was optimal when plates were coated with 10 μg LI-1/ml (200 μl/well). This is about twice the concentration required to achieve the densest IgG packing on the available surface area (14). Lower concentrations (<2 μg/ml) reduced the signal significantly, higher concentrations did not improve it (not shown). LI-1-HRP concentration was optimal at 3 μg LI-1-HRP/ml (Fig. 2A). Extinction was maximal, for example, when 200 μl of IFN-α (100 ng/ml) (5% dimers) and 50 μl LI-1-HRP (3 μg/ml) were added to mictotiter plates that contained 1 μg LI-1 per well. This corresponds to a molar ratio of LI-1:IFN-α:LI-1-HRP of about 6:1:0.75. Less LI-1-HRP decreased the sensitivity of the assay, since not all available binding sites

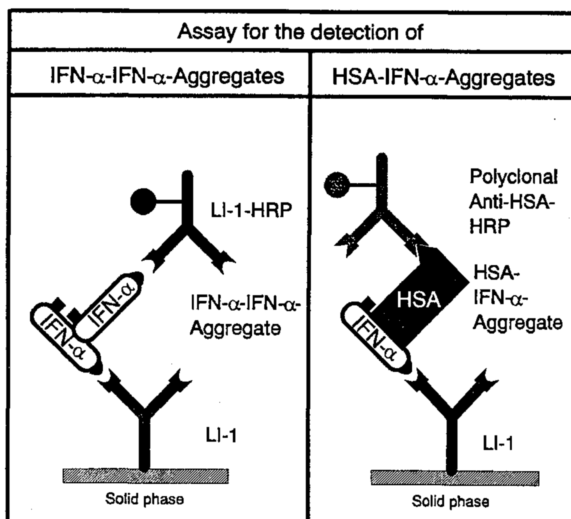

Fig. 1. Schematic representation of ELISAs for the detection of IFN-α-IFN-α and HSA-IFN-α aggregates. IFN-α-IFN-α aggregates are detected using the same monoclonal anti-IFN-α antibody (LI-1) as capture and detection antibodies. HSA-IFN-α aggregates are captured by the anti-IFN-α antibody LI-1 and IFN-α-bound HSA is identified by a polyclonal anti-HSA antibody. For simplicity, aggregates are illustrated at a 1:1 molar ratio. (HRP = Horseradish peroxidase).

