Immunogenicity of Recombinant Human Interferon Beta Interacting with Particles of Glass, Metal, and Polystyrene

MIRANDA M.C. VAN BEERS,[1,2] FRANCESCA GILLI,[3] HUUB SCHELLEKENS,[2] THEODORE W. RANDOLPH,[4] WIM JISKOOT[1]

[1]Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands

[2]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

[3]Clinical Neurobiology Unit, Neuroscience Institute Cavalieri Ottolenghi, University Hospital San Luigi Gonzaga, Orbassano, Italy

[4]Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado

Received 19 November 2011; revised 2 August 2011; accepted 10 August 2011

Published online 14 September 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22744

ABSTRACT: Aggregates play a major role in the immunogenicity of recombinant human interferon beta (rhIFNβ), a protein used to treat multiple sclerosis. A possible cause of aggregation is interaction between therapeutic protein and surfaces encountered during processing, storage, and administration. Moreover, proteins may adsorb to particles shed from these surfaces. In this work, we studied the immunogenicity of recombinant human interferon beta-1a (rhIFNβ-1a) interacting with glass microparticles, stainless steel microparticles, and polystyrene nanoparticles. At physiological pH, rhIFNβ-1a readily adsorbed to the particles, while the degree of adsorption was influenced by the ionic strength of the phosphate buffer. Front-face fluorescence showed that the tertiary structure of rhIFNβ-1a slightly changed upon adsorption to glass. The interaction with stainless steel microparticles resulted in increased levels of aggregates in the free protein fraction. Furthermore, protein adsorbed to stainless steel microparticles was more difficult to desorb than protein adsorbed to glass. Incubation with stainless steel considerably enhanced the immunogenicity of rhIFNβ-1a in transgenic mice immune tolerant for human interferon beta. The protein fraction adsorbed on stainless steel particles was responsible for this. In conclusion, rhIFNβ-1a adsorbs to common hydrophilic surface materials, possibly increasing the immunogenicity of the protein. © 2011 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:187–199, 2012

Keywords: adsorption; fluorescence spectroscopy; immunogenicity; microparticles; nanoparticles; protein aggregates; protein structure; recombinant human interferon beta; transgenics

INTRODUCTION

Interferon beta is a cytokine produced by macrophages and epithelial and fibroblast cells with anti-inflammatory, antitumor, and antiviral functions. Recombinant human interferon beta (rhIFNβ) has been established as a therapeutic protein in the treatment of multiple sclerosis (MS), an immune-mediated demyelinating disease of the central nervous system.[1,2] Chronic administration of the protein reduces the frequency of exacerbations characteristic of relapsing–remitting forms of MS. Nevertheless, a number of patients do not respond to rhIFNβ therapy. Therapeutic response depends on patient characteristics such as baseline expression of type I interferon, type I-interferon-induced genes, and other genetic factors.[3,4]

Some patients initially respond to rhIFNβ therapy, but show a decline in the effectiveness of the drug over time during long-term administration. This can be attributed to the formation of neutralizing antibodies (NABs), which usually recognize an epitope close to the receptor binding site of the protein, thereby inhibiting activity of the protein.[2] The

Abbreviations used: ANS, 8-anilino-1-naphthalene-sulfonic acid; BAB, binding antibody; hIFNβ, human interferon beta; i.p., intraperitoneally; MS, multiple sclerosis; MxA, myxovirus resistance protein A; NAB, neutralizing antibody; rhIFNβ, recombinant human interferon beta; rhIFNβ-1a, recombinant human interferon beta-1a; rhIFNβ-1b, recombinant human interferon beta-1b; RSA, random sequential adsorption; TRU/mL, 10-fold reduction units per mL.

Correspondence to: Wim Jiskoot (Telephone: +31-71-5274314; Fax: +31-71-5274565; E-mail: w.jiskoot@lacdr.leidenuniv.nl)

Journal of Pharmaceutical Sciences, Vol. 101, 187–199 (2012)

appearance of NABs is often preceded by formation of binding antibodies (BABs). BABs against rhIFNβ typically appear after 9–18 months of treatment.[5] They do not interfere with the intrinsic biological activity of the protein, but may affect clearance and, thereby, the biological effect of the protein. Avonex® [recombinant human interferon beta-1a (rhIFNβ-1a), Biogen Idec, Cambridge, Massachusetts], Rebif® (rhIFNβ-1a, EMD Serono, Rockland, Massachusetts), and Betaseron/Betaferon® [recombinant human interferon beta-1b (rhIFNβ-1b), Bayer, Montville, New Jersey] are three of the commercial interferon products for MS therapy. The products are all immunogenic, albeit to different extents. Betaseron/Betaferon® (Bayer) induces the highest percentage of patients developing antibodies, whereas Avonex® (Biogen Idec) shows the lowest immunogenicity.[6] Among the multiple factors influencing immunogenicity, in particular, aggregation of a therapeutic protein is known to increase its potential to elicit an immune response.[7–9] Recombinant human interferon beta-1b aggregates likely cause the high clinical immunogenicity of Betaseron/Betaferon® (Bayer).[6,10] Indeed, transgenic mice immune tolerant for human interferon beta (hIFNβ) formed antibodies against Betaseron/Betaferon® (Bayer),[11,12] whereas aggregate-free rhIFNβ-1b produced by high hydrostatic pressure treatment was not immunogenic in these mice.[13]

Aggregates may comprise therapeutic protein or therapeutic protein associated with other substances such as excipients. For instance, recombinant human interferon alpha forms immunogenic aggregates with human serum albumin,[14] which is used as a stabilizer in some protein pharmaceuticals including Betaseron/Betaferon® (Bayer). A therapeutic protein may also interact with the surface of materials encountered during manufacture, fill and finish, storage, or administration of the protein. For example, interaction of interleukin-2 with silicone rubber delivery tubing reduced its activity by 97%,[15] adsorption of factor VIII onto polyvinyl chloride infusion bags decreased its activity by 42%,[16] silicone oil syringe lubricant induced aggregation of several model proteins,[17] silica nanoparticles caused aggregation of recombinant human platelet-activating factor acetylhydrolase,[18] tungsten leachates induced protein precipitation,[19] interaction of an immunoglobulin G (IgG) 2 with Teflon containers during freeze–thawing resulted in aggregation,[20] and stainless steel microparticles caused aggregation of a monoclonal antibody.[21] Tyagi et al.[22] demonstrated that stainless steel particles were shed from a filler pump, causing aggregation of a monoclonal antibody. Also, glass particles are known to be a major source of particulate matter in parenteral solutions.[23] Interaction of protein with particles can lead to adsorption of the protein to the particles[24] and/or surface-induced aggregation of free protein in solution. Adsorption to the particle may occur in a monolayer[24] or multilayer, possibly resulting in unfolding of the protein or other physical or chemical changes in the structure.

In previous work we had characterized aggregates of rhIFNβ-1a and showed that removal of these aggregates eliminated immunogenicity in transgenic, immune tolerant mice.[25] In this work we studied the immunogenicity of rhIFNβ-1a incubated with glass and stainless steel microparticles and polystyrene nanoparticles. Glass and stainless steel are materials to which rhIFNβ-1a is exposed during production and packaging in vials and prefilled syringes. We used polystyrene nanoparticles as model particles because of their known ability to increase the immunogenicity of peptide and protein vaccines.[26,27] The aim of this research was to gain insight in the adsorption behavior of rhIFNβ-1a onto different hydrophilic surfaces and how this relates to the immunogenicity of the protein. Therefore, the interaction of rhIFNβ-1a with glass, stainless steel, and polystyrene particles, as well as the effects thereof on protein structure and aggregation, was characterized. Moreover, the immunogenicity of rhIFNβ-1a incubated with particles was studied using hybrid transgenic mice that are immune tolerant for hIFNβ.[12,25]

MATERIALS AND METHODS

Recombinant Human Interferon Beta

Recombinant human interferon beta-1a was supplied by Biogen Idec, Inc. (Cambridge, Massachusetts) as 0.26 or 1.0 mg/mL solution in 20 mM sodium acetate buffer and 154 mM arginine at pH 4.8. Before use, these solutions were dialyzed in a 3.5 kDa molecular weight cutoff Slide-A-Lyzer Cassette (Perbio Science, Etten-Leur, The Netherlands) against 100 mM sodium phosphate buffer and 200 mM sodium chloride at pH 7.2. This solution is further referred to as bulk rhIFNβ-1a and its phosphate buffer as 300 mM phosphate buffered saline (PBS).

Nanoparticles and Microparticles

Glass microparticles were prepared from borosilicate syringe barrels (Becton Dickinson, Franklin Lakes, New Jersey) by ball milling as described by Bee et al.[24] This resulted in ground glass particles with a mean size of $1.1 \pm 0.1\,\mu m$ (SD), a specific surface area of $5.20 \pm 0.03\,m^2/g$, and a ζ-potential of $-41 \pm 1\,mV$, as measured by Bee et al.[24] Metal microparticles (Ametek, Inc., Eighty Four, Pennsylvania) consisted of stainless steel alloy P316L, and their mean size was $14 \pm 1\,\mu m$ (SD), specific surface area was $0.15 \pm 0.01\,m^2/g$, and ζ-potential was $-11 \pm 1\,mV$, as measured by Bee et al.[24] Carboxylate polystyrene nanoparticles (Polybeads®) were obtained from

Brunschwig Chemie (Amsterdam, The Netherlands) as a 2.78% (w/v) latex suspension in deionized water. The polystyrene particles had a diameter of 0.209 ± 0.011 μm (SD) and a density of 1.05 g/mL. Their specific surface area as predicted by the formula for the surface of a solid sphere is 27 ± 1 m^2/g.

High-Performance Size-Exclusion Chromatography

Samples (100–200 μL) were analyzed with a TSKgel Super SW2000 column and Super SW guard column (Sigma-Aldrich, Munich, Germany), and chromatograms were recorded with a Shimadzu SPD-6AV UV detector (Shimadzu, Duisburg, Germany) at a wavelength of 280 nm. A Waters 515 high-performance liquid chromatography pump and 717 Plus autosampler (Waters, Etten-Leur, The Netherlands) were operated at a flow rate of 0.35 mL/min. The mobile phase consisted of 300 mM PBS with 0.05% (w/v) sodium azide and 0.1% (w/v) sodium dodecyl sulfate (SDS), and was filtered through a 0.2-μm filter prior to use.

Adsorption of rhIFNβ-1a to Particles

Recombinant human interferon beta-1a was adsorbed to nano- and microparticles by incubating triplicate, 1 mL samples of 40 μg/mL protein with various amounts of particles in 1.5-mL polypropylene microcentrifuge tubes on a roller mixer (Boule 820, Stockholm, Sweden) at 15 rpm for 30 min at room temperature. The dispersions were centrifuged at 17,000 g at 4°C for 10 min (or 20–30 min for polystyrene particles, until the supernatant was clear). The supernatant (200 μL) was analyzed by high-performance size-exclusion chromatography (HP-SEC) to determine the free protein fraction in solution. The free protein fraction of rhIFNβ-1a was calculated by normalizing the total area of all peaks observed between 6.5 and 11 min in the HP-SEC chromatograms obtained for samples containing particles to the total peak area observed for samples without added particles. The fraction of adsorbed protein was then calculated by mass balance. Adsorption isotherms were prepared by measuring the fraction of rhIFNβ-1a adsorbed after mixing with various amounts of particles in 150 mM PBS (50 mM sodium phosphate and 100 mM sodium chloride at pH 7.2) or in 300 mM PBS (100 mM sodium phosphate and 200 mM sodium chloride at pH 7.2).

Aggregation State of Free Protein Fraction

High-performance size-exclusion chromatography was used to assess the aggregation state of the free protein fraction. Samples (4 mL) of 300 μg/mL rhIFNβ-1a were incubated with 110 mg glass, 2270 mg metal, or 4.9 mg polystyrene particles per milligram protein in 150 mM PBS. Particle amounts were selected based on measured adsorption isotherms (see *Results* section). After centrifugation, 100 μL of the supernatant was analyzed in duplicate by HP-SEC.

Desorption of Protein from the Particles

The reversibility of adsorption of rhIFNβ-1a to the particulate materials was studied using three different desorption solutions: 300 mM PBS, 20 mM sodium acetate buffer with 150 mM arginine and 0.04 mM Tween 20 at pH 4.8 (acetate), or 0.1% (w/v) sodium dodecyl sulfate in water. Samples (250 μL) of 100 μg/mL rhIFNβ-1a were incubated with 110 mg glass, 2270 mg metal, or 4.9 mg polystyrene particles per milligram protein in 10 mM sodium phosphate and 20 mM NaCl at pH 7.2. After 30 min of incubation, 250 μL of one of the desorption solutions or 250 μL of water as a control was added and the samples were reincubated for 30 min in triplicate. After centrifugation, 200 μL of the supernatant was analyzed by HP-SEC to assess how much rhIFNβ-1 had been desorbed.

Front-Face Fluorescence of Protein Adsorbed on Glass

The tertiary structure of rhIFNβ-1a adsorbed on glass was characterized with front-face fluorescence spectroscopy according to Bee et al.[24] A SLM-Aminco spectrofluorometer (SLM-Aminco, Urbana, Illinois) was operated at 25°C and bandwidths of 4 nm were used. Fluorescence spectra of dispersions (2 mL) of rhIFNβ-1a adsorbed on glass particles were obtained at a 53° angle of incidence to avoid scattering signals. The dispersions were stirred during the measurements to prevent a decrease in the intensity due to settling. For front-face fluorescence measurements, bulk rhIFNβ-1a (68 μg/mL) was adsorbed onto 450 mg glass per milligram protein in 150 mM PBS. To remove nonadsorbed protein, the dispersion was centrifuged three times, and each time the pellet was washed by replacing the supernatant with 150 mM PBS and subsequent vortexing. According to HP-SEC, the adsorbed protein concentration was 63 μg/mL and no detectable levels of free protein were present after washing. Protein control samples were prepared by heating 68 μg/mL bulk rhIFNβ-1a in 150 mM PBS for 10 min at 90°C or by incubation with 6 M guanidine hydrochloride.

Tryptophan Fluorescence

Intrinsic tryptophan fluorescence was measured by excitation at λ = 295 nm and recording the emission from 300 to 380 nm. All samples were measured in a front-face setup. Sample spectra were corrected with corresponding blank spectra of 150 mM PBS with or without glass particles or 6 M guanidine hydrochloride.

8-Anilino-1-Naphthalene-Sulfonic Acid Fluorescence

8-Anilino-1-naphthalene-sulfonic acid (ANS) fluorescence spectra were measured after incubation of rhIFNβ-1a samples with ANS (Sigma–Aldrich) in 150 mM PBS for 2 h while rotating at 8 rpm. A rhIFNβ-1a to ANS molar ratio of 1:10 was applied according to Fan et al.[28] All spectra were recorded from 400 to 600 nm in a front-face setup after excitation at 372 nm. The weak emission spectra of ANS in the corresponding buffers with or without particles or guanidine hydrochloride were used as blanks. Emission intensities at $\lambda = 476$ nm were used to calculate ANS fluorescence intensity relative to that of bulk rhIFNβ-1a.

Tryptophan Fluorescence Quenching

Tryptophan fluorescence was quenched by adding increasing amounts of acrylamide (Sigma–Aldrich) to the protein samples. Surface-exposed tryptophan residues are quenched by acrylamide to a greater extent than buried tryptophan residues, and can, therefore, be used to understand relative tertiary structure of the protein.[29] The quenching of rhIFNβ-1a adsorbed on glass particles was measured in a front-face setup, whereas that of the protein control solutions without particles was measured in normal mode using a 90° geometry and an inner filter correction of 0.23 M^{-1} acrylamide.[24,29] Tryptophan residues were excited at $\lambda = 295$ nm and 5 μL aliquots of 7 M acrylamide were added to 2 mL samples. After mixing with a pipette, fluorescence intensities were scanned from 300 to 380 nm. The Stern–Volmer constant was derived from a plot of the fluorescence intensity at $\lambda =$ 337 nm upon increasing the acrylamide concentration and the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher (corrected for dilution), respectively, $[Q]$ is the quencher concentration (in M), and K_{SV} is the Stern–Volmer constant (in M^{-1}).

Immunogenicity in a Transgenic, Immune Tolerant Mouse Model

Adsorption Samples

In the first mouse study, the immunogenicity of rhIFNβ-1a adsorbed on glass, metal, and polystyrene particles was tested, using bulk rhIFNβ-1a and Betaferon®–rhIFNβ-1b as controls with demonstrated low and high immunogenicity, respectively.[25] Dispersions were prepared by incubating 28 mL of 55.5 μg/mL rhIFNβ-1a in a 50-mL plastic centrifuge tube with 110 mg glass, 2270 mg metal, or 4.9 mg polystyrene per milligram protein in 150 mM PBS. After incubation, HP-SEC showed that the glass sample contained 78% adsorbed protein, whereas the metal and polystyrene samples contained 71% and 46% adsorbed protein, respectively. The entire sample, including free protein and particles with adsorbed protein, was used for injection. At the end of the *in vivo* experiment, it was confirmed by HP-SEC that the protein concentration as well as the mono-, di-, and oligomer composition of the free protein fraction in the adsorption samples had not changed.

For the second mouse study, 50 mL of 55.5 μg/mL rhIFNβ-1a was incubated in a 50-mL plastic centrifuge tube with 2270 mg metal per milligram protein in 150 mM PBS. The dispersion was left at 4°C for 20 min to allow the metal particles to settle and, subsequently, 40 mL of supernatant was removed. The supernatant, containing 66% of the original protein concentration and no visible metal particles, was used for injection. The remaining supernatant (10 mL) was removed from the precipitate and discarded. The precipitate, containing 34% of the original protein fraction according to the mass balance, was resuspended in 50 mL of 150 mM PBS. The resuspended precipitate was used for injection. No free protein was detected in this sample when tested for desorption by HP-SEC after the *in vivo* study.

Mouse Breeding

Heterozygous C57Bl/6 transgenic mice immune tolerant for hIFNβ developed by Hermeling et al.[11] were bred at the Central Laboratory Animal Institute (Utrecht University, Utrecht, The Netherlands). The strain was maintained by crossing the transgenics with wildtype C57Bl/6 mice obtained from Janvier (Bioservices, Uden, The Netherlands). The genotype of the offspring was determined by polymerase chain reaction (PCR) showing the presence or absence of the hIFNβ gene in chromosomal DNA isolated from ear tissue. Transgenic C57Bl/6 mice were crossed with wildtype FVB/N mice obtained from Janvier and their C57Bl/6 × FVB/N hybrid offspring were genotyped using PCR. Both transgenic C57Bl/6 × FVB/N hybrid mice and their nontransgenic (wildtype) littermates, evaluated previously as a mouse model for hIFNβ,[12] were used.

In Vivo Studies

The animal experiments were approved by the Institutional Ethical Committee. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available *ad libitum*. For the first *in vivo* study, blood was drawn from the cheek pouches (submandibularly) of 40 wildtype and 40 transgenic mice before starting the treatment.[11,12] Eight mice per group were injected intraperitoneally (i.p.) with 5 μg of bulk, metal-incubated, glass-incubated, or polystyrene-incubated rhIFNβ-1a, or 5 μg of Betaferon®–rhIFNβ-1b on days

0–4, days 7–11, and days 14–18. Blood was collected submandibularly just before treatment with rhIFNβ on days 7 and 14. On day 21, all mice were sacrificed by bleeding through cardiac puncture under isoflurane anesthesia. For the second *in vivo* study, again blood was drawn from 40 wildtype and 40 transgenic naïve mice, and the same blood collection and injection schedule as described above was used. Ten mice per group were injected i.p. 5 days per week with 5 μg bulk rhIFNβ-1a, 1.7 μg rhIFNβ-1a fully adsorbed on metal particles, 3.3 μg of non-adsorbed rhIFNβ-1a without metal particles, or 5 μg of Betaferon®–rhIFNβ-1b. Blood samples were collected in lithium heparin gel tubes and centrifuged for 10 min at 3000 *g*, and the obtained plasma was stored at –80°C until analysis.

BAB Assay

Titers of BABs against rhIFNβ were measured in plasma by direct enzyme-linked immunosorbent assay according to the protocol described in detail by Hermeling et al.[11] Plates were coated with bulk rhIFNβ-1a. Absorbance values were measured with a BioTek EL808 microplate reader (Beun de Ronde, Abcoude, The Netherlands) at a wavelength of 405 nm and a reference wavelength of 490 nm. The 100-fold diluted plasma samples were screened and defined positive if the background corrected absorbance values were ten times higher than that of the pretreatment plasma.[11,12] The titer of anti-hIFNβ IgG in positive plasma was determined by plotting the absorbance values of a serial dilution against ^{10}log dilution. The plots were fitted to a sigmoidal dose-response curve using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California). The reciprocal of the dilution of the half-maximal effective concentration was considered the titer of the plasma sample.[11,12,25] A standard anti-hIFNβ sample consisting of the pooled plasma of mice treated for 3 weeks with Betaferon®–rhIFNβ-1b was added to each plate, and its average ^{10} log titer was 2.65 ± 0.09 (SD; $n = 29$). Nonresponders were given a titer of 10 and statistical analyses were performed with a Kruskal–Wallis nonparametric one-way analysis of variance and Dunn's multiple comparison test.

NAB Assay

Neutralizing antibody levels in the plasma samples of day 21 were assessed in a bioassay based on the inhibition of induction of myxovirus resistance protein A (MxA) gene expression in an A549 cell line, as previously described.[30] The type of rhIFNβ (1a or 1b) used in the assay was the same as the type used for injecting the animal. Both MxA and a control housekeeping gene derived messenger RNA (eukaryotic 18S ribosomal RNA) were detected with a real-time reverse transcription-PCR multiplex assay. Neutralizing activity was expressed in 10-fold reduction units per mL (TRU/mL). Plasma samples with a neutralizing activity below 130 TRU/mL were considered negative.

RESULTS

Adsorption of rhIFNβ-1a at Different Ionic Strengths

In the absence of particles, about 15% of rhIFNβ-1a could not be recovered with HP-SEC, which is in line with previous observations, probably due to aggregates too large to enter the column or unfolded protein irreversibly attached to the column material.[25] The addition of increasing amounts of glass, metal, or polystyrene particles to solutions of rhIFNβ-1a resulted in a decrease in free rhIFNβ-1a for all particle types (Fig. 1). Depletion of free protein from the solution can be attributed to the adsorption of protein onto the particle surface. Incubation and centrifugation of rhIFNβ-1a in the absence of particles did not affect the percentage of nonrecovered protein (not shown). Protein adsorption occurred in both 150 and 300 mM PBS, although the level of adsorption was lower at higher ionic strength of the buffer. This suggests that electrostatic forces play a role in the adsorption of rhIFNβ-1a. The particles are hydrophilic and negatively charged at pH 7, as expected from their ζ-potential (glass and metal) and high degree of carboxylation (polystyrene), whereas rhIFNβ-1a is positively charged according to its isoelectric point.[32] At higher ionic strength, charge shielding reduces the attractive charge–charge interactions between protein and the particles. The adsorption of rhIFNβ-1a to polystyrene particles was less affected by ionic strength, suggesting that other types of interactions also play a role.

Random Sequential Adsorption Model for rhIFNβ-1a

Theoretical linear adsorption isotherms were calculated from random sequential adsorption (RSA) model (Fig. 1). This is a model for irreversible random adsorption of hard discs onto a surface, assuming that the discs form a monolayer and do not overlap.[31] According to the model, the surface area per milligram protein that is needed to create exactly one monolayer of rhIFNβ-1a on the particles (the adsorption footprint) can be predicted as follows:

$$\text{Adsorption footprint } (\text{m}^2/\text{mg}) = \frac{A \times N_\text{A}}{1000 M \times \theta} \quad (2)$$

where A is the projected area of the adsorbed rhIFNβ-1a molecule on the surface (m^2), N_A is the Avogadro constant, M is the molecular weight of rhIFNβ-1a (22,500 g/mol), and θ is the assumed packing efficiency (0.547 for RSA).[31] A is calculated from the surface area of a disc of 4-nm diameter, which is the

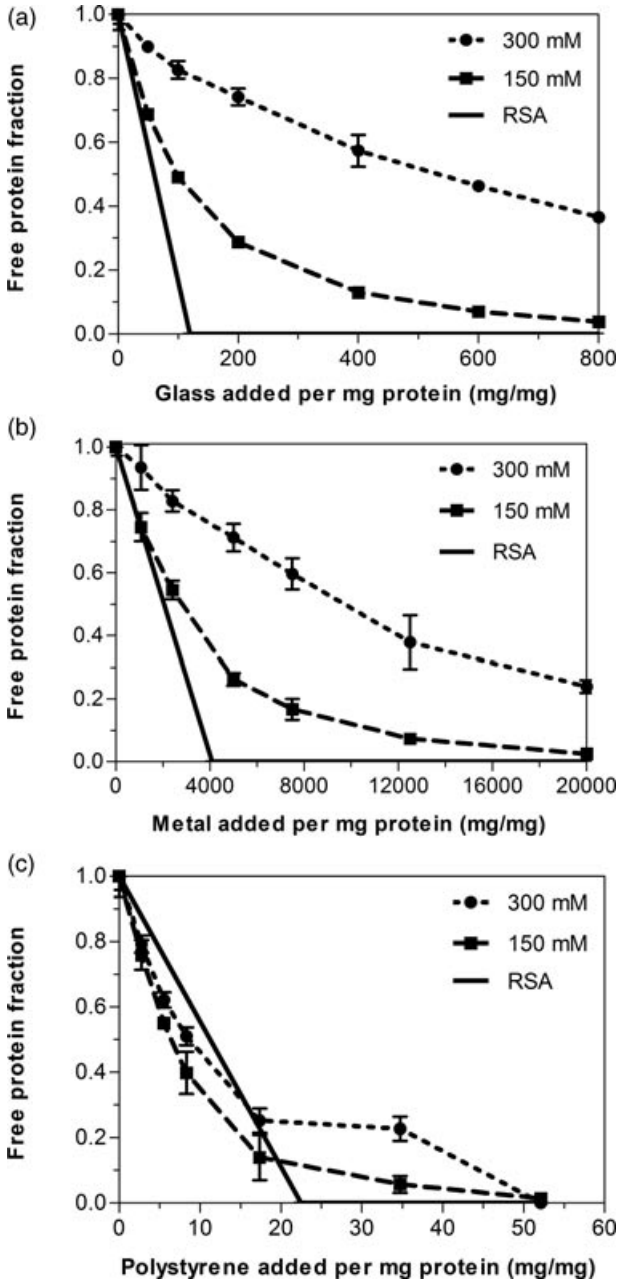

Figure 1. Adsorption isotherms of rhIFNβ-1a on particles of (a) glass, (b) metal, and (c) polystyrene in 50 mM sodium phosphate buffer with 100 mM NaCl at pH 7.2 (150 mM PBS) or in 100 mM sodium phosphate buffer with 200 mM NaCl at pH 7.2 (300 mM PBS). Theoretical linear isotherms are presented according to the RSA model, assuming that 54.7% of the particle surface is covered by protein.[31]

maximum estimated hydrodynamic diameter of a rhIFNβ-1a monomer according to its crystal structure.[33] Thus, the predicted adsorption footprint for rhIFNβ-1a is 0.61 m^2/mg, which corresponds to 120 mg glass particles (Fig. 1a), 4100 mg metal particles (Fig. 1b), or 23 mg polystyrene particles (Fig. 1c) per milligram protein.

Evaluating rhIFNβ-1a Adsorption with the RSA Model

The measured adsorption isotherms in Figure 1 were used to calculate adsorption footprints and corresponding packing efficiencies in 150 and 300 mM PBS (Table 1). For each particle type and buffer strength, the measured footprint was larger than that predicted by the RSA model; thus, packing efficiencies of proteins on particles were lower than the theoretical maximum surface packing of 0.547. This could be due to reversible or nonrandom (preferential) adsorption, unfolding of the protein, or agglomeration of the particles. Moreover, all adsorption isotherms (Fig. 1) showed considerable curvature in contrast with the high degree of linearity observed previously for a monoclonal antibody in 10 mM sodium acetate (pH 5.0) adsorbing to glass and metal.[24] This suggests a relatively low affinity between rhIFNβ-1a and the particles, or adsorption of multiple rhIFNβ-1a species with different affinities. Increasing the ionic strength of the buffer using 300 mM PBS instead of 150 mM PBS further reduced the protein coverage on the particles (Table 1). At lower ionic strength and low particle amounts, the surface coverage of rhIFNβ-1a on the particles approached maximum RSA packing levels (Fig. 1). Maximum packing efficiencies were reached by incubating rhIFNβ-1a with 15 mg or less polystyrene particles per milligram protein (Fig. 1c). It should be noted, though, that the polystyrene particles are highly porous. According to the manufacturer, a polystyrene bead with numerous single-chain polymers resembles a ball of wool, and the formula for the surface of a solid sphere perhaps underestimates its rhIFNβ-accessible surface area.

Selecting rhIFNβ-1a Adsorption Conditions

Dispersions with relatively low particle amounts (110 mg glass, 2270 mg metal, and 4.9 mg polystyrene) per milligram protein in 150 mM PBS were used for further characterization and *in vivo* immunogenicity testing. By applying these conditions, the formation of a monolayer of randomly distributed rhIFNβ-1a molecules on the surface of the particles with a high packing density of almost 55% was achieved. Only the samples for fluorescence analysis of the adsorbed protein were prepared with higher amounts of glass (450 mg) per milligram protein, which was needed to increase the adsorbed protein concentration and thereby improve the signal-to-noise ratio.

Aggregation State of the Free Protein Fraction

The free protein fraction was analyzed with HP-SEC to examine whether incubation with glass, metal, or polystyrene changed the monomer and oligomer composition of bulk rhIFNβ-1a (Table 2). Incubation with glass particles did not induce significant changes in the percentage of monomer (82% monomer vs. 85%

Table 1. Footprint Values Based on Linear Regression of the Adsorption Isotherms of rhIFNβ-1a on Glass, Metal, and Polystyrene Particles as Shown in Figure 1, with Corresponding Packing Efficiencies Derived from Equation (2)

	Glass		Metal		Polystyrene	
	150 mM[a]	300 mM[b]	150 mM[a]	300 mM[b]	150 mM[a]	300 mM[b]
Measured footprint ± SD (mg/mg protein)	590 ± 10	1200 ± 30	13,000 ± 400	23,000 ± 2000	35 ± 2	41 ± 1
Corresponding packing efficiency (θ)	0.10	0.05	0.15	0.10	0.35	0.30
Goodness of fit (r^2)	0.75	0.96	0.71	0.96	0.71	0.81

[a] In a buffer of 50 mM sodium phosphate and 100 mM NaCl at pH 7.2.
[b] In a buffer of 100 mM sodium phosphate and 200 mM NaCl at pH 7.2.

monomer in bulk rhIFNβ-1a) and oligomer, suggesting that there was no preferential adsorption of one of the (monomeric or oligomeric) species. The interaction with metal particles led to an increase of 11% in the oligomer content of the soluble fraction, which could be due to either the formation of new aggregates or preferential adsorption of rhIFNβ-1a monomer to the metal surface. In contrast, the free protein fraction, after incubation with polystyrene, contained 5% more monomer than the starting solution, thus indicating that the rhIFNβ-1a oligomers were more prone to adsorption to the polystyrene surface than the monomers.

Desorption of Protein from the Particles

Mixtures of rhIFNβ-1a and particles were incubated in a buffer of low ionic strength (10 mM sodium phosphate and 20 mM NaCl at pH 7.2), resulting in large fractions of adsorbed protein, ranging from 67% to 98% (Table 2). Desorption of rhIFNβ-1a was studied by adding three different desorption solutions. The addition of a high ionic strength buffer (300 mM PBS) resulted in 55% of the adsorbed protein desorbing from the glass particles, whereas the same buffer induced considerably less desorption of protein from metal and polystyrene particles. Probably, the electrostatic interaction of rhIFNβ-1a with glass particles was weaker than with metal and polystyrene particles, and/or other types of interactions played a role for metal and polystyrene.

Incubation of the rhIFNβ-1a–particle mixtures in a low-pH acetate buffer induced only moderate desorption, with the highest percentage desorbing from the metal particles (35%). At lower pH, the higher positive net charge of the protein increases its solubility. Moreover, hydrogen ions in the buffer could react with oxide or hydroxide groups on the surface of metal particles, resulting in less electrostatic interaction between the particles and rhIFNβ-1a.

The addition of SDS induced extensive (87%–99%) desorption of rhIFNβ-1a from all particle types. The anionic detergent possibly caused rhIFNβ-1a to unfold and apply a negative charge to the protein, thereby effectively desorbing it from the negatively charged particle surfaces. SDS was most effective on rhIFNβ-1a adsorbed on glass, with nearly all protein desorbing. The degree of desorption is indicative of the type and strength of interaction between rhIFNβ-1a and different particle surfaces. Overall, the glass particles showed the weakest and polystyrene the strongest interaction with the protein.

Front-Face Fluorescence of Protein Adsorbed on Glass

Intrinsic Tryptophan Fluorescence

Intrinsic fluorescence spectroscopy performed at an excitation wavelength of 295 nm gives information on the environment of the three tryptophan residues in rhIFNβ-1a at positions 22, 79, and 143. Maximum tryptophan fluorescence of the starting material, bulk rhIFNβ-1a, was detected at 339.7 nm. Peak

Table 2. Adsorption and Desorption Characteristics of rhIFNβ-1a Incubated with Nano- and Microparticles of Glass, Metal, and Polystyrene

		Glass	Metal	Polystyrene
Composition of the free protein fraction (% ± SD)[a]	Monomer	82 ± 1	74 ± 0	90 ± 1
	Oligomer	18 ± 2	26 ± 1	10 ± 2
Fraction (% ± SD) of adsorbed protein[b]	Adsorbed	98 ± 0	80 ± 2	67 ± 4
Fraction (% ± SD) of adsorbed protein desorbing after incubation with[c]	300 mM PBS	55 ± 4	32 ± 5	28 ± 7
	Acetate	22 ± 3	35 ± 4	22 ± 7
	SDS	99 ± 3	87 ± 4	90 ± 8

[a] Samples were incubated in 50 mM sodium phosphate and 100 mM NaCl at pH 7.2 (cf. composition of bulk rhIFNβ-1a was 85 ± 1% monomer and 15 ± 1% oligomer).
[b] Samples (250 μL) were incubated in 10 mM sodium phosphate and 20 mM NaCl at pH 7.2 and reincubated after addition of 250 μL of water.
[c] Samples (250 μL) were incubated in 10 mM sodium phosphate and 20 mM NaCl at pH 7.2 and reincubated after addition of 250 μL desorption solution (300 mM PBS, acetate, or SDS; see text for details). Percentages were calculated from the free protein fraction after desorption minus the free protein fraction after adsorption, and divided by the fraction of adsorbed protein.

fluorescence of rhIFNβ-1a adsorbed on glass showed a 4.7-nm blue shift relative to that of bulk rhIFNβ-1a (Fig. 2a and Table 3). Apparently, the tryptophan residues of rhIFNβ-1a became less exposed to the solvent upon