Antibody Response to Aggregated Human Interferon Alpha2b in Wild-type and Transgenic Immune Tolerant Mice Depends on Type and Level of Aggregation

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ABSTRACT: The aim of this study was to determine the sensitivity of transgenic immune tolerant mice for the type and level of aggregation of recombinant human interferon alpha2b (rhIFNα2b). RhIFNα2b was aggregated by metal-catalyzed oxidation or by incubation at elevated temperature and various pHs. Native rhIFNα2b was mixed with oxidized rhIFNα2b at different ratios to obtain samples with different aggregation levels. The preparations were characterized by UV and fluorescence spectroscopy, gel permeation chromatography (GPC), dynamic light scattering (DLS), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting, and ELISA. The immunogenicity was evaluated in wild-type mice and transgenic mice immune tolerant for hIFNα2. Sera were analyzed by ELISA for the presence of rhIFNα2b-specific antibodies. The oxidized and aged preparations widely differed regarding the level and nature of aggregates. All preparations containing aggregates increased the immune response in the wild-type mice as compared to native rhIFNα2b and were able to break the tolerance of the transgenic mice. The more native-like the conformation of the aggregated proteins, the more immunogenic the preparations were in the transgenic mice. The native-like aggregates prepared via metal catalysis induced a dose-dependent loss of tolerance in the transgenic mice. In conclusion, the transgenic mouse model can be used to screen rhIFNα2b formulations for low levels of immunogenic aggregates obtained under accelerated storage conditions.

Keywords: immunology; interferon alpha2b; protein aggregation; protein structure; proteins; transgenic mice; immune tolerant

INTRODUCTION

Immunogenicity is being recognized as a potential, serious problem for the use of therapeutic proteins. The incidence and level of antibody formation against therapeutic proteins is dependent on a number of factors,1,2 including patient and protein characteristics, formulation and route of administration. The presence of aggregates in a protein formulation is an important factor known to increase the immunogenicity of a therapeutic protein.3–7
Prediction of immunogenicity of therapeutic proteins would be advantageous, but at present no fully validated predictive approaches are available. In conventional animals, in principle, all human proteins are foreign and will, therefore, induce a classical immune response. Mice transgenic for the gene of the human therapeutic protein share immune tolerance for this protein with humans. Therefore, repeated administration of the protein to immune tolerant transgenic mice will more closely resemble the patient situation, since the immunogenicity seen in patients is mostly due to breaking of tolerance.

Recombinant human interferon alpha2a/b (rhIFNα2) is a protein used for the treatment of a variety of malignancies and viral diseases. Aggregates of rhIFNα2a were shown to break the tolerance of transgenic mice, immune tolerant for hIFNα2. Recently, we showed that the characteristics of rhIFNα2b aggregates are crucial for the breaking of tolerance in these mice. In particular, it was demonstrated that not all aggregates of rhIFNα2b are able to break the tolerance of the transgenic mice.

Only aggregates consisting of rhIFNα2b with preserved conformation, obtained by metal catalyzed oxidation, were shown to break the tolerance of transgenic mice after intraperitoneal (i.p.) administration. The aggregates accompanying this oxidation, rather than the oxidized methionines, were shown to be responsible for breaking of the immune tolerance. The reason for the immunogenicity of the aggregates was probably their repetitive antigen presentation, since a spacing of 5–10 nm between more than 10 repeating epitopes is a potent way to induce high levels of antibodies, even to self proteins. Apparently, metal catalyzed oxidized rhIFNα2b meets these requirements.

To rapidly screen the stability of formulated proteins, accelerated stability studies under stress conditions, such as extreme pHs and temperatures, are often performed. Although these studies are not always predictive for long-term stability, the degradation products obtained are probably more relevant than artificially obtained structural variants, such as the degraded rhIFNα2b products previously reported. The purpose of the present work was to study the structure–immunogenicity relationship of more relevant degradation products and to gain more insight into the sensitivity of our mouse model to the level of aggregation. Therefore, metal catalyzed oxidized rhIFNα2b was mixed with native rhIFNα2b at different ratios and these preparations, as well as the samples incubated at elevated temperatures and different pHs, were evaluated for their level of aggregation, protein structure, and immunogenicity in both the wild-type and the transgenic mice.

MATERIALS AND METHODS

Degradation of rhIFNα2b

RhIFNα2b was a gift from AlfaWassermann, Italy. Oxidized rhIFNα2b was prepared as described previously. In short, rhIFNα2b (300 μg/mL in 10 mM sodium phosphate buffer (PB), pH 7.4) was oxidized in the presence of metal ions and ascorbic acid for 3 h at room temperature. The oxidation reaction was stopped by the addition of EDTA and the sample was dialyzed against 10 mM PB, pH 7.4. To obtain preparations with different levels of aggregates, native rhIFNα2b was mixed with oxidized rhIFNα2b in different ratios (preparation A, 1:0; B, 3:1; C, 1:1; D, 1:3, and E, 0:1 (v/v)). The total protein concentration was equal in all preparations.