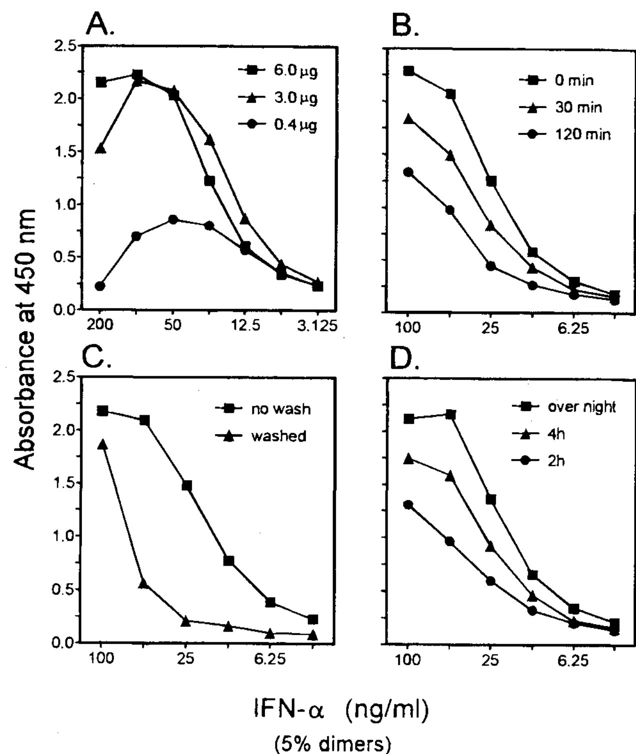

Fig. 2. Parameters affecting the sensitivity of the IFN-α-IFN-α aggregate ELISA. Serial dilutions of IFN-α containing 5% of IFN-α dimers were added to anti-IFN-α-antibody coated microtiter plates (LI-1) and the influence of subsequent assay modifications on the sensitivity of the ELISA was determined. *A. Amount of LI-1-HRP.* Three different concentrations of LI-1-HRP were added, and the ELISA was developed after an over night incubation at RT. *B. Timing of LI-1-HRP addition.* LI-1-HRP was mixed directly with the IFN-α sample (0 h) or was added after 30 min or 120 min incubation at RT. *C. Washing step.* IFN-α dilutions were mixed with LI-1-HRP and incubated for 16 h (no wash). Alternatively, IFN-α dilutions were first incubated with LI-1-coated wells for 6 h, wells were washed and LI-1-HRP was added for 16 h (wash). *D. Time of incubation with LI-coated wells.* LI-1-HRP was mixed with the IFN-α sample and the mixture was incubated with the LI-1-coated wells for 2 h, 4 h or overnight (16 h) at RT.

in IFN-oligomers are saturated by the antibody. Higher LI-1-HRP concentrations also decrease the sensitivity since binding sites in aggregates recognized by plastic bound LI-1 are already occupied by LI-1-HRP. Similarly, excess IFN-α inhibits the binding of IFN-α aggregates to the surface-bound antibody ("prozone effect" = lower extinction at higher IFN-α concentrations). Therefore, IFN-α starting concentration was adjusted to 0.1 μg IFN-α/ml (approx. 25'000 IU IFN-α/ml) in subsequent ELISAs.

The timing of addition of LI-1-HRP to the sample also affected assay sensitivity (Fig. 2B). LI-1-HRP added immediately to samples in LI-1-coated plates, rendered the assay 2 times and 4 times more sensitive than LI-1-HRP added after 30 min and 2 h, respectively. This was surprising, since simultaneous incubation of LI-1-HRP with IFN-α-IFN-α aggregates was expected to inhibit, at least partially, aggregate binding to LI-1-coated wells. An explanation might be that oligomers initially bind via only one site to LI-1-coated plates but subsequently slowly bind through more than one site to LI-1. This would reduce the number of binding sites available for LI-1-HRP and hence the sensitivity of the assay. This might also explain the about 4-fold decrease in sensitivity when LI-1-coated plates were incubated first with the sample for 8 h and washed before LI-1-HRP was added (Fig. 2C).

For optimal results, an overnight incubation of LI-1-HRP and sample with LI-1-coated plates seems to be required. Reduction of the incubation time to 4 h and 2 h, decreased the sensitivity 2 and 4 times respectively (Fig. 2D).

Calculations based on the known amount of aggregates in the standard, showed that the ELISA can detect IFN-α-IFN-α aggregates in samples down to about 1 ng. The assay was linear in the range of about 5–50 ng of IFN-α/ml and intraassay deviations for the standards and samples were 3–7%

Correlation between aggregate titers determined by IFN-α-IFN-α ELISA and aggregates measured by HPLC size exclusion chromatography were good between the two assays up to about 7% of aggregates in the formulation (Fig. 3). Aggregates in this range were predominantly dimers and smaller oligomers of IFN-α as indicated by their elution profil in HPLC chromatograms (data not shown). In contrast, at higher percentages of aggregates this correlation was lost. Potential reasons might be (A) removal of larger aggregates by the prefilter of the HPLC column, (B) disruption of antigen-antibody interactions by shearing forces during the washing processes in ELISA, or (C) inaccessibility of some binding sites inside larger IFN-α aggregates for LI-1-HRP, which would result in an underestimation of the amount of aggregated IFN-α in a sample in ELISA.

