A Role for Protein Misfolding in Immunogenicity of Biopharmaceuticals*

Received for publication, June 22, 2006, and in revised form, September 27, 2006 Published, JBC Papers in Press, November 29, 2006, DOI 10.1074/jbc.M605984200

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For largely unknown reasons, biopharmaceuticals evoke potentially harmful antibody formation. Such antibodies can inhibit drug efficacy and, when directed against endogenous proteins, cause life-threatening complications. Insight into the mechanisms by which biopharmaceuticals break tolerance and induce an immune response will contribute to finding solutions to prevent this adverse effect. Using a transgenic mouse model, we here demonstrate that protein misfolding, detected with the use of tissue-type plasminogen activator and thioflavin T, markers of amyloid-like properties, results in breaking of tolerance. In wild-type mice, misfolding enhances protein immunogenicity. Several commercially available biopharmaceutical products were found to contain misfolded proteins. In some cases, the level of misfolded protein was found to increase upon storage under conditions prescribed by the manufacturer. Our results indicate that misfolding of therapeutic proteins is an immunogenic signal and a risk factor for immunogenicity. These findings offer novel possibilities to detect immunogenic protein entities with tPA and reduce immunogenicity of biopharmaceuticals.

Over the past decades, the use of therapeutic proteins has become common practice in medicine and as their use is very promising, many more biopharmaceuticals are under development (1, 2). Unfortunately, a major drawback of protein therapeutics is the risk of antibody formation (3–7). These immunogenicity problems are of concern regarding therapeutic efficacy and patient safety (5, 8). For example, drug-induced neutralizing antibodies to erythropoietin (EPO)† result in pure red cell aplasia (9), whereas drug-induced acquired anti-factor VIII (FVIII) antibodies worsen the pathology associated with hemophilia (10). As more and more recombinant therapeutic proteins become licensed for marketing, the incidence of immunogenicity problems is expected to rise.

Initially, when mainly proteins from animal origin were used for therapy, it was thought that their foreign (non-self) nature was the main cause of immunogenicity. Unexpectedly, however, both human plasma derived as well as recombinant human protein therapeutics such as EPO (11) and FVIII (12) also elicit immune responses. This suggests that the molecular characteristic evoking antibody responses is at least more complex than being self or non-self to the human immune system. Several additional factors contributing to immunogenicity have been proposed, including contaminants or impurities, protein aggregation (13), chemical degradation and protein modification, such as differences in glycosylation or oxidation (14, 15) to explain the induction of antibodies.

Protein misfolding is an intrinsic and problematic property of proteins, which underlies a variety of degenerative diseases, such as Alzheimer disease. These diseases are characterized by the occurrence of fibrillar deposits, classically termed amyloid, containing aggregates of misfolded proteins. Whereas the term amyloid is classically used to classify these fibrillar deposits, aggregation of proteins, irrespective of amino acid sequence, results in formation of amyloid-like properties with similar common features (16). Amyloid can be defined histochemically by affinity for amyloid-specific dyes, but also morphologically when 6–10-nm filaments are seen by microscopy. X-ray diffraction experiments can confirm the presence of cross-β structure, a structural element characteristic for amyloid. We have reported that amyloid proteins, positive for the amyloid markers described above, also are able to bind and activate tissue-type plasminogen activator (tPA) in vitro (17, 18). Concomitantly, it has been reported that denatured protein aggregates have the same capacity, whereas native proteins do not bind tPA (19, 20). Therefore, tPA can serve as a fast novel tool for detection of misfolded protein with amyloid-like properties (17, 21). Protein misfolding can be accelerated by a number of environmental factors, including protein modifications such as glycation, deamidation, or oxidation (17, 22, 23), interaction of proteins with surfaces, such as mica (24) or negatively charged phospholipids (25), or conditions such as heating (26), lyophilization (27), sonication (28), packaging materials (26), and others (29). Amyloids and proteins with amyloid-like properties activate the immune system, which can lead to the formation of (auto-) antibodies (30, 31).

Because protein misfolding is an intrinsic problematic propensity of proteins we hypothesized that protein misfolding processes in general could underlie immunogenicity of bio-

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†The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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¶The abbreviations used are: EPO, erythropoietin; tPA, tissue-type plasminogen activator; ThT, thioflavin T; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; rhIFNα2b, human recombinant interferon α2b.
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pharmaceuticals. We investigated whether the common structural features of misfolded therapeutic proteins are detectable by markers for amyloid formation. We exposed biopharmaceuticals to denaturing conditions and investigated markers for amyloid formation in the preparations. Moreover, we analyzed several purchased biopharmaceuticals for the presence of misfolded proteins using these common markers for amyloid properties. Both experiments pointed out that biopharmaceuticals, like any other protein, are amyloidogenic and that misfolding, detected by amyloid markers, takes place in several preparations. These markers, however, are not necessarily specific for fibrillar amyloid, but also for smaller misfolded protein species. Next, we tested the main hypothesis that misfolded protein, detectable by these amyloid markers, is causative for increased risk of having immune reactions against biopharmaceuticals. We compared the immunogenicity of experimentally misfolded protein solutions to control solutions, containing native protein. The titer of antibody responses against the native protein was taken as a measure for immunogenicity. Although further study is required to identify the exact immunogenic misfolded species that mediates this mechanism, our experiments confirmed the hypothesis that misfolding leads to biopharmaceutical immunogenicity.

EXPERIMENTAL PROCEDURES

**Thioflavin T and Congo Red Fluorescence Measurements**

Fluorescence of Thioflavin T (ThT; Sigma, T-3516) and Congo Red (Aldrich Chemical Company Inc.) was measured on a Hitachi F-4500 spectrophotometer at an excitation wavelength of 435 nm and emission wavelength of 485 nm for ThT and an excitation wavelength of 550 nm and emission wavelength of 595 nm. Five μl of the various protein solutions were diluted in either 1 ml of 25 μM ThT in 50 mM glycine buffer, pH 9.0, or 1 ml of 25 μM Congo Red in phosphate-buffered saline (PBS), pH 7.2, and incubated for 30 min at room temperature. Fluorescence was measured in triplicate with an integration time of 5 s per reading. Background fluorescence of both protein in buffer and dye solution were subtracted from the total fluorescence signal. Five μg/ml of aggregated amyloid β (Aβ “dutch type” E22Q, residues 1–40; DAEFRHDSGYEVH-HQKLVFFAEDVGSNKGAIGLMVGGV), produced by Peptide Synthesis Facility of the Dutch Cancer Institute NKI) was used as a positive control in all fluorescence assays. This positive control was prepared by dissolving lyophilized Aβ peptide at 50 mg/ml in a 1:1 mixture of trifluoroacetic acid and 1,1,1,3,3,3-hexafluoro-2-propanol. After evaporating the solvent, the pellet was resuspended at 10 mg/ml in H2O and incubated at 37 °C for 72 h.

**tPA Binding Assay**

Nunc Immobilizer plates (Nalge Nunc, number 436013) were coated with 50 μl containing 5 μg/ml of sample protein (unless indicated otherwise) in 100 mM NaHCO3, pH 9.6, 0.05% (m/v) NaN3 for 1 h at room temperature. Plates were washed twice with Tris-buffered saline, pH 7.2, containing 0.1% Tween 20 (TBST) and blocked with PBS containing 1% Tween 20 for 1 h at room temperature. Plates were washed twice with TBST and incubated, in duplicate, with a concentration series of either tPA (Actilyse, Alteplase; Boehringer-Ingelheim, Alkmaar, The Netherlands) or a truncated form of tPA (Reteplase; Rapisyn, Roche Diagnostics GmbH, Mannheim Germany), lacking the amyloid binding domain, diluted in PBS containing 0.1% Tween 20 (PBST). Incubations were performed for 1 h at room temperature in the presence of 10 mM ε-amino caproic acid. ε-Amino caproic acid is a lysine analogue and is used to avoid potential binding of tPA to lysine-containing ligands via its kringle2 domain. Plates were washed five times with TBST and incubated with antibody 374b α-tPA (American Diagnostics, Instrumentation Laboratory, Breda, The Netherlands) or a truncated form of tPA (Reteplase; Reteplase, Glostrup, Denmark) diluted 1:3000 in PBST for 30 min at room temperature. Plates were washed five times with PBS, 0.1% Tween 20, and stained with 100 μl/well of tetramethyl-benzidine substrate (BISOsource Europe, Nivelles, Belgium). The reaction was terminated with 50 μl/well of 2 m H2SO4 and substrate conversion was read at 450 nm on a Spectramax340 microplate reader. Curves were fitted with a one-site binding model (GraphPad Prism version 4.02 for Windows, Graphpad Software) from which Kd and Bmax were determined.

**tPA Activation Assay**

Exiqon Peptide Immobilizer plates were blocked for 1 h with PBS, 1% Tween 20 and rinsed twice with distilled water. The conversion of the chromogenic substrate S-2251 (Chromogenix, Italy) by plasmin was kinetically measured at 37 °C on a Spectramax 340 microplate reader at a wavelength of 405 nm. The assay mixture contained 400 pm tPA, 100 μg/ml plasminogen (purified from human plasma), and 415 μM S-2251 in HEPES-buffered saline, pH 7.4. Denatured γ-globulins (100 μg/ml) with amyloid-like structure was used as reference and positive control. Lyophilized γ-globulins (Sigma) were dissolved in a 1:1 volume ratio of 1,1,3,3,3-hexafluoro-2-propanol and trifluoroacetic acid and subsequently dried under air. Dried γ-globulins were dissolved in H2O to a final concentration of 1 mg/ml and kept at room temperature for at least 3 days and subsequently stored at −20 °C. Maximal tPA activating capacity was determined from the linear increase seen in each activation curve and expressed as a percentage of the standardized positive control. To confirm tPA dependence of plasmin generation, all samples were assayed for their ability to convert plasminogen into plasmin in the absence of tPA.

**Analyses of Protein Therapeutics**

Protein therapeutics were obtained from the local hospital pharmacy and analyzed within the expiry limits as stated by the manufacturers. Five μl of the various protein therapeutics were tested for their ability to enhance both ThT and Congo Red fluorescence. tPA activating capacity of the protein therapeutics was determined in 1:10 diluted samples (unless indicated otherwise). tPA binding ELISAs were performed by coating protein therapeutics 1:10 in 100 mM NaHCO3, pH 9.6, 0.05% (m/v) NaN3.
Dependency Immune Reactivity Toward Amyloid-Like Properties

For testing of dose-dependent immune reactivity toward amyloid-like properties before and after treatment by tPA activation assay at 100 µg/ml protein and ThT fluorescence enhancement assay at 25 µg/ml protein. For this purpose, 5 mg/ml glucagon (Glucagen; Novo Nordisk Farma B.V., Alphen aan de Rijn, The Netherlands) and water (acidified) were available at Universiteit Utrecht, The Netherlands. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available at Universiteit Utrecht, The Netherlands. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available at Universiteit Utrecht, The Netherlands. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available at Universiteit Utrecht, The Netherlands.

Inducing Protein Misfolding in Solutions of rhIFNα2b and Ovalbumin

Unformulated human recombinant interferon α2b (rhIFNα2b, kindly provided by Alfa Wasserman, Italy) was incubated at 300 µg/ml with 4 mM ascorbic acid, 40 µM CuCl2 for 3 h at room temperature, buffered by 10 mM sodium phosphate buffer, pH 7.2. This method for metal-catalyzed oxidation has been reported to result in oxidation of methionine residues in rhIFNα2b (32). There are 6 methionines present in interferon α2b. The reaction was stopped with 1 mM EDTA and dialyzed overnight against 4 liters of PBS. For testing of dose-dependent immune reactivity toward amyloid-like properties in rhIFNα2b, mixtures of unmodified rhIFNα2b and oxidized rhIFNα2b were prepared containing 0, 25, 50, 75, and 100% oxidized rhIFNα2b (total protein concentration of rhIFNα2b was equal in all samples). A solution of 1 mg/ml ovalbumin (Sigma) in 67 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0, was gradually heated from 30 to 85 °C over a period of 12 min and afterward cooled on ice.

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Antibody Titer Determinations

rhIFNα2b—Sera were analyzed for IgG antibodies against native rhIFNα2b, coated in microtiter plates. Micronol high-binding 96-well plates were coated with 100 µl of native rhIFNα2b (2 µg/ml in PBS) per well for 1 h. The wells were drained and washed 4 times with 300 µl of PBST. After washing, the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 200 µl of 2% bovine serum albumin in PBS for 1 h. The wells were drained and washed 2 times with 300 µl of PBST. After the last wash, wells were carefully tapped dry on a tissue. Sera (diluted 100-fold with 2% bovine serum albumin in PBS) were added to the wells and the plates were incubated for 1 h. The plates were washed 4 times with 300 µl of PBST. After the last wash, wells were carefully tapped dry on a tissue. Because most immunogenicity problems of biopharmaceuticals are mediated by neutralizing the IgG, we chose to measure IgG titers against rhIFNα2b. Peroxidase-labeled anti-mouse IgG (Sigma) was added to the wells and the plates were incubated for 1 h. Plates were drained and washed 4 times with 300 µl of PBST and twice with 300 µl of PBS. After the last wash, wells were carefully tapped dry on a tissue. ABTS (2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) substrate (Roche) was added and the absorbance was recorded after 30 min on a Novapath microplate reader (Bio-Rad) at 415 nm and a reference wavelength of 490 nm. All incubations were in covered plates at room temperature with constant orbital shaking. Sera were defined positive when the absorbance of the 1:100 dilution minus the background was 3 times higher than the average absorbance value of the pretreatment sera minus the background. Antibody titers of the positive sera were determined by adding the sera in 3-fold 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, Graphpad software). The dilution needed to obtain 50% of the maximum absorbance was taken as the titer of the serum.

Ovalbumin—Sera were analyzed for antibodies against native ovalbumin coated in microtiter plates. Micronol high-binding 96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 50 µl of native ovalbumin (5 µg/ml in 100 mM NaHCO3, pH 9.6, 0.05% NaN3) per well for 1 h. Then the wells were drained and washed 2 times with 300 µl of PBST. After washing, the wells were tapped dry on a tissue. Wells were blocked by incubating with 200 µl of 1× Roche blocking reagent (Roche) in PBS for 1 h. The wells were drained and washed twice with 300 µl of PBST. After the last wash, wells were tapped dry on a tissue. Antibody titers were determined by adding pooled sera of each experimental group (n = 5) in 3-fold serial dilutions (starting from 1:10, 50 µl/well) to plates coated with native ovalbumin. The plates were washed 4 times with 300 µl of PBST. After the last wash, wells were tapped dry on a tissue. RAMPO, diluted 1:3000 in PBST, was added to the wells and incubated for 1 h. Plates were drained and washed 4 times with 300 µl of PBST and twice with 300 µl of PBS. After the last wash, wells were tapped dry on a tissue. The plates were stained for ~5 min using 100 µl/well of tetramethylbenzidine substrate.
Immunogenicity of Unfolded Biopharmaceuticals

(BIOSOURCE Europe, Nivelles, Belgium), the reaction was stopped with 50 μL/well of 2 M H2SO4 and read at 450 nm on a Spectramax340 microplate reader. Analysis of obtained data were performed as described above.

RESULTS

Various Biopharmaceuticals Display Amyloid-like Properties upon Exposure to Conditions of Stress, Indicating Protein Misfolding—During manufacturing and storage, biopharmaceuticals may also become exposed to various conditions of stress that can potentially underlie protein misfolding and the formation of amyloid-like properties. To mimic stability testing we examined whether exposure of biopharmaceuticals to conditions of stress, such as low pH or heat, induced amyloid-like properties. Fig. 1 shows that amyloid-like properties are adopted by Etanercept, Glucagon, Abciximab, and Infliximab upon exposure to these harsh denaturing conditions. Thus, like any protein, biopharmaceuticals can adopt common amyloid-like properties during misfolding and this phenomenon can be enhanced upon storage or under conditions of stress.

The Presence of Misfolded Protein with Amyloid-like Properties in Various Biopharmaceuticals—Next, we examined whether proteins with amyloid-like properties are present in marketed biopharmaceuticals. As indicators for amyloid-like properties we measured the fluorescence of ThT, Congo Red, and binding and activation of tPA, all qualitative measures for the presence of amyloid cross-β structure conformation in proteins in solution (17, 18). As shown in Table 1, several biopharmaceuticals showed significant potential to enhance fluorescence of thioflavin T and/or Congo Red, indicating the presence of amyloid-like structure. These biopharmaceuticals also bound tPA with high affinity and activated tPA-mediated plasminogen activation. These findings indicate that misfolded proteins with amyloid-like properties are present in various marketed therapeutic proteins.

Storage Increases the Level of Misfolded Protein in Biopharmaceuticals—Most protein pharmaceuticals can be stored for prolonged periods of time without losing their bioactivity. However, because proteins have the intrinsic propensity to lose their unique native structure, some fraction of proteins may gradually lose their structure and degrade. We examined the effect of storage on the level of protein with amyloid-like structure in a number of biopharmaceuticals, i.e., insulin, human albumin, and somatropin, proteins previously known to be able to form aggregates or amyloid under certain conditions. Fig. 2 shows that the level of protein with amyloid-like properties increases when these biopharmaceuticals were examined closer to their expiration date. These findings show that protein misfolding is time-dependent in biopharmaceuticals, when measured by elevated markers for amyloid structure.

TABLE 1

The presence of misfolded protein with amyloid-like properties in various biopharmaceuticals

Content of protein with amyloid-like properties in biopharmaceuticals was determined by enhancement of ThT and Congo Red fluorescence, binding of tPA and tPA-dependent plasminogen activation (% of standardized positive control). Biopharmaceuticals containing the highest levels of amyloid-like properties are listed at the top.

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<thead>
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<th>Fluorescence</th>
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<td></td>
<td>ThT</td>
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<td>Rmax (OD450 nm)</td>
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<td>A.U. ± S.D.</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1970 ± 5</td>
<td>978 ± 2</td>
<td>1.228</td>
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<tr>
<td>Somatropin</td>
<td>1317 ± 10</td>
<td>429 ± 2</td>
<td>0.9369</td>
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<tr>
<td>Insulin zinc suspension</td>
<td>387 ± 72</td>
<td>79 ± 6</td>
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<tr>
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<tr>
<td>Epoietin Alfa</td>
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<td>19 ± 3</td>
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<td>67 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>y-Globulin</td>
<td>25 ± 2</td>
<td>0 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>Glucagon</td>
<td>48 ± 1</td>
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* Plasma purified drug products.

a ND, not determined.

FIGURE 1. Various biopharmaceuticals display amyloid-like properties upon exposure to conditions of stress, indicating protein misfolding. N, native; D, denatured. Etanercept, Glucagon, Abciximab, and Infliximab were exposed to denaturing conditions (see ”Experimental Procedures”) and subsequently analyzed for the presence of amyloid-like properties, using ThT fluorescence (A) and tPA activation assay (B); expressed as percentage of standardized positive control.

TABLE 1

The presence of misfolded protein with amyloid-like properties in various biopharmaceuticals

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a ND, not determined.
We then examined whether a biopharmaceutical solution, containing misfolded protein, could trigger antibody formation and break tolerance. We chose to use metal-catalyzed oxidized rhIFNα2b as a model protein because it is known that this modification induces unfolding and aggregation of rhIFNα2b, which correlates with its immunogenicity (32, 33). As shown in Fig. 3, oxidation of rhIFNα2b induces amyloid-like properties as exemplified by the finding that oxidized rhIFNα2b greatly enhances ThT fluorescence, and facilitates the binding and activation of tPA. Native rhIFNα2b does not enhance ThT fluorescence and hardly binds or activates tPA (Fig. 3, A–C, respectively). These findings indicate that oxidation of rhIFNα2b induces amyloid-like properties, which is indicative for protein misfolding. The generation of IgG antibodies in wild-type mice against a foreign antigen (rhIFNα2b) was found to be significantly accelerated when oxidized rhIFNα2b with amyloid-like properties was injected, compared with the native protein (Fig. 3D). In contrast, mice transgenic for rhIFNα2b remained tolerant to native protein (Fig. 3E). In hIFNα2b transgenic mice injected with rhIFNα2b with amyloid-like properties, tolerance was broken and a potent IgG response was induced (Fig. 3E). These findings strongly indicate that protein misfolding, detectable by markers for amyloid, induces an immune response.

Next, we examined whether the immunogenic potential of rhIFNα2b was dependent on the level of misfolded rhIFNα2b with amyloid-like properties. We injected mice transgenic for hIFNα2b with preparations in which native and oxidized rhIFNα2b were mixed to obtain varying doses of protein with amyloid-like properties. ThT and tPA activating potential of the mixtures was determined and found to be gradually increased (Fig. 4, A and B). Again, in mice injected with solely unmodified rhIFNα2b no breaking of tolerance was observed (Fig. 4C). In mice injected with increasing doses of rhIFNα2b with amyloid-like properties, both the number of mice in which tolerance was broken rose from 2 to all 5 and their respective antibody titers were increased from a mean IgG titer of 22 to a titer of more than 230 (Fig. 4C). These observations demonstrate that immune responses against misfolded proteins, with amyloid-like properties, are dependent on the extent of protein misfolding that has occurred in a sample. This concentration effect was found to influence both the magnitude of raised antibody titers and incidence of tolerance breaking.
Besides formation of misfolded protein, oxidation of proteins also leads to modification of amino acids. To investigate whether immunogenicity could be induced by misfolded protein with amyloid-like properties in the absence of amino acid modifications, we used ovalbumin, an immunological model protein, that has been shown to form amyloid-type cross-β structure after gradual heating (35). As expected, the gradual heating of ovalbumin in solution led to both large increases in the enhancement of ThT fluorescence (Fig. 5) and increased in the capacity to activate tPA (Fig. 5B). Mice immunized with this heat-denatured ovalbumin developed 2 weeks after immunization with a 5-fold higher general antibody titer, compared with mice injected with freshly dissolved ovalbumin (Fig. 5C). This further indicates that immunogenicity is caused by common structural characteristics, i.e. amyloid-like properties, without the need of chemical protein modification.

DISCUSSION

The advent of recombinant technology has enabled the large scale production of biopharmaceuticals, such as FVIII, EPO, IFN, and various monoclonal antibodies. The use of these biopharmaceuticals is very promising and the number of biopharmaceuticals is expected to rise rapidly (1). Unfortunately, the generation of antibodies against therapeutic proteins has posed a mystifying problem for biopharmaceutical manufacturers, medical practitioners, and scientists. We now show that adoption of generic amyloid-like proper-

**FIGURE 4.** Breaking of tolerance by misfolded IFNα2b is dose-dependent. Native and oxidized rhIFNα2b were mixed in a ratio of 1:0, 3:1, 1:1, –1:3, and 0:1. This resulted in increasing levels of protein with amyloid-like properties between samples, as confirmed by the enhanced fluorescence of ThT (A) and determination of tPA activating capacity (B; expressed as percentage of standardized positive control). Antibody generation in transgenic mice increased as the level of injected with amyloid-like properties increased (C). The values represent antibody titers of individual mice (3 mice per group) 14 days after the start of the experiment.

**FIGURE 5.** Oxidation-independent misfolding of ovalbumin leads to an enhanced immune response. Amyloid-like properties were determined by the thioflavin-T fluorescence enhancement assay (25 μg/ml protein) (A) and tPA-dependent plasmin generation assay (100 μg/ml protein). Antibody titers in BALB/c mice after 14 days (C), preimmune sera contained no α-OVA antibodies (not shown). nOVA, native ovalbumin; dOVA, heat denatured ovalbumin.
ties, a hallmark for misfolded proteins, is a potential cause of drug-induced immunogenicity.

So far, several seemingly unrelated factors have been described to influence biopharmaceutical immunogenicity. For instance, post-translational modifications such as oxidation, deamidation, and aggregation influence protein immunogenicity (14, 15). Strikingly, specific modifications in formulations and packaging have also been correlated to biopharmaceutical immunogenicity (36, 37). Most of these factors may influence the structural properties of a therapeutic protein molecule (23, 26). Metal-catalyzed oxidation of rhIFNα2b was described to lead to increased immunogenicity (32). We found that metal-catalyzed oxidation of rhIFNα2b resulted in a conformational change accompanied by adoption of amyloid-like properties, indicative for protein misfolding, that was far more immunogenic in vivo than its native counterpart and broke immune tolerance. We also found that, in general, biopharmaceuticals can have amyloid-like properties and that these properties can be induced upon storage or conditions of stress. These data indicate that misfolded proteins with amyloid-like properties can be responsible for enhanced immunogenicity of biopharmaceuticals and breaking of tolerance. Based on our results, we propose a unifying mechanism by which individual immunogenic factors, such as oxidation or formulation changes, via the formation of misfolded protein with common amyloid-like properties, ultimately lead to an (enhanced) immune response. Protein misfolding in biopharmaceutical solutions is expected to lead to a heterogeneous distribution of aggregates (39). Similarly, our preparations of misfolded rhIFNα2b and ovalbumin may very well contain multiple species of aggregates and protein in both native and misfolded conformations. However, once misfolding is induced, markers for amyloid increase and this dose-dependently correlates to the immunogenic potential of the preparation. Additional investigations are required to isolate the exact immunogenic misfolded protein species, but at present time it is difficult to predict and control the stability of fibrils and oligomeric species, especially in vivo. Hopefully, assays using antigen-presenting cells will help to answer this question and further elucidate the underlying mechanism.

Our results point to a common mechanism by which the immune system perceives misfolded proteins. We hypothesize that this lies in the changed conformation of the protein backbone itself. This implies that the innate immune system may be activated by recognition of the amyloid-like properties of misfolded protein. Indeed, several cellular receptors for the amyloid-like protein fold have been identified: scavenger receptor A, CD36, receptor for advanced glycation end products, low density lipoprotein receptor-like protein, and scavenger receptor B type I (17). Moreover, these receptors are expressed on dendritic cells and could initiate an immune response against these proteins (34, 38).

Drug formulation is an important factor in the development of biopharmaceuticals. This also affects immunogenicity, which, for example, has been observed with a specific formulation of EPO, that was far more immunogenic than its previous formulation (36, 37). One of the main problems in formulation development is maintaining protein stability, because chemical modification, adsorption, and most importantly, aggregation phenomena are generally difficult to prevent (14, 29). Our findings indicate that various biopharmaceuticals have a tendency to misfold, which may result in the generation of immunogenic proteins with amyloid-like properties in various drug products that are on the market. At this moment, it is difficult to predict those conditions that will preserve the native non-immunogenic fold of a protein pharmaceutical. Identification of conditions, compounds, excipients, and materials that induce amyloid-like properties in therapeutic proteins during production and/or storage may be valuable for improving the quality of biopharmaceuticals by reducing immunogenicity related adverse effects. Monitoring the formation of amyloid-like properties in a biopharmaceutical may even have predictive value toward pre-clinical determination of expiry dates for a drug, because it seems to be a marker for an immunogenic protein entity. Hereto, it is required to pinpoint the exact markers for amyloid-like properties that correlate best with immunogenicity and further develop sensitive and robust tools to detect these misfolded protein species. This may ultimately lead to development of better and safer drugs.

Acknowledgments—We thank Miranda van Berkel, Frank Miedema, Huub Schellekens, Bonno Bouma, and Peter Lenting for helpful discussion and Tamara Stevens, Mieke Poland, and Bettina Schiks for valuable assistance. We are very grateful to Alfa Wasserman for kindly providing rhIFNα2b.

REFERENCES
Immunogenicity of Unfolded Biopharmaceuticals