Accelerated degradation products were obtained by incubating rhIFNα2b solutions (300 μg/mL) of pH 4.0 (10 mM sodium acetate buffer), pH 7.2 (10 mM PB), and pH 9.0 (10 mM Tris (hydroxymethyl) aminomethane) at 50°C. The ionic strength of the solutions was adjusted to 0.14 M with sodium chloride. Preliminary tests showed that after 2 weeks of incubation at pH 7.2 and 50°C, the protein was partly aggregated. However, after 2 weeks of incubation at pH 4.0 and 9.0, hardly any protein was detectable by gel permeation chromatography (GPC). Therefore, the solutions were incubated at 50°C for 1 week (pH 4.0 and 9.0) or 2 weeks (pH 7.2). After incubation, the solutions were dialyzed against 10 mM PB, pH 7.4 and stored at −20°C.

Characterization

Unless stated otherwise, all dilutions were made in 10 mM PB, pH 7.4. Protein concentrations were determined with a modified Lowry method with bovine serum albumin (BSA) (Sigma, Zwijndrecht, The Netherlands) as a standard.

UV-Spectroscopy

UV spectra (200–450 nm) of the samples (100 μg/mL) were recorded on a Perkin Elmer Lambda
2 UV/VIS spectrophotometer in 1 cm quartz cuvettes.

**Dynamic Light Scattering (DLS)**

Samples (100 µg/mL) were analyzed with DLS at an angle of 90° to obtain an average diameter of the particles (Z-ave) and a polydispersity index (PDI), which is a measure for the heterogeneity of the sample. A Malvern CGS-3 apparatus equipped with a He-Ne (633 nm) JDS Uniphase laser, an optical fiber based detector, and an ALV/LSE-5003 correlator was used.

**Gel Permeation Chromatography**

A Superdex 200 10/300 GL column (Amersham, Roosendaal, The Netherlands) was used to analyze the aggregate composition of the samples (100 µg/mL). The mobile phase consisted of 50 mM PB, pH 7.2, and 200 mM sodium chloride, was passed through a 0.2-µm filter, and was delivered to the column at a flow rate of 0.50 mL/min by a Waters (Etten-Leur, The Netherlands) 2695 controller equipped with an autosampler. Chromatograms were recorded with a multi wavelength fluorescence detector (model 2475, Waters) or a photodiode array detector (model 2996, Waters). The column was calibrated with protein standards obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The samples were filtered over a 0.2-µm cellulose acetate filter prior to analysis.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE)**

The gels consisted of a separating gel containing 15% (w/v) acrylamide and 0.1% (w/v) SDS, and a stacking gel containing 5% (w/v) acrylamide and 0.1% (w/v) SDS. Gels of 0.75 mm thickness were run under reducing (sample buffer containing 5% (v/v) β-mercaptoethanol) and non-reducing conditions at 200 V 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 first boiled for 5 min. A low range molecular weight standard (Biorad) was included on the gels. Protein bands were visualized by silver staining (Biorad).

**Fluorescence Spectroscopy**

Emission spectra of the samples (300–450 nm, 1-nm steps; 330–345 nm, 0.1-nm steps) and emission spectra of native rhIFNα2b in the presence of increasing amounts of guanidine hydrochloride (300–450 nm, 0.5-nm steps) were measured on a Fluorolog III fluorimeter. Excitation was at 295 nm. Slits were set at 5 nm. Samples (protein concentration: 55 µg/mL) were measured at 25°C and stirred during measurement. Integration time per data point was 0.1 s and the average of 10 scans was taken. The buffer spectrum was subtracted. Second-derivative spectra were calculated to study changes in tertiary structure, according to Kumar et al.17 Smoothed normalized spectra were derivatized twice and after each derivatization, the spectra were smoothed. Smoothing was performed by taking the average of 13 neighboring points, with a simplified least squares procedure as described by Savitzky and Golay.18 All calculations were performed with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA).