Development of an ELISA for HSA-IFN-α-2a Aggregates

An ELISA for the identification of HSA-IFN-α aggregates in HSA-containing marketed IFN-α formulations was established with artificial HSA-IFN-α aggregates using the monoclonal anti-IFN-α antibody LI-1 as capture and a HRP-labelled polyclonal goat anti-human HSA antibody as detection antibody (Fig. 1). Other ELISA set-ups, such as replacement of LI-1 by LI-9 or by a polyclonal rabbit anti-human IFN-α antibody, reversal of the ELISA (anti-HSA antibody as capture antibody and LI-1-HRP as detection antibody), or use of a monoclonal anti-HSA antibody always resulted in less sensitive assays. Under optimized conditions, the HSA-IFN-α ELISA had a detection limit of about 2 ng HSA-IFN-α aggregates per ml. The assay was specific for HSA-IFN-α aggregates, since (A) dilutions of HSA or of IFN-α did not give a signal, (B) large quantities of free IFN-α inhibited the binding of HSA-IFN-α aggregates to LI-1, and (C) HSA-IFN-α aggregates were not detected without LI-1-coating of the wells (Fig. 4).

Assay sensitivity was affected by a number of factors: (A) Coating of microtiter plates and detection of bound HSA-IFN-α aggregates were optimal at LI-1 and anti-HSA antibody concentrations of 10 μg/ml and a 1:4000 dilution, respectively (200 μl/well). This was determined basically as described for the IFN-α-IFN-α aggregate ELISA. The selected final anti-HSA-HRP concentration represents a compromise, since non-specific background increased with antibody concentration and

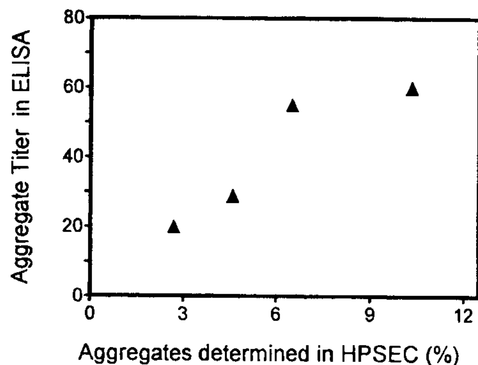

Fig. 3. Correlation between the percentage of aggregates determined by HPSEC and the IFN-α-IFN-α aggregate titer in ELISA. HSA-free IFN-α formulations were applied onto a BIO-SIL TSK 125 HPLC size exclusion column and the percentage of IFN-α aggregates in the formulations was calculated from the AUC of IFN-α present in peaks eluting before the IFN-α monomer peak and the amount of IFN-α applied to the column. In parallel, the samples were adjusted to the same protein concentration and were analysed in the IFN-α-IFN-α ELISA. The mid-point of the titration curves were determined and the aggregate titers were plotted against the percentage of IFN-α aggregates determined by HPLC analysis.

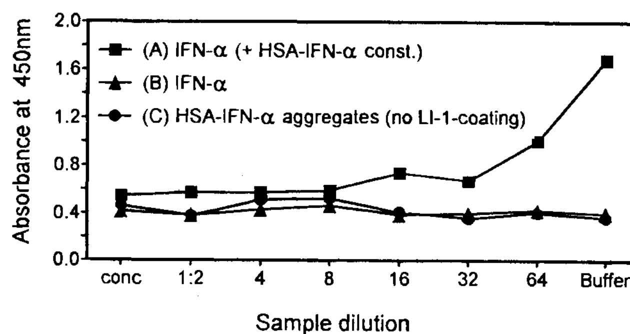

Fig. 4. Specificity of the HSA-IFN-α aggregate ELISA. Microtiter plates were coated either with the anti-IFN-α antibody LI-1 (A, B) or with saturation buffer (C). LI-1-coated wells were subsequently incubated in a competition assay with serial dilutions of IFN-α (starting concentration, 10 μg/ml, 200 μl/well) for 2 h at RT. Wells were washed and incubated with a constant amount of HSA-IFN-α aggregates (1 μg/ml, 200 μl/well) (A) or with buffer (B). Alternatively, HSA-IFN-α aggregates were serially diluted on saturated wells (C). After incubation for 16 h at room temperature, wells were washed and bound material was detected by a polyclonal goat anti-HSA-HRP antibody as described in the Material and Methods section.

