# Oxidation, aggregation and immunogenicity of therapeutic proteins

Torosantucci, R.

# Chapter 7

## Immune mechanisms underlying immunogenicity of aggregated recombinant human interferon alpha-2a in immune tolerant mice

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## Abstract

**Purpose.** To investigate the immune mechanism responsible for the immunogenicity of aggregated recombinant human interferon alpha-2a (rhIFNα) and to study if the presence of aggregated rhIFNβ increases rhIFNα's immunogenicity.

**Methods.** Transgenic mice immune tolerant for human interferon alpha were treated with native or aggregated rhIFNα. After a washout, the mice were rechallenged with aggregated or native rhIFNα to study immunological memory. Mice depleted from $CD4^+$ T-cells were used to test for $CD4^+$ T-cell involvement in immunogenicity. Furthermore, the mice were treated with a formulation containing aggregated rhIFNβ and native rhIFNα to test whether aggregated rhIFNβ acts as an adjuvant for rhIFNα.

**Results.** Native rhIFNα did not elicit antibodies, demonstrating the immune tolerant status of the transgenic mice. Aggregated rhIFNα was immunogenic in immune tolerant mice but did not induce immunological memory. Blocking $CD4^+$ T-cells abolished the antibody response against aggregated rhIFNα. Aggregated rhIFNβ did not increase immunogenicity of native rhIFNα.

**Conclusions.** An atypical immune response with T-cell dependent and T-cell independent characteristics appears to be involved in the formation of antibodies elicited by aggregated rhIFNα, in line with previous results obtained with rhIFNβ. Moreover, highly aggregated rhIFNβ does not act as adjuvant for native rhIFNα.

## Introduction

The number of recombinant human protein drugs entering the market is expanding greatly. Due to their intrinsic low toxicity and high versatility they are excellent drugs to treat various diseases [1]. Nonetheless, protein drugs do have a major disadvantage, namely immunogenicity [2], i.e., during treatment some patients will form anti-drug antibodies (ADAs). Those ADAs can interfere with kinetics, compromise efficacy, lead to infusion reactions and even cause life-threatening side-effects [3-5]. So, immunogenicity poses a serious health risk and increases costs of therapy.

In order to lower immunogenicity, either by developing products with minimal immunogenicity, by adjusting treatment regimen, or by identifying patients at risk, more insight into the underlying immune mechanisms is needed. Immune tolerant mouse models have proven to be very suitable to assess which product-related characteristics, such as aggregates, lead to ADA formation and to study the immune processes responsible for this [6-9]. We have previously shown that for highly aggregated recombinant human interferon beta-1b (rhIFNβ, Betaferon®) the immune response leading to ADA formation shares characteristics of both a T-cell independent response, i.e., involvement of marginal zone B-cells and no apparent immunological memory formation, and characteristics of a T-cell dependent immune response, i.e., involvement of $CD4^+$ T-cells [10].

The primary aim of this study was to determine if such an atypical immune response would also be responsible for the ADA formation against another highly aggregated and immunogenic protein, metal catalyzed oxidized recombinant human interferon alpha-2a (rhIFNα). We therefore performed a set of experiments in an immune tolerant mouse model for human interferon alpha-2a, in which we studied the presence of an immunological memory response upon rechallenge with either native or aggregated rhIFNα and the involvement of $CD4^+$ T-cells in the formation of ADAs. In addition, we studied whether immunogenic, highly aggregated rhIFNβ, when mixed with native rhIFNα, would induce or enhance an ADA response against native rhIFNα.

## Materials and Methods

### Materials

RhIFNα was provided as liquid formulation (1.5 mg/mL in 25 mM ammonium acetate, 120 mM NaCl, acetic acid, pH 5) by Hoffmann-La Roche (Basel, Switzerland). Betaferon® (Bayer Schering Pharma AG, Berlin, Germany) was reconstituted with 0.54% NaCl, according to the instructions in the package insert.

Tris, glycine, SDS, L-ascorbic acid, copper (II) chloride, disodium hydrogen phosphate, hydrogen peroxide and EDTA were purchased from Sigma–Aldrich, Schnelldorf, Germany. Slide-A-Lyzer dialysis cassettes, molecular weight cut-off 10 kDa, were purchased from Thermo Fisher Scientific, Breda, the Netherlands. All chemicals were of analytical grade and used without further purification. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, Marlow, UK) prior to use. Lithium heparin gel tubes were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, the Netherlands).

### Preparation of native and aggregated rhIFNα formulations

RhIFNα, as received from the provider, was dialyzed against 10 mM sodium phosphate buffer, pH 7.4 (PB). Aggregation of rhIFNα was induced by metal catalyzed oxidation (hereafter refer to as aggregated rhIFNα) [11], performed after dilution of rhIFNα to 0.3 mg/mL, and by subsequent incubation with 40 μM $CuCl_2$ (previously prepared in 400 μM stock solution in PB) for 10 minutes. Subsequently, ascorbate (previously prepared in 400 mM stock solution in PB) was added to a final concentration of 4 mM. After three hours the reaction was quenched by adding 100 mM EDTA (previously dissolved in PB) to a final concentration of 1 mM, as previously reported [12-13]. The aggregated samples were dialyzed using 10 kDa cut-off dialysis cassettes against PB for 24 hours. Both formulations were subsequently characterized for protein concentration and structure (see below).