**Analysis of Native Epitopes**

**Western Blotting.** SDS–PAGE gels were blotted onto a nitrocellulose sheet with a Scie-Plas semi-dry blotter (Scie-Plas, Warwickshire, UK). Blots were blocked with 1% (w/v) non-fat milk powder in 0.005% (w/v) Tween 20 in phosphate buffered saline (PBS) for 1 h at room temperature with constant orbital shaking. After washing with 0.005% (w/v) Tween 20 in PBS and with water, the blots were incubated with a polyclonal rabbit anti-rhIFNα2b serum in 0.1% (w/v) non-fat milk powder in 0.005% (w/v) Tween 20 in PBS for 1 h at room temperature. Blots were washed with 0.005% (w/v) Tween 20 in PBS and with water. Blots were incubated with peroxidase labeled anti-rabbit IgG (Sigma) in 0.1% (w/v) non-fat milk powder in 0.005% (w/v) Tween 20 in PBS for 1 h at room temperature. Blots were washed with 0.005% (w/v) Tween 20 in PBS and with water. Blots were incubated in a solution of 4-chloro-1-naphtol (Sigma) in methanol (15% (v/v)), water, and H2O2 (0.015% (v/v)). After color development, the blots were stored overnight in the dark in water to increase the intensity of the bands.

**ELISA for rhIFNα2b.** Microlon 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands)
were coated with 100 μL of a monoclonal antibody against rhIFNα2b (diluted 1:2500; Pierce, Etten-Leur, The Netherlands) in 10 mM PBS for 1 h. The wells were drained and washed four times with 300 μL wash buffer (0.1% (w/v) Tween 20 in PBS). After each wash, the plates were tapped dry on a tissue. Wells were blocked with 200 μL 2% (w/v) BSA in PBS for 1 h. The wells were drained and washed two times with 300 μL wash buffer. After each wash, the plates were tapped dry on a tissue. Samples were added in twofold serial dilutions (starting at 200 ng/mL) to the wells, containing 2% (w/v) BSA in PBS. Plates were incubated for 1 h. The wells were drained and washed four times with 300 μL wash buffer. After each wash, the plates were tapped dry on a tissue. Wells were incubated with a polyclonal rabbit antiserum against rhIFNα2b for 1 h. The wells were drained and washed four times with 300 μL wash buffer. After each wash, the plates were tapped dry on a tissue. Peroxidase labeled antirabbit IgG was added to the wells and the plates were incubated for 1 h. The wells were drained and washed four times with 300 μL wash buffer and two times with 300 μL PBS. After each wash, the plates were tapped dry on a tissue. 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Roche, Almere, The Netherlands) was added and the absorbance was recorded after 30 min on a Novapath microplate reader (Biorad) at a wavelength of 415 nm and a reference wavelength of 490 nm. All incubation steps were performed at room temperature with constant orbital shaking. During all incubation steps, the plates were covered. The absorbance values were plotted against log protein concentration of the samples.

Immunogenicity

Animal Experiments

The animal experiments were approved by the Institutional Ethical Committee. Wild-type (FVB/N) mice were obtained from Charles River laboratories. Transgenic mice were bred at the Central Laboratory Animal Institute. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available ad libitum.

Groups of five mice received 10 μg of the different protein preparations subcutaneously on days 0–4, 7–11, and 14–18. Blood was taken from the vena saphena on days 0, 7, and 14 just before injection of the interferon preparations and on day 21. The blood samples were incubated on ice for 2 h. Sera were collected after centrifugation and stored at −20°C.

Sera were analyzed for the presence of antibodies by an ELISA as described below. It was shown before, by i.p. administration of ovalbumin following the same immunization scheme as described above, that the tolerance of the transgenic mice is specific for hIFNα2 and not due to a general immune suppression.10

Antibody Assay

Sera were analyzed by ELISA for antibodies against native rhIFNα2b. Microlon 96-well plates (Greiner) were incubated with 100 μL native rhIFNα2b (2 μg/mL in PBS) per well for 1 h. Then the wells were drained and washed four times with 300 μL wash buffer (0.1% (w/v) Tween 20 in PBS). After washing, the wells were carefully tapped dry on a tissue. Wells were blocked with incubating with 200 μL 2% (w/v) BSA in PBS for 1 h. The wells were drained and washed two times with 300 μL wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Sera (diluted 100-fold with 2% (w/v) BSA in PBS) were added to the wells and the plates were incubated for 1 h. The plates were washed four times with 300 μL wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Peroxidase-labeled anti-mouse IgG (Sigma) was added to the wells and the plates were incubated for 1 h. Plates were washed and twice with 300 μL wash buffer. After the last wash, wells were carefully tapped dry on a tissue. ABTS-substrate was added and the absorbance was recorded after 30 min on a Novapath microplate reader at 415 nm and a reference wavelength of 490 nm. All incubations were performed at room temperature with constant orbital shaking. Sera were arbitrarily defined positive if the absorbance of the 1:100 dilution minus the background was three times higher than the average absorbance value of the pre-treatment sera minus the background.

Antibody titers against native rhIFNα2b of the positive sera were determined by adding the sera in threefold serial dilutions (starting from 1:10) to plates coated with native rhIFNα2b. The other steps of the ELISA procedure were as described above and the absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows). The dilution needed to obtain 50% of the maximum absorbance was taken as the titer of
the serum. When the absorbance of the most concentrated dilution (1:10) was too low for obtaining a good fit, the titer was defined to be smaller than 10. Each plate contained a standard serum (rabbit-anti rhIFNα2b), which showed a titer (±SD) of 6.8 (±0.6) × 10^3.

RESULTS

Characterization

UV Spectroscopy

Oxidized rhIFNα2b showed a slightly increased optical density at wavelengths where rhIFNα2b does not absorb light (>320 nm) (Fig. 1) and a decrease in A_{280}/A_{260} as compared to native rhIFNα2b, indicating the presence of aggregates in this solution. The aged samples also showed a decrease in A_{280}/A_{260}, as well as an increased optical density above 320 nm, indicative of the presence of aggregates.

GPC

GPC was used to analyze the samples for the presence of soluble aggregates (Fig. 2). Native rhIFNα2b contained mostly monomeric protein and a small dimer peak. Oxidized rhIFNα2b showed a large drop in monomer content, whereas significant amounts of dimers, trimers, and higher molecular weight products were formed (Fig. 2A). The samples incubated at pH 4.0 and 7.2 showed only a small amount of monomeric rhIFNα2b (Fig. 2B). The sample incubated at pH 9.0 did not show any major protein peak at all (Fig. 2B). The percentages of recovered fractions are listed in Table 1. Differences between the total peak area of the samples and that of native rhIFNα2b can be explained by the presence of insoluble aggregates and/or protein fragments, and is referred to as the non-recoverable fraction in Table 1.

SDS–PAGE

Fragmentation and formation of covalent aggregates was analyzed by SDS–PAGE (Fig. 3A and B). Different amounts of aggregates were visible in all samples, except in native rhIFNα2b. Most of the aggregates were covalently linked via disulfide bonds, except for the sample incubated at pH 9.0, which showed a considerable amount of non-reducible aggregates (see Fig. 3A and B). The
samples incubated at pH 7.2 and 9.0 showed a precipitate not able to enter the stacking gel, as was visible during electrophoresis under non-reducing conditions, but not under reducing conditions, thus indicative of large disulfide-mediated aggregates. The sample incubated at pH 4.0 showed, besides aggregates, some fragmentation.

**DLS**

Native rhIFNα2b did not scatter enough light to obtain reliable results about its particle size. The

<table>
<thead>
<tr>
<th>Sample</th>
<th>Z-ave (μm)</th>
<th>PDI</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Trimer</th>
<th>Oligomer</th>
<th>Non-recov. Fraction</th>
<th>Conf. Changes</th>
<th>Native Epitopes</th>
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<tr>
<td>Native</td>
<td>nd</td>
<td>nd</td>
<td>99%</td>
<td>1%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
<td>100 ± 27%</td>
</tr>
<tr>
<td>PH 4.0</td>
<td>0.40</td>
<td>0.16</td>
<td>13%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>86%</td>
<td>++</td>
<td>41 ± 9%</td>
</tr>
<tr>
<td>PH 7.2</td>
<td>0.41</td>
<td>0.32</td>
<td>47%</td>
<td>1%</td>
<td>nd</td>
<td>2%</td>
<td>49%</td>
<td>+</td>
<td>90 ± 2%</td>
</tr>
<tr>
<td>PH 9.0</td>
<td>2.2</td>
<td>0.36</td>
<td>Nd</td>
<td>nd</td>
<td>nd</td>
<td>2%</td>
<td>98%</td>
<td>++</td>
<td>1.4 ± 0%</td>
</tr>
<tr>
<td>Oxidized</td>
<td>0.38</td>
<td>0.75</td>
<td>41%</td>
<td>11%</td>
<td>3%</td>
<td>10%</td>
<td>34%</td>
<td>+</td>
<td>104 ± 11%</td>
</tr>
</tbody>
</table>

*Percentages were calculated based on GPC peak areas relative to the total peak area in GPC of native rhIFNα2b: \( \frac{A_{\text{peak}}}{A_{\text{total}}} \times 100\% \).

b|Non-recoverable fraction: percentages were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFNα2b: \( \frac{A_{\text{peak}} - A_{\text{native}}}{{A_{\text{total}} - A_{\text{native}}}} \times 100\% \).

c|Conformational changes, as shown by fluorescence.

d|Shown by ELISA: percentages were calculated based on the absorbance in the ELISA for rhIFNα2b of the dilution containing 25 ng/mL protein relative to the absorbance obtained with native rhIFNα2b. Values represent average ± range, \( n = 2 \).

e|nd, not detectable.

Explanation of used symbols: -, not detected; +, mild; ++, extensive.

**Figure 3.** SDS–PAGE (A: non-reducing, B: reducing) and Western blot of an SDS–PAGE gel run under non-reducing conditions (C) of native rhIFNα2b (N), the samples aged at the different pHs, and oxidized rhIFNα2b (O). Numbers on the left represent band positions (in kDa) of the molecular weight markers.
other samples showed diameters between approximately 0.4 and 2 μm with relatively high polydispersity indices (Tab. 1), indicating the presence of aggregates heterogeneous in size. The sample incubated at pH 4.0 contained visible particles, which sedimented to the bottom of the measuring tube and were, therefore, missed by the DLS analysis.

**Fluorescence Spectroscopy**

Fluorescence spectroscopy at an excitation wavelength of 295 nm gives information about the environment of the two tryptophans in rhIFNα2b at positions 76 and 140. The emission spectra are shown in Figure 4A and the peak maxima listed in Table 2. Oxidized rhIFNα2b and the samples incubated at pH 4.0 and 9.0 showed a red-shifted emission maximum as compared to native rhIFNα2b, indicating for at least one of the tryptophans a more hydrophilic environment. The shift shown by the samples incubated at pH 4.0 and 9.0 was large, indicative of either a non-native protein structure with incomplete unfolding or a large fraction of (partly) unfolded protein. RhIFNα2b in 5 M guanidine chloride (completely

![Figure 4. Emission spectra (A) and second-derivative spectra (B) of native rhIFNα2b (closed squares), aged samples incubated at pH 4.0 (triangles), pH 7.2 (diamonds), and pH 9.0 (circles) and oxidized rhIFNα2b (open squares). Second-derivative spectra (C) of rhIFNα2b in guanidine hydrochloride (GnHCl). a: 0 M, b: 3.4 M, c: 3.6 M, d: 3.8 M, e: 4.0 M, f: 4.2 M, g: 4.4 M, and h: 4.6 M GnHCl. RhIFNα2b in GnHCl below 3.4 M GnHCl gave identical spectra as rhIFNα2b in 0 M GnHCl. RhIFNα2b in GnHCl above 4.6 M GnHCl gave identical spectra as rhIFNα2b in 4.6 M GnHCl. The fluorescence intensity is an arbitrary unit.](https://doi.org/10.1002/jps)
unfolded) had an emission maximum at 356 nm. Moreover, the spectrum obtained with the sample incubated at pH 9.0 showed extreme light scattering. The sample incubated at pH 7.2 did not show a shift in emission maximum. Kumar et al.\textsuperscript{17} showed that small changes in second-derivative emission spectra around 325 nm, with similar intensities around 335 nm, are indicative of small changes in tertiary structure of the protein, even when the emission spectra do not show a shift in peak maximum. The second-derivative spectra of native, oxidized rhIFN\(\alpha_2b\), and the sample incubated at pH 7.2 are shown in Figure 4B. The second-derivative spectra of the samples incubated at pH 4.0 and 9.0 were not calculated since the emission spectra already showed differences in the tryptophan environment for these samples. For comparison, the second-derivative spectra of rhIFN\(\alpha_2b\) with increasing concentrations of guanidine hydrochloride are shown in Figure 4C. Guanidine hydrochloride is a chaotropic agent, known to unfold proteins. The structure of rhIFN\(\alpha_2b\) in less than 3.4 M guanidine hydrochloride was unchanged, as compared to rhIFN\(\alpha_2b\) without guanidine hydrochloride. Starting at 3.4 M, the protein progressively unfolded with increasing concentrations of guanidine hydrochloride, as can be seen by the decrease of the negative second-derivative peak around 325 nm, while the intensity around 337 nm remained constant. Eventually, a complete loss of the peak around 325 nm occurred (Fig. 4C), indicating that the two tryptophans had similar, relatively hydrophilic environments.\textsuperscript{17} When the concentration of guanidine hydrochloride was further increased, the second-derivative spectrum showed differences in the region 350-375 nm, yielding spectra that resemble the spectrum of N-acetyl-L-tryptophanamide (NATA) in water.\textsuperscript{17}

The second-derivative spectrum of oxidized rhIFN\(\alpha_2b\) showed an intensity around 325 nm in between that of the reference spectra of rhIFN\(\alpha_2b\) in 3.2 M and 3.4 M guanidine hydrochloride. The intensity around 325 nm in the second-derivative spectrum of the sample incubated at pH 7.2 was near that of the reference spectrum of rhIFN\(\alpha_2b\) in 3.6 M guanidine hydrochloride. This indicates that both samples, oxidized rhIFN\(\alpha_2b\) and the sample incubated at pH 7.2, contained protein with slightly altered tertiary structures as compared to native rhIFN\(\alpha_2b\), or a small fraction of (partly) unfolded protein in the presence of excess “native” protein.

### Analysis of Native Epitopes

**Western Blotting.** The reactivity of the bands in the SDS–PAGE gel with a polyclonal anti-rhIFN\(\alpha_2b\) serum is shown in Figure 3C. The monomers of all samples reacted with the polyclonal antiserum, albeit only faintly for the pH 9.0 sample. Also, at least part of the dimers as well as the aggregates present in the samples reacted with the polyclonal antiserum.

**ELISA for rhIFN\(\alpha_2b\).** The reactivity of the samples with a monoclonal antibody was also tested in an ELISA (Tab. 1), which avoids the denaturation step needed for SDS–PAGE and subsequent Western blotting. The sample incubated at pH 9.0 did not react at all in the ELISA. This indicates the loss of the epitope recognized by the monoclonal antibody and/or the loss of epitopes recognized by the polyclonal antiserum, which may be due to conformational changes, chemical modifications, and/or aggregation. The absorbance values obtained with a concentration of 25 ng/mL were within the linear part of the dose-response curve for all samples, except for the sample incubated at pH 9.0. This allows the differences in the absorbance to be explained by a difference in antigenicity (Tab. 1). The binding of oxidized rhIFN\(\alpha_2b\) was comparable to that of native rhIFN\(\alpha_2b\), indicating that the major (immunodominant) epitopes were preserved. The sample incubated at pH 7.2 showed a small decrease in binding, indicating some loss of epitopes as compared to native rhIFN\(\alpha_2b\). The samples incubated at pH 4.0 and pH 9.0 showed drastically reduced reactivities, indicating a major loss of epitopes.

### Summarized Characteristics

The main characteristics of the samples as determined by the analyses discussed above are summarized in Table 1. Oxidized rhIFN\(\alpha_2b\)

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### Table 2. Results of the Emission Spectra (330–345 nm)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intensity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Emission Maximum (nm)</th>
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</thead>
<tbody>
<tr>
<td>Native</td>
<td>7.3</td>
<td>337.1</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>10</td>
<td>340.9</td>
</tr>
<tr>
<td>pH 7.2</td>
<td>8.0</td>
<td>337.2</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>5.2</td>
<td>344.2</td>
</tr>
<tr>
<td>Oxidized</td>
<td>6.8</td>
<td>338.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Arbitrary unit.
showed the same characteristics as previously described\textsuperscript{10}: partly aggregated, a slightly changed tertiary structure and preservation of native epitopes. The samples incubated at pH 4.0 and 7.2 showed partly aggregated protein. The sample incubated at pH 4.0 was partly denatured and had lost about 60\% of its native epitopes. The sample incubated at pH 7.2 showed a slight change in tertiary structure and retained most of its native epitopes. The sample incubated at pH 9.0 showed a highly aggregated sample, with a complete loss of native epitopes.

**Mixtures of Native and Oxidized rhIFNa2b**

Native and oxidized rhIFNa2b were mixed to obtain preparations with different levels of aggregates. The aggregation level of the preparations as determined by GPC is shown in Figure 5. Preparations B, C, D, and E consisted of different amounts of insoluble protein, as well as soluble monomers, dimers, trimers, and larger aggregates. The compositions of the mixtures B, C, and D, as measured by GPC, were close to the calculated compositions of the preparations based on measured compositions of unmixed preparations A and E, indicating that the mixing itself had not induced dramatic shifts in aggregate level.

**Immunogenicity**

Native rhIFNa2b induced serum antibodies in the wild-type mice (Figs. 6A and 7A), as was expected. For all groups, none of the mice showed detectable antibodies at day 7 (results not shown), but on day 14 and day 21, all mice were positive. Oxidized rhIFNa2b and the degraded samples showed an increased immune response as compared to native rhIFNa2b in the wild-type mice at day 21. The preparations containing mixtures of native and oxidized rhIFNa2b led to the formation of antibodies (Fig. 7A). On day 14, all groups showed comparable antibody titers, but on day 21

**Figure 5.** Measured composition of the preparations obtained by mixing native and oxidized rhIFNa2b. Peak area percentages were calculated based on GPC peak areas relative to the total peak area in GPC of native rhIFNa2b: $\frac{\text{AUC}_{\text{peak}}}{\text{AUC}_{\text{native, total}}} \times 100\%$. The percentages of the non-recoverable fraction were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFNa2b: $\frac{\text{AUC}_{\text{sample, total}} - \text{AUC}_{\text{native, total}}}{\text{AUC}_{\text{native, total}}} \times 100\%$.

**Figure 6.** Anti-rhIFNa2b IgG titers of the wild-type (A) and transgenic (B) mice after administration of native (Native) rhIFNa2b, aged samples incubated at pH 4.0 (4.0), pH 7.2 (7.2), or pH 9.0 (9.0) and oxidized rhIFNa2b (Oxidized). The values represent average titers (+SEM) of positive mice. All wild-type mice were positive for antibodies. The numbers above the bars represent the number of positive mice out of total mice. ND: not detectable. $^* p < 0.05$, $^{***} p < 0.001$ as compared to native rhIFNa2b (one-way parametric ANOVA). No statistical test could be performed to compare the titers of the transgenic mice, since not all mice showed detectable antibody levels.
preparations C, D, and E had induced significantly higher antibody titers as compared to native rhIFNα2b. Remarkably, elevating the aggregate dose beyond 2.5 μg per injection did not further enhance the immune response in the wild-type mice.

The transgenic mice were immune tolerant for native rhIFNα2b (Figs. 6B and 7B). The aggregate-containing samples were able to break the tolerance of the transgenic mice to a different extent (Fig. 6B): Oxidized rhIFNα2b induced the highest antibody levels (on both day 14 and day 21), while the sample incubated at pH 9.0 induced the lowest level of antibodies. For the most immunogenic sample, oxidized rhIFNα2b, the immune response was shown to be aggregate dose dependent (Fig. 7B). With the lowest amount of aggregates (preparation B contained 4% of oligomers and 10% of insoluble aggregates, i.e., 1.4 μg aggregates/injection), two out of five mice showed detectable antibody titers on day 21 (Fig. 7B). With an increasing aggregate dose (preparations C–E), the number of mice producing antibodies and the average antibody titers gradually increased, whereas the onset of antibody production tended to be earlier (Fig. 7B).

DISCUSSION

In this study, we have evaluated the sensitivity of the mouse models for aggregate type and level of aggregation. RhIFNα2b was used as a model protein since an immune-tolerant mouse model was available for this protein. RhIFNα2b can cause immune alterations, which might implicate the results obtained. However, rhIFNα2b has no activity in mice. Moreover, Braun et al.9 have shown that no differences in immunogenicity were observed when murine IFNα2b was co-administered with rhIFNα2b. To obtain relevant degradation products, rhIFNα2b solutions were aged without excipients under stress conditions, that is, at elevated temperature and different pHs. The solutions were incubated below the melting temperature of rhIFNα2b, which is around 55–60°C under all conditions tested.20 For comparison, rhIFNα2b was oxidized by metal catalysis, which was shown before to lead to a product capable of breaking the immune tolerance of transgenic mice.10 Aggregation was observed in all samples. For the samples incubated at elevated temperature, this was probably due to unfolding of the protein caused by acidic and alkaline conditions and/or elevated temperatures,20–25 and observed in the present study by

Figure 7. Anti-rhIFNα2b IgG titers of the wild-type (A) and transgenic (B) mice after administration of the mixed samples. The values represent average titers (+SEM) of positive mice. All wild-type mice were positive for antibodies. The numbers above the bars represent the number of positive mice out of total mice. ND: not detectable. **p < 0.01, ***p < 0.001 as compared to native rhIFNα2b (one-way parametric ANOVA). No statistical test could be performed to compare the titers of the transgenic mice, since not all mice showed detectable antibody levels.
fluorescence spectroscopy. When the protein unfolds, hydrophobic surfaces become exposed and their interaction may lead to aggregation. In addition, disulfide scrambling, which is most favorable at moderate to high pH, was at least in part involved in the aggregation process, as visualized by SDS–PAGE.

Besides physical degradations also chemical degradation could have occurred (e.g., deamidation, disulfide bond formation, oxidation, and hydrolysis), which may account for the lower reactivity with the monoclonal antibody and polyclonal antiserum for the samples aged at pH 4.0 and 9.0. Hydrolysis predominantly occurs at mild acidic conditions, which explains the fragmentation seen in the sample incubated at pH 4.0. Complete chemical analysis of the samples was not pursued and would be very difficult, because the samples have high levels of aggregation and are very heterogeneous in composition.

All samples, except native rhIFN-α2b, contained aggregates. In general, the presence of aggregates is an important risk factor for inducing an antibody response. Aggregation and chemical degradation may lead to conformational changes and the presentation of new epitopes. Aggregation may also lead to the type of multimeric epitope presentation that can break immune tolerance. The B-cell receptor is capable of responding without T-cell help to epitope patterns meeting certain criteria, such as spacing of 5–10 nm. All samples, except native rhIFN-α2b, were able to break tolerance in transgenic mice. The degraded samples were less immunogenic in the transgenic mice than oxidized rhIFN-α2b. The immunogenicity of the degraded samples seemed not to be related to the level of aggregation of the samples, but to the presence of native-like protein in the aggregates, because the sample incubated at pH 9.0 contained the highest amount of non-native-like aggregates (see Tab. 1) and was the least immunogenic. This was in line with previous results. Considering repetitive antigen presentation as an explanation for breaking tolerance, the low immunogenicity of the sample incubated at pH 9.0 can be explained by the destruction of native epitopes in this sample. A similar phenomenon was previously observed for boiled rhIFN-α2b. The sample stored at pH 7.2 showed aggregation but also partial preservation of native epitopes (Tab. 1), which may explain that it showed earlier breaking of the immune tolerance of the transgenic mice as compared with the pH 9.0 sample (Fig. 6B).

In the wild-type mice, all degraded samples (including oxidized rhIFN-α2b) were more immunogenic than native rhIFN-α2b. All degraded samples showed the same level of immunogenicity in the wild-type mice, but the transgenic mice showed differences in the immunogenicity of the different degraded samples (including oxidized rhIFN-α2b). This can be explained by the different mechanisms that play a role in the antibody formation. In the wild-type mice, a classical immune response takes place, whereas in the transgenic mice, it is breaking of tolerance that leads to the formation of antibodies. In a classical immune response, the antigen is taken up by antigen presenting cells (APC) and digested to peptides that are presented on the surface of the APC in combination with major histocompatibility complex (MHC) class II molecules. T-cells will recognize the peptides in combination with the MHC molecule and activate B-cells into making antibodies. The mechanism of breaking tolerance is not exactly known, but the previously mentioned multimeric epitope presentation, which is T-cell independent, is considered to be important. The aggregates in the degraded samples could act as a danger signal, recruiting APC to the injection site, which might explain the increased immunogenicity in the wild-type mice of the degraded samples as compared to native rhIFN-α2b.

In the present study, rather extreme degradation conditions were used, leading to nearly complete disappearance of native protein in the samples incubated at pH 4.0 and 9.0. More subtle changes will occur if the protein is stored for shorter incubation periods, or at lower temperatures. As we showed native-like aggregates to be more immunogenic than rigorously denatured ones, samples containing moderately altered protein species may be more immunogenic than largely denatured proteins. Therefore, it would be interesting to study the kinetics of the degradation process of rhIFN-α2b under the conditions used in this study (i.e., various pHs and high temperature) and the relation between degree of degradation and immunogenicity more quantitatively.

In this study, it was also demonstrated that the immune response induced by rhIFN-α2b preparations depended on the level of aggregation: the more aggregates were present, the more immunogenic the formulations were. Still, as little as 14% of aggregated protein (i.e., 1.4 μg per injection) was capable of breaking the immune tolerance in some of the transgenic mice. Marketed rhIFN-α2 formulations have been reported to contain less...
than 1% (i.e., 0.04–0.24 μg per administered dose) aggregated protein, which is close to the lowest aggregate dose tested in this study. In Betaseron, a recombinant human interferon beta formulation, 60% of the protein (150 μg per administered dose) is high-molecular-weight material (>600 kDa). We showed before that Betaseron is immunogenic in a transgenic animal model, immune tolerant for human interferon beta. Qualitative links have been made between the clinic and animal experiments: (a) Betaseron, being immunogenic in patients, was able to break the tolerance of transgenic mice and (b) aggregates of HSA and rhIFNa2b were immunogenic in patients and were able to break the tolerance of transgenic mice. Here, we showed that transgenic mouse models may be sufficiently sensitive to detect immunogenic aggregates typically present in commercial protein formulations, describing a more quantitative link between the clinic and the animal experiments. However, the effect of excess unmodified protein on the immune response against small amounts of aggregates has to be established yet. Also, to investigate which aggregation products are responsible for the immunogenicity, the different aggregated protein fractions (dimers, trimers, oligomers, insoluble fraction) should be isolated and their structure and immunogenicity tested in wild-type and transgenic mice. Future studies should also include experiments to elucidate the mechanism of antibody formation.

CONCLUSIONS

In this study, we investigated the immunogenicity of rhIFNa2b aged at accelerated conditions. The immunogenicity of the degradation products was studied in wild-type and transgenic immune tolerant mice. Aggregates containing protein molecules with a native-like conformation seem to be the main factor responsible for the increased immunogenicity observed. Moreover, the antibody response against rhIFNa2b is dependent on the level of aggregation. Since breaking of tolerance is the mechanism most likely to be responsible for the development of antibodies in patients, the transgenic mouse model is more relevant for the prediction of immunogenicity in humans than the wild-type animal model. Our results indicate that transgenic animal models may help to establish maximum allowable aggregate levels in commercial protein formulations.

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