lowered the assay sensitivity significantly (data not shown). (B) A washing step between sample incubation and addition of detection antibody increased the sensitivity of the HSA-IFN-α ELISA and eliminated the "pro-zone" effect (HSA concentration >> anti-HSA-HRP concentration) observed at high HSA concentrations. (C) The blocking agent used to inhibit non-specific binding of protein components to the plates was critical. Among the tested blocking agents, BSA, ovalbumin, casein, gelatin, and milk powder, the latter yielded the highest signal to background ratio (data not shown). (D) Sensitivity of the ELISA increased with incubation time. The signal to background ratio was highest when samples were incubated with LI-1 and anti-HSA-HRP antibody with LI-1-bound HSA-IFN-α aggregates for 16 h and 4 h, respectively. Under these conditions, sensitivity, linear range and intraassay deviations of the ELISA were similar to the IFN-α-IFN-α ELISA.

Analysis of Marketed IFN-α Formulations

Currently, the IFN-α market is dominated by four products: Berofor®, Intron A®, Roferon A® and Wellferon®, which differ in production process/technique, physical state, and formulation. Although their formulations are different, all these products use HSA as a stabilising agent, albeit in different amounts (Table I). Tested IFN-α products were for clinical use and were all analyzed 1–1.5 years before the expiration date indicated on the vials. When anlaysed in the IFN-α-IFN-α and HSA-IFN-α aggregate ELISA, HSA-IFN-α aggregates were present in all IFN-α products, although to a different extent. In addition, Intron A® and Wellferon®, also contained IFN-α-IFN-α aggregates (Fig. 5). A rough estimate of the amount of aggregates present in marketed formulations (based on the aggregate standards used) indicated that less than 1% of the IFN-α is present in an aggregated form. Determination of the size of the aggregates in two selected products, Intron A® and Roferon A®, by HPLC size exclusion chromatography revealed that most IFN-α-IFN-α aggregates eluted between the IFN-α monomer (19KD) and the horseradish peroxidase (44KD) peak (Fig. 6). This indicated that they were predominantly IFN-α dimers. The HSA-IFN-α aggregates peaked in the range of BSA (65KD), which suggests that most of them are formed at a 1:1 ratio. However, the observed HSA-IFN-α peak was relatively broad. This could be due to an insufficient resolution of the gelfiltration column in this range. Alternatively, other species of aggregates

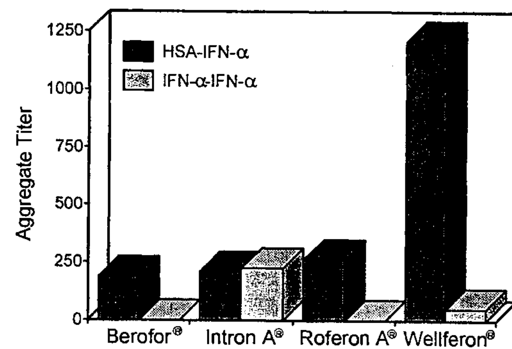

Fig. 5. IFN-α protein aggregates in marketed IFN-α products. Lyophilized IFN-α products were reconstituted as recommended by the manufacturer and all samples were adjusted to a starting concentration of 50,000 IU IFN-α/ml. Serial dilutions of the IFN-α products were added to LI-coated microtiter plates and IFN-α-IFN-α and HSA-IFN-α aggregates were determined by ELISA as described.