**Mixture of rhIFNα and Betaferon®**

The mixture of native rhIFNα with Betaferon® was obtained by mixing 0.66 mL of rhIFNα (0.15 mg/mL in PB) with 0.33 mL of Betaferon® 0.15 mg/mL, yielding final concentrations of 0.1 mg/mL of rhIFNα and 0.05 mg/mL rhIFNβ, pH 7.0. The formulation was subsequently analyzed by SEC (size exclusion chromatography) and SDS-PAGE (sodium dodecyl sulfate epolyacrylamide) to verify the non-aggregated state of rhIFNα in the mixture.

**Analytical characterization of the formulations**

*UV/VIS absorption spectroscopy*

UV/VIS absorption measurements were performed with an Agilent 8453 UV/VIS spectrophotometer (Waldbronn, Germany), which included a Peltier element for temperature control and a magnetic stirrer. The Peltier element was stirred by an Agilent 89090A controller. Quartz cells with a path length of 1 cm were used for all measurements. Scans were taken from 200-900 nm with 1 nm intervals. The protein concentration was determined by measuring the absorbance at 278.5 nm, using an extinction coefficient (0.1 %; 1 cm; 278.5 nm) of 1.06 [14].

*Far-UV circular dichroism (far-UV CD) spectroscopy*

Far-UV CD spectra were recorded from 190 to 250 nm using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1-mm path length quartz cuvette at 20 °C using a scan rate of 100 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of 6 repeated scans, background corrected with the corresponding buffer spectrum. The CD signals were converted to molar differential extinction coefficient, Δε.

*Intrinsic steady state fluorescence*

Intrinsic fluorescence was measured in 96-well plates using the plate reader unit of the FS920 fluorescence spectrometer (Edinburgh Instruments, Livingston , UK). All the formulations were diluted to a protein concentration of 0.1 mg/ml to avoid inner filter effects. The concentration after dilution was

confirmed with a BCA assay. Tryptophan residues were selectively excited at 295 nm. Emission spectra were recorded from 305 to 500 nm using a step size of 2 nm, gain of 115, Z-position of 20 mm, number of flashes 100 with a frequency of 400 Hz. Triplicates for each sample were analyzed.

*Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

Acrylamide gradient gels (4-20%, tris-HCl), were run under reducing (sample buffer containing 5% (v/v) β-mercaptoethanol) and non-reducing (sample buffer without β-mercaptoethanol) conditions at room temperature. The electrophoresis buffer was 25 mM tris(hydroxymethyl)aminomethane, 192 mM glycine, and 0.1% (w/v) SDS. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, the Netherlands). Samples analyzed under reducing conditions were boiled for 2 min before application to the gel to favor the reduction of disulfide bonds. A low-range molecular weight standard (Biorad) was included on the gel for determination of molecular weight.

*Western blotting*

Protein bands in SDS-PAGE gels were blotted onto a polyvinylidene difluoride immuno blotting membrane with a mini trans-blot electrophoretic transfer cell (Biorad, Veenendaal, the Netherlands). Blots were blocked for 2 hours at room temperature with 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) with constant orbital shaking. After washing with 0.1% (v/v) Tween 20 in PBS and with water, the blots were incubated with polyclonal rabbit anti-hIFNα (PBL Biomedical Laboratories, Piscataway, NJ, US) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water and further incubated with peroxidase goat anti-rabbit IgG (Sigma-Aldrich) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Afterwards blots were again washed with 0.1% (v/v) Tween 20 in PBS and with water, and then incubated in a solution of 4-chloro-1-naphtol (Sigma- Aldrich) in methanol (20% (v/v)), water, and $H_2O_2$ (0.015%

(v/v)). After color development the blots were stored overnight to increase the intensity of the bands.

*Size-exclusion chromatography (SEC)*

Samples (100 µg protein/mL) were analyzed by SEC with a TSK-GEL 3000 column (TOSOH BIOSCIENCE GmbH, Stuttgart, Germany), using a mobile phase of 50 mM sodium phosphate and 200 mM sodium chloride, pH 7.2, filtered through a 0.2-µm filter prior to use, at a flow rate of 0.50 mL/min by a Waters 2695 controller equipped with an autosampler and a Waters 2996 photodiode array detector (Waters, Milford, MA, USA). The column was calibrated by analyzing protein molecular weight standards obtained from Sigma-Aldrich (i.e., thyroglobulin, BSA, ovalbumin, α-chymotrypsin, and myoglobin).

**Mouse studies**

*Breeding*

Heterozygous FVB/N transgenic (TG) mice immune tolerant for hIFNα and their non-transgenic (NTG) littermates were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). The genotype of the offspring was determined by PCR showing the presence or absence of the hIFNα gene in chromosomal DNA isolated from ear tissue. Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available *ad libitum,* and all animal experiments were performed according to Institutional Ethical Committee Regulations of Utrecht University, the Netherlands.

*Immunogenicity and immunological memory response*

TG and NTG mice were injected intraperitoneally (i.p.) with 10 µg of native rhIFNα or aggregated rhIFNα (n= 24 per group) for 5 days per week during 3 consecutive weeks (days 0-4, days 7-11 and days 14-18). After an injection-free period of 6 weeks, half of the TG and NTG mice (n=12) were rechallenged with 10 µg native rhIFNα and the other half (n=12) were rechallenged with 10 µg aggregated rhIFNα on 2 consecutive days (days 63 and 64). Blood was collected via cheek puncture before the start of the experiment, on different

days during the 3 treatment weeks (days 7, 11, 14 and 18), during washout (days 21, 28 and 42) and before rechallenge (days 56, 58 and 60). At each blood sampling time point, blood was collected before injection to prevent interference of ADA-drug complexes in antibody determination, and was taken from 7 out of 24 mice per group per time point to prevent oversampling. At 13 days after rechallenge (day 77) all mice were sacrificed and blood was isolated. Plasma was obtained from blood after centrifugation in lithium heparin tubes (3000 g, 10 min) and stored at -20 °C until further analysis.