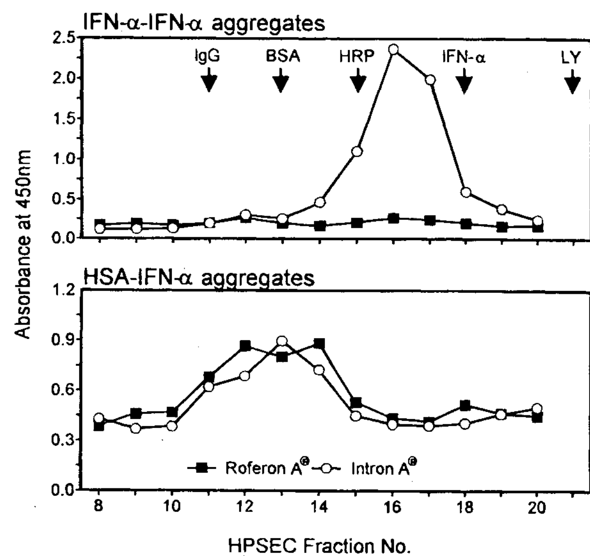

Fig. 6. HPSEC analysis of marketed IFN-α products. Freshly reconstituted Roferon A® and Intron A® were subjected to HPLC size exclusion chromatography and 0.5 ml fractions were collected. Fractions were analyzed for the presence of IFN-α-IFN-α and HSA-IFN-α aggregates by ELISA. The column was pre-calibrated with the indicated marker proteins.

Table I. Characteristics and Formulations of the Analyzed Marketed IFN-α Products[a]

	Recombinant			Non-recombinant
Product name	Berofor®	Intron A®	Roferon A®	Welferon®
Source	E. coli	E. coli	E. coli	Cell culture supernatant
Number of subtypes	1 (IFN-α2c)	1 (IFN-α2b)	1 (IFN-α2a)	≈22
Presentation	Lyophilisate	Lyophilisate	Lyophilisate	Liquid
Interferon	6 μg	3 MIU (15 μg)	3 MIU	3 MIU
Human-Albumin	~20 mg/ml[b]	1 mg/ml	5 mg/ml	1.5 mg/ml
Other excipients (per ml)		2.27 mg Na_2HPO_4 0.55 mg NaH_2PO_4 20 mg Glycine	9 mg NaCl	Glycine Tris Saline

[a] The exact composition of some of the formulations was not available.
[b] Determined by a BioRad total protein assay.

may be present in formulations, for example, IFN-α bound to small soluble HSA aggregates which can form under certain conditions (15,16). The presence of HSA-IFN-α aggregates with an IFN-α to HSA ratio of >1 is not very likely, since (a) These types of aggregates would probably be detected by the IFN-α-IFN-α aggregate ELISA and (b) HSA is present about 100–500 times in excess of the tested formulations.

The reason for the relatively high amount of HSA-IFN-α aggregates in Wellferon compared to other products is not obvious. However, Wellferon is the only liquid formulation and the only IFN-α product that contains a mixture of IFN-α subtypes. Thus it seems possible that either liquid formulations favour HSA-IFN-α aggregate formation or that all or some of the IFN-α subtypes present in Wellferon have a higher tendency to interact with HSA.

Factors Affecting IFN-α Aggregation in Test Formulations

To identify some parameters that influence IFN-α aggregation in marketed products, IFN-α-2a test-formulations were prepared that varied in pH (3.5, 5.5, and 7.5), constituents (HSA or saccharose), production process (lyophilisation or liquid formulation), storage temperature (−80°C, 4°C, and 30°C with 75% relative humidity), and storage time (0, 1 and, 3 months).

Total IFN-α recovery and formation of both IFN-α-IFN-α and HSA-IFN-α aggregates were affected by all tested parameters, although to varying extents. Recovery of IFN-α was higher from HSA- than from saccharose-containing formulations and was better from lyophilisates than from liquid formulations. Lower storage temperatures or shorter storage times also yielded better IFN-α recoveries. Loss of IFN-α in saccharose formulations was so significant that they could not be analyzed further for the presence of aggregates (data not shown). In contrast, HSA-containing formulations stabilized IFN-α much better. In these formulations small amounts of IFN-α-IFN-α and of HSA-IFN-α aggregates were already formed during preparation (Fig. 7). Freezing to −80°C did not induce significant additional aggregation (not shown). However, upon lyophilisation and, much more pronounced after storage of the lyophilisates for 1 month at 30°C, both types of aggregates increased significantly in a pH dependent manner. IFN-α-IFN-α aggregates peaked at pH 5.5. This could be due to the pH of IFN-α2a of about 5.7, which might result in a decreased solubility of IFN-α at that pH and hence could facilitate IFN-α aggregation during lyophilization. Similarly, human growth hormone (hGH) was found to aggregate during lyophilisation (Eckhardt *et al.*, 1991). The tremendous increase in HSA-IFN-α aggregates at pH 7.5 in lyophilisates might be attributed to the formation of covalent bonds between HSA and IFN-α by certain chemical reactions that have a pH optimum in that range. The exact nature of the chemical bonds in these aggregates is not yet clear. At least two different types seem to be present, since only a part of the aggregates was sensitive to reduction in immunoblotting studies (data not shown). Moreover, the chemical bonds formed and the size of aggregates in formulations may vary depending on IFN-α subtype, formulation constituents, and/or production process used.

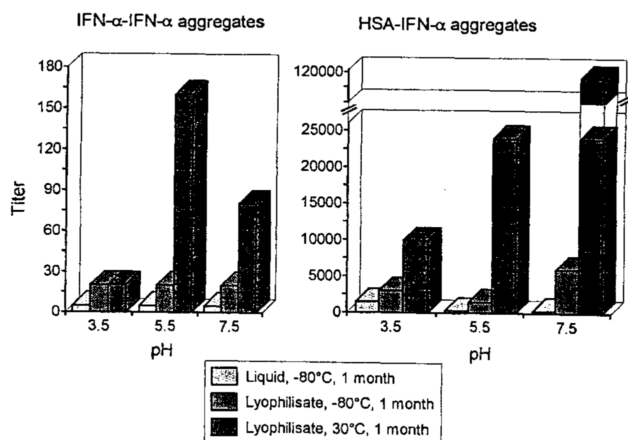

Fig. 7. Identification of factors affecting IFN-α aggregate formation in formulations. Interferon alpha (IFN-α2a, 18 MIU/ml) was formulated in 10 mM phosphate buffered saline, pH 3.5, 5.5 or 7.5 containing 1% benzyl alcohol as preservative and 0.5% human serum albumin. Liquid formulations were either directly frozen at −80°C or were lyophilized and stored at −80°C or 30°C. After storage for 1 month, lyophilisates were reconstituted and all samples were analyzed for the presence of IFN-α-IFN-α and HSA-IFN-α aggregates by ELISA.

CONCLUSIONS

The ELISAs for the detection of IFN-α-IFN-α and HSA-IFN-α aggregates are easy to perform, highly specific, work in the presence of large quantities of IFN-α monomers or of HSA and do not require a pretreatment of samples that might cause modifications in analyzed samples. The ELISAs are more sensitive than other commonly used assays for the detection of aggregates and allow the detection of IFN-α or HSA-IFN-α aggregates down to 1–2 ng. The principles of the ELISAs may be easily adapted to other proteins and may aid in developing similar aggregate assays for other therapeutic proteins in development or clinical use.

To present, the ELISAs have been used exclusively for the detection of aggregates in formulations and for comparative studies. For quantification, IFN-α-IFN-α and HSA-IFN-α aggregate standards identical to those present in formulations will have to be established. In addition, it has to be tested further to see if LI-1 has the same affinity for all types of aggregates that may form in formulations—although at present no differences have been observed in its binding to aggregates in IFN-α formulations, to glutaraldehyde cross-linked aggregates, or to a number of IFN-α subtypes.

The ELISAs were found to be very helpful in the detection of small, soluble aggregates which are believed to preceed the formation of larger insoluble aggregates. In test formulations, it was found that the final content of both types of IFN-α aggregates depends on a number of different parameters, such as formulation constituents, the lyophilisation process, the storage time, or the storage temperature. Therefore, the ELISAs should be able to detect the formation of aggregates in a very early phase of the production or formulation phase of IFN-α, long before they become detectable in HPSEC or SDS-PAGE analysis. This may help to identify, validate, and prevent aggregate-inducing steps early during formulation development. Although these small amounts of aggregates (<1%) will not significantly affect the clinical efficacy of the products, they might contribute to immunological reactions. They represent modified IFN-α molecules which could be recognized by the immune system as foreign molecules and elicit an antibody response to IFN-α.

This hypothesis is currently under investigation and data will be presented in a subsequent publication.

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