*Involvement of CD4⁺ T-cells in the formation of ADAs*

TG and NTG mice (n=18 per group) were treated with 10 µg aggregated rhIFNα or 5 µg ovalbumin adsorbed to aluminum hydroxide gel (Sigma-Aldrich BV, Zwijndrecht, the Netherlands) in 100 µl PBS (ova) for 5 days per week during 3 consecutive weeks (days 0-4, days 7-11 and days 14-18). Ova is a T-cell dependent antigen and served as control for a CD4⁺ T-cell immune response. As control for a T-cell independent immune response, TG and NTG mice were treated with Pneumovax® (n=18 per group), according to Scheikl and colleagues [15]. Primary i.p. immunization was performed with 1 µg of Pneumovax® in 100 µl PBS on day 0, followed by a second i.p. injection with another 1 µg of Pneumovax® on day 11.

To study involvement of the CD4⁺ T-cells, half of the mice (n=9 per group) received 3 i.p. injections of rat anti-CD4 antibody GK 1.5 (150 µg in 100 µl PBS) (Bioceros, Utrecht, the Netherlands) before the start of treatment. Depletion was maintained by administration of 150 µg GK 1.5 every 3 to 4 days during the 3 treatment weeks. Blood was collected via cheek puncture before the start of the experiment and during treatment (days 9 and 16) from 3 or 6 mice out of 9. On day 23 all mice were sacrificed and blood was isolated. During the experiment, 2 TG and 2 NTG mice died due to fighting. Numbers of remaining mice can be found in Figure 5.

To confirm depletion of CD4⁺ T cells, additional groups of mice (n=22) were treated with GK 1.5 or PBS following the same procedure as described before. During every treatment week, some of these mice were sacrificed and single-cell suspensions of spleens were tested by flow cytometry for depletion by a non-competing anti-CD4 monoclonal RM4-4 antibody (BD Bioscience, the Netherlands). Measurements were taken using a FACSCanto II® (BD

Bioscience) and analysis was performed with the FACSDiva software v6.1.1 (BD Bioscience). Depletion efficiency was on average >95% throughout the experiment (data not shown).

*Immunogenicity of rhIFNα when co-administered with Betaferon®*

TG and NTG mice were injected i.p. with 10 µg of (i) native rhIFNα, (ii) aggregated rhIFNα, or (iii) the mixture of native rhIFNα and Betaferon® (n=6 per group) for 3 weeks, 5 days per week (days 0-4, days 7-11 and days 14-18). Blood was drawn via cheek puncture from 3 out of 6 mice per time point to prevent oversampling, before the start of the experiment and on days 9 and 14. On day 21, all mice were sacrificed and blood was isolated. Plasma was obtained after centrifugation in lithium heparin tubes (3000 g, 10 min) and stored at -20°C until further analysis.

*Determination of antibody titers by enzyme-linked immunosorbent assay (ELISA)*

Plasma was analyzed for anti-rhIFNα antibodies, anti-rhIFNβ antibodies (Betaferon®) and anti-ova antibodies by ELISA, as described in detail by Hermeling et al [7, 16]. In the case of Pneumovax®, anti-pneumococcal polysaccharide antibodies were detected by the following method. Mouse plasma was preadsorbed to pneumococcal cell wall polysaccharide (CWPS) antigens to capture non-specific antibodies against CWPS, a known impurity of the Pneumovax® vaccine [15]. In brief, mouse plasma was diluted 1:100 in PBS, mixed with 2 µg of CWPS and the mixture was incubated for 30 min at room temperature. Adsorbed samples (100 µl) were added to 0.05 µg/well Pneumovax® coated microtiter plates, which were then incubated for two hours followed by incubation with primary antibody and secondary antibody. After each step plates were washed 3 times with washing buffer (0.05 % (w/v) Tween 20 in PBS). After adding the secondary antibody a color reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine (Roche, Almere, the Netherlands) and stopped after 10 minute incubation time by adding 0.18 M sulfuric acid. Optical density values for all ELISAs were measured at 450 nm wavelength with a microplate reader (Novopath; Biorad, the Netherlands) and data analysis was performed with GraphPad Prism 4.03 software (San Diego,

CA, USA). Samples were defined positive if their mean absorbance values were at least three times higher than the 95th percentile value of negative control plasma. OD450-log dilution plots were fitted to a sigmoidal dose-response curve and the reciprocal of the dilution corresponding to 50% of the maximal signal (EC50 value) was defined as the titer. Negative samples were assigned a titer of 0, and were taken along in data-analyses.

*Statistics*

For the immunological memory experiment, differences in anti-rhIFNα antibody titers between initial treatments were assessed using a nonparametric Kruskal-Wallis test. The same test was used to assess difference in ADAs before and after rechallenge. First an overall effect of time (before and after rechallenge) was assessed. If this overall effect was significant, further tests were performed to specify which groups differed. For the $CD4^+$ T-cell depletion study and co-administration of native rhIFNα and Betaferon® experiment also a nonparametric Kruskal-Wallis test was used to assess statistical difference in antibody titers between groups. For all comparisons an overall effect of treatment (all time points combined) was determined; if statistically different, further statistical testing was performed to determine differences in titers per time point. A p value $\leq 0.05$ was considered significant.

## Results and Discussion

### Physicochemical characterization of native rhIFNα, aggregated rhIFNα and the rhIFNα and Betaferon® mixture

Two batches of native rhIFNα and aggregated rhIFNα were prepared. Reported analytical data represent the average ± lower/upper value for the two batches and are summarized in Table I. Our results are in fair agreement with those reported for rhIFN alpha-2b (which contains Arg in position 23 instead of Lys, as in rhIFN alpha-2a used in the present study) by Hermeling et al. [16] and are briefly discussed below.

The mixture of native rhIFNα with Betaferon® was prepared in duplicate and analyzed by SEC and SDS-PAGE to verify the monomeric nature of rhIFNα.

*Characteristics of native and aggregated rhIFNα*

The chromatographic behavior of the aggregated protein indicated a substantial reduction in the monomer content, next to a large fraction of higher molecular weight species, when compared to native rhIFNα, which contained 99% of monomeric protein besides a small amount of dimer according to SEC (Table I). SDS-PAGE showed an apparent molecular weight of about 17 kDa under reducing and non-reducing conditions for native rhIFNα, whereas aggregated rhIFNα was characterized by the presence of higher molecular weight species, which appeared to be mainly mediated by non-reducible covalent bonds (Figure 1, panel a and b). Western blotting indicated that that the aggregated protein still contained native epitopes (Figure 1, panel c).

**Table I.** Summary of SEC results and spectroscopic features of rhIFNα and its aggregated form.

| Sample | SEC | | | UV | | Far-UV CD | | | Fluorescence maximum | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Monomer[1] (soluble fraction) | Oligomers[2] (soluble fraction) | Insoluble fraction[3] | OD ratio 280/260 nm | OD at 350 nm | 208 nm (Δε) | 222 nm (Δε) | Δε ratio 208/222 nm | λ nm | Intensity (a.u.) |
| Native rhIFNα | 99.0 ± 0.6 | 1.1 ± 0.6 | 0 | 1.85 ± 0.03 | 0.006 ± 0.002 | -5.34 ± 0.97 | -5.03 ± 0.96 | -1.06 ± 0.01 | 326 ± 1 | 19.7 ± 0.7 |
| Aggregated rhIFNα | 55.3 ± 8.5 | 44.7 ± 8.5 | 0 | 1.10 ± 0.15 | 0.22 ± 0.07 | -3.12 ± 1.28 | -2.89 ± 1.16 | -1.08 ± 0.01 | 330 ± 1 | 7.9 ± 1.3 |

[1] Percentages were calculated based on SEC peak areas relative to the total peak area of native rhIFNα: $AUC_{peak}/AUC_{native,\ total} \times 100$.

[2] For the aggregated protein due to the broad overlapping peaks, an exact estimation of the molecular weight of the multimers was not achievable.

[3] The insoluble fraction was defined as the fraction that was not recovered by SEC; percentages were calculated from the total peak area in SEC and the total peak area of native rhIFNα: $(AUC_{native,\ total} - AUC_{sample,\ total})/AUC_{native,\ total} \times 100$.

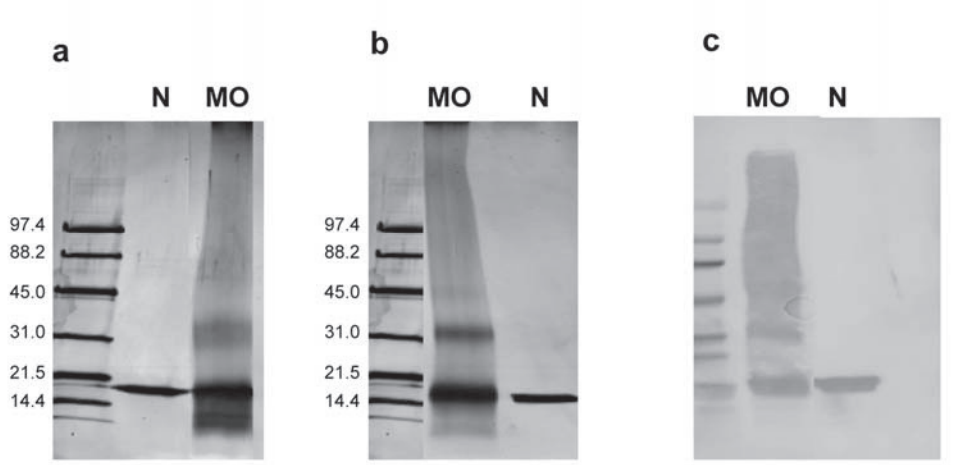


**Figure 1**. SDS-PAGE of native (N) and aggregated by metal catalyzed oxidation (MO) rhIFNα-2a under (a) reducing conditions and (b) non-reducing conditions, and (c) Western Blotting obtained from a gel run under non-reducing conditions. Numbers on the left indicate molecular weight (kDa) of the markers.

UV spectra of aggregated rhIFNα showed a decrease in the A280/A260 ratio, besides an increase in the optical density at 350 nm, providing further evidence of the presence of aggregates in the stressed formulation via metal catalysis [17]. Intrinsic steady state fluorescence suggested a more hydrophilic environment of the tryptophan residues in the stressed sample, where a lower intensity and a red-shifted spectrum (emission maximum 330 ± 1 nm), was recorded in comparison to the native protein (emission maximum 326 ± 1 nm). Far-UV CD measurements pointed to a decrease in α-helical content for aggregated rhIFNα but no major changes towards other secondary structures, since no statistical difference between the 208/222 nm intensity ratio of native and stressed rhIFNα was measured (Table I).

*Characteristics of the rhIFNα and Betaferon® mixture*

The association state of rhIFNα in the formulation containing rhIFNα and Betaferon® was investigated by SEC. The chromatograms show that rhIFNα was maintained in its monomeric state (Figure 2). Furthermore, no changes in the soluble fraction of Betaferon® were observed before and after the addition of rhIFNα (Figure 2), highlighting that mixing of rhIFNα with rhIFNβ did not lead to major changes in the aggregation status of either of the two proteins.

Using SDS-PAGE, it was confirmed that rhIFNα recovered from the rhIFNα and Betaferon® mixture, was indeed in its monomeric non-aggregated state, as demonstrated by the detection of a single band with the same molecular weight of native rhIFNα (data not shown). Similarly, Betaferon® alone and in combination with rhIFNα showed similar bands. Based on Western blotting, native rhIFNα and rhIFNα recovered from the combined rhIFNα and Betaferon® solution (not shown) contained intact epitopes.

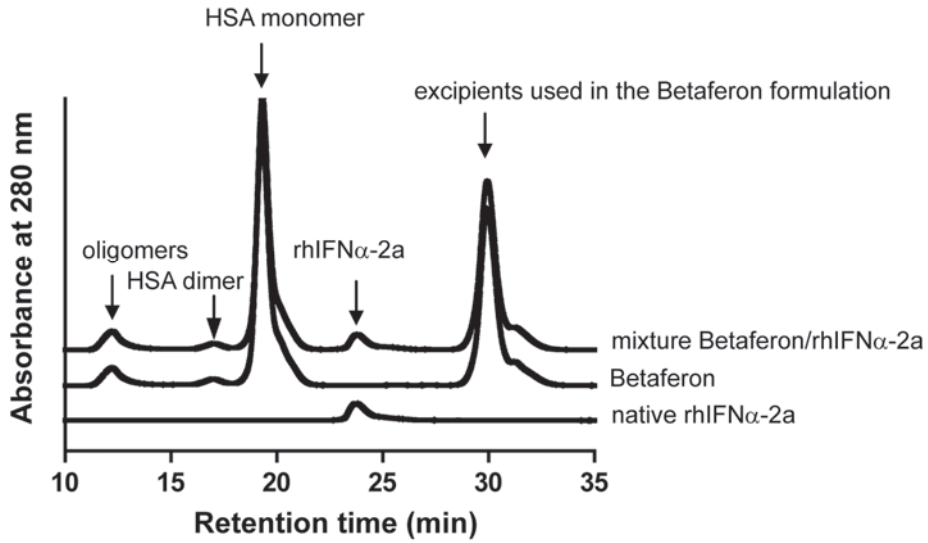


**Figure 2.** SEC of native rhIFNα, Betaferon and the mixture Betaferon/rhIFNα. The identity of the peaks from left to right is: oligomers, HSA dimer, HSA monomer, rhIFNα-2a, excipients used in the Betaferon formulation. Note that no rhIFNβ peak could be identified, probably because the protein is highly aggregated [18].

## Mouse studies

*Immunogenicity of native and aggregated rhIFNα*

TG and NTG mice were treated with either native rhIFNα or aggregated rhIFNα for 3 weeks followed by a treatment-free period of 6 weeks (washout). Native rhIFNα caused an ADA response in all NTG mice (Figure 3a), while it was very poorly immunogenic in the TG mice (2 out of 24 mice had detectable antibody levels, each at only 2 time points (t= 18 and t=42 days), illustrating the immune tolerant status of these mice towards rhIFNα. Since the two TG mice with detectable antibody levels displayed similar antibody responses as the NTG mice at only two time points, the antibody titers were considered statistical outliers and these two mice were excluded from further analyses.

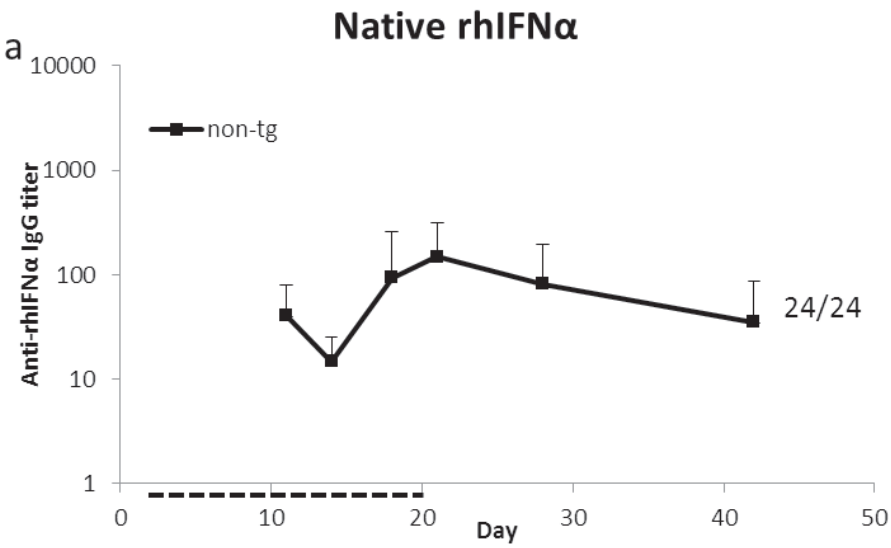


**Figure 3a.** Anti-rhIFNα IgG titers of NTG (non-tg, black lines) mice treated with native rhIFNα during the initial three treatment weeks (dashed line below x-axis) and washout period. Two out of 24 TG mice treated with native rhIFNα showed detectable antibody titers on days 18 and 42, these mice were considered outliers and are not presented. The number of antibody positive mice out of the total number of mice per group is given. Data represent mean + standard deviation per time point (n= 24).

In contrast, aggregated rhIFNα caused an antibody response in both the NTG (22 out of 24 responders) and TG mice (21 out of 24 responders) (Figure 3b). For the NTG mice, treatment with aggregated rhIFNα gave higher titers than treatment with native rhIFNα (p=0.002, Figure 3).

These results show that native rhIFNα is poorly immunogenic in the TG mice and that by aggregation immunogenicity is strongly enhanced. This corresponds to our previous findings with recombinant human interferon alpha-2b [16, 19], and to our recent results with human monoclonal IgG [8], showing that metal catalyzed oxidation of therapeutic proteins results in aggregation and enhanced immunogenicity.

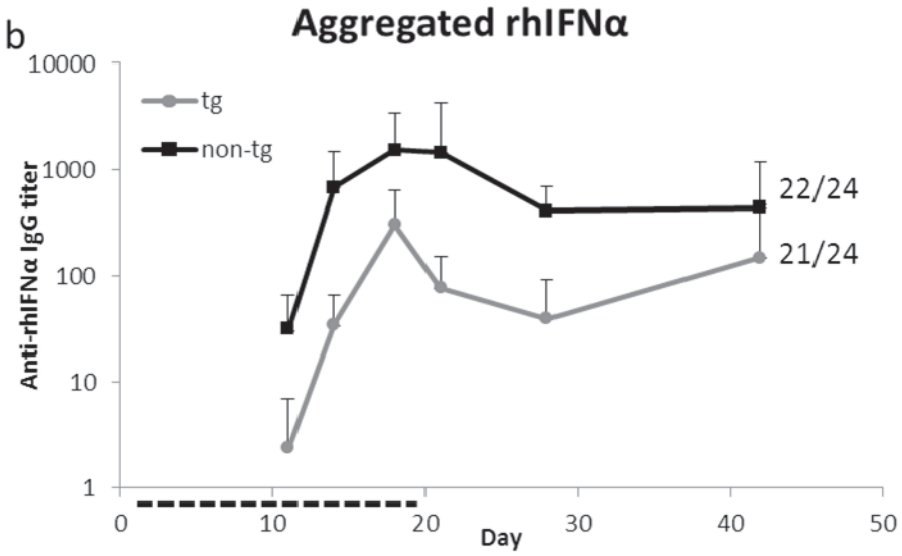


**Figure 3b.** Anti-rhIFNα IgG titers of TG (tg, grey lines) and NTG (non-tg, black lines) mice treated with aggregated rhIFNα during the initial three treatment weeks (dashed line below x-axis) and washout period. The number of antibody positive mice out of the total number of mice per group is given. Data represent mean + standard deviation per time point (n= 24).

*Immunological memory response*

NTG mice displayed low antibody titers before rechallenge independent of the initial treatment, while TG mice had detectable titers before rechallenge only when initially treated with aggregated rhIFNα (Figure 4). NTG mice initially treated with native rhIFNα showed increased antibody levels after rechallenge with native and aggregated rhIFNα (overall effect, p=0.038, Figure 4, panel a). This was most apparent in NTG mice initially treated and rechallenged with native rhIFNα, (p<0.001). NTG mice initially treated with aggregated rhIFNα had higher antibody titers after rechallenge with both rhIFNα products (overall effect p<0.001, Figure 4, panel b); this effect was most pronounced in mice rechallenged with aggregated rhIFNα (p=0.004). In TG mice initially treated with aggregated rhIFNα, no statistically significant increase in antibody titers was present upon rechallenge with native or aggregated rhIFNα (overall effect, p=0.077, Figure 4, panel b).

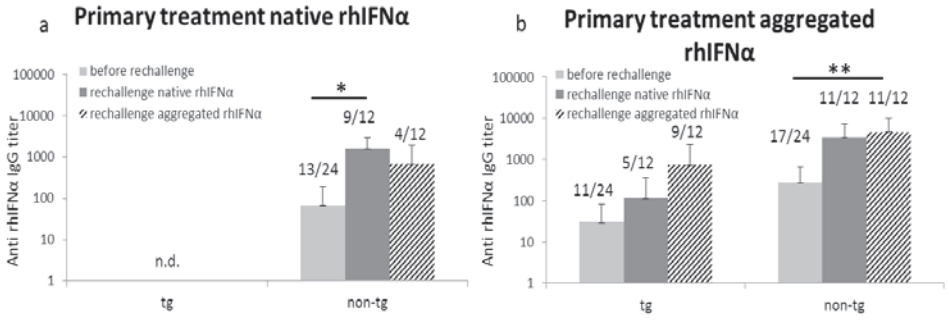


**Figure 4.** Anti-rhIFNα IgG titers of TG (tg) and NTG (non-tg) mice receiving primary treatment with native rhIFNα (a) and aggregated rhIFNα (b). Bars show titers before rechallenge (light grey) and after rechallenge with native rhIFNα (dark grey) or with aggregated rhIFNα (striped). Data represent mean + standard deviation. Above each bar the number of antibody positive mice out of the total number of mice per group is given. * indicates p<0.05. ** indicates p<0.01. n.d.=not detectable.

These results suggest that for NTG mice a memory response is present when either native or aggregated rhIFNα is given as primary treatment, and when either of the two products is given during rechallenge. Interestingly, antibody titers after rechallenge appear to be higher when the same product is given during initial treatment and rechallenge. This indicates that there is some

level of specificity in the immunological memory response and that there are distinct features in native or aggregated rhIFNα responsible for this specificity. This could be due to differences in exposed epitopes between aggregated and native rhIFNα and/or to the formation of new epitopes in aggregated rhIFNα as a result of the oxidation; it was recently shown for insulin that the same oxidation mechanism used to create rhIFNα aggregates leads to numerous chemical changes and cross-links in insulin [13].

For the TG mice there was no statistically significant immunological memory response observed, although there is a trend towards increased antibody levels after rechallenge (Figure 4, panel b). This apparent lack of immunological memory is similar to our previous observations with rhIFNβ in immune tolerant mice for hIFNβ [10]. However, studies assessing the presence of memory T- and B-cells should be performed to provide more definite proof on the presence or absence of immunological memory formation.

*Involvement of CD4⁺ T-cells in the formation of ADAs*

To study whether CD4⁺ T-cells are involved in the antibody response against aggregated rhIFNα, TG and NTG mice depleted from their CD4⁺ T-cells were treated with aggregated rhIFNα (Figure 5, panel a). Control groups of TG and NTG mice were treated with ova (a T-cell dependent antigen, Figure 5, panel b) or Pneumovax® (a T-cell independent antigen, Figure 5, panel c). CD4⁺ T-cell depletion in both TG and NTG mice treated with aggregated rhIFNα resulted in an almost abolished antibody response (overall effect, $p=0.009$ for TG and $p=0.003$ for NTG) with most apparent effects at the end of testing. Only 1 out of 8 NTG mice showed detectable antibody levels after depletion. As expected, CD4⁺ T-cell depletion also diminished antibody titers against the T-cell dependent antigen ova in both TG and NTG mice (overall effect $p<0.001$ for both), while the antibody response against the T-cell independent antigen Pneumovax® remained unaffected (overall effect $p=0.408$ for TG and $p=0.741$ for NTG).

These results indicate that the antibody response against aggregated rhIFNα is dependent on CD4⁺ T-cells. Together with the previous observation of an apparent lack of immunological memory, it appears that the immune mechanisms underlying immunogenicity of aggregated rhIFNα resemble the mechanisms underlying immunogenicity of Betaferon® [10]; both appear to lack

immunological memory and both involve CD4+ T-cells. However, it has to be noted that for the antibody response against Betaferon®, early involvement of marginal zone B-cell in particular led to the hypothesis of a T-cell independent trigger of immunogenicity, and of ancillary T-cell involvement in actual antibody production [10].

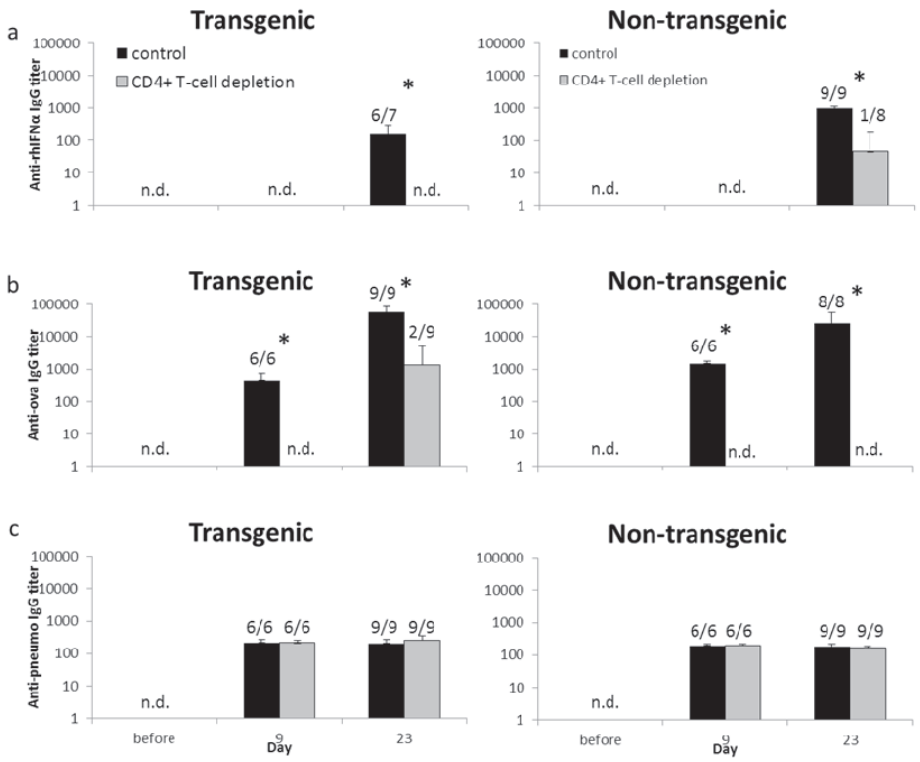


**Figure 5.** Total anti-rhIFNβ IgG titers (a), total anti-ova IgG titers (b) and total anti-pneumo IgG titers (c) of TG and NTG mice treated with aggregated rhIFNα, ovalbumin or Pneumovax®, respectively. Data represent mean + standard deviation per time point. Above each bar the number of antibody positive mice out of the total number of sampled mice per group is given. Black bars represent control mice with CD4+ T-cells, grey bars indicate mice depleted from CD4+ T-cells. * shows statistical difference in antibody titers between control mice and mice without CD4+ T-cells ($p<0.05$). n.d.= not detectable.

Nonetheless, this is the first study showing that the immune mechanisms underlying immunogenicity of two highly aggregated proteins might be comparable. Moreover, this and the previous study [10] indicate the importance of clinical studies looking into the immune mechanisms in humans involved in immunogenicity of therapeutic proteins. Sparse patient data on re-induction of treatment with interferon beta and monoclonal antibodies indicate that immunological memory might also be absent in patients [20], but this needs to be confirmed in larger patient cohorts.

*Immunogenicity of rhIFNα when co-administered with Betaferon®*

TG and NTG mice were treated with either native rhIFNα, aggregated rhIFNα or the combined rhIFNα and Betaferon® solution. Figure 6 shows the corresponding anti-rhIFNα IgG titers of the TG and NTG mice. For the TG mice, only aggregated rhIFNα was capable of inducing ADAs (overall effect $p<0.0001$, Figure 6a), which was most apparent on day 21. Native rhIFNα and the combined rhIFNα and Betaferon® solution did not cause detectable ADA formation in these mice.

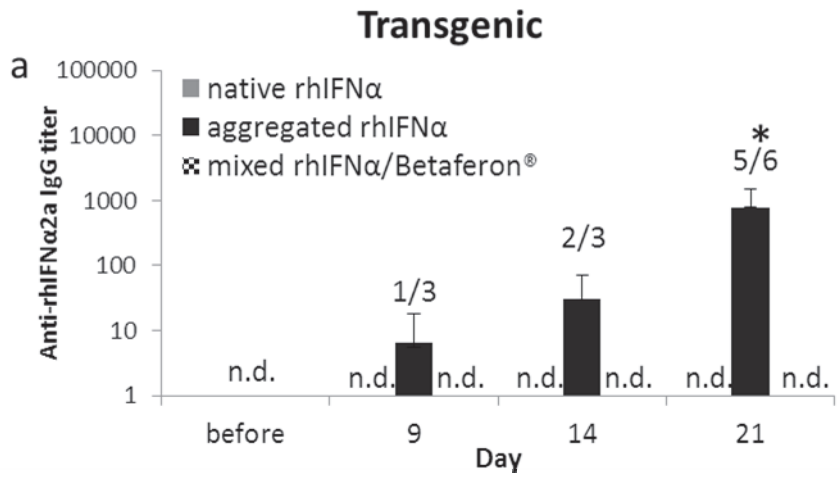


**Figure 6a.** Anti-rhIFNα IgG titers of TG mice treated with either native rhIFNα (grey bars), aggregated rhIFNα (black bars) or the mixture of rhIFNα/Betaferon® (black and white pattern). Above each bar the number of antibody positive mice out of the total number of sampled mice per group is given. Data represent mean + standard deviation per time point. * indicates $p<0.05$ between the three treatment groups. n.d = not detectable.

For the NTG mice (Figure 6b) ADAs were formed for all 3 treatments, however no differences in ADA titers between treatments were found (p=0.122). This confirms some of our previous studies [19] in which NTG mice, in contrast to TG mice, could not discriminate between poorly and highly immunogenic formulations.

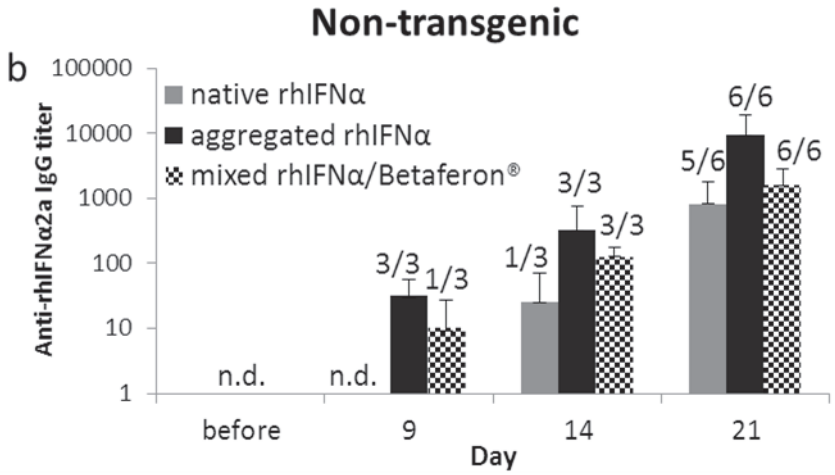


**Figure 6b.** Anti-rhIFNα IgG titers of NTG mice treated with either native rhIFNα (grey bars), aggregated rhIFNα (black bars) or the mixture of rhIFNα/Betaferon® (black and white pattern). Above each bar the number of antibody positive mice out of the total number of sampled mice per group is given. Data represent mean + standard deviation per time point. n.d = not detectable.

Both TG and NTG mice treated with combined rhIFNα and Betaferon® solution formed antibodies against rhIFNβ (data not shown), confirming that immune tolerance was specific for rhIFNα.

These findings show that Betaferon® does not trigger immunogenicity of rhIFNα, although it causes the formation of anti- rhIFNβ antibodies. It can therefore be concluded that activation of the immune pathways involved in immunogenicity of Betaferon® does not lower the threshold for immunogenicity of native rhIFNα. Betaferon® is therefore not functioning as adjuvant. It would be interesting to study if the lack of stimulatory effect by Betaferon® is also applicable to other therapeutic proteins, and if well-known

adjuvants are actually capable of enhancing immunogenicity of native rhIFNα and other non-immunogenic proteins in the immune tolerant mouse model.

In the current experiment we co-administered the two protein species in one formulation, however they were not chemically linked, as was confirmed by SEC (Figure 2). Several studies have shown that depending on the immunostimulatory molecules, linkage might be important in exerting their effect [21-22]. It might therefore be worth studying immunogenicity of rhIFNα when covalently bound to immunogenic rhIFNβ aggregates.

## Conclusion

The immune mechanisms underlying immunogenicity of aggregated rhIFNα resemble the immune mechanisms underlying immunogenicity of Betaferon®; an apparent lack of immunological memory and involvement of $CD4^+$ T-cells. Immunogenic and highly aggregated Betaferon® does not act as adjuvant for non-immunogenic rhIFNα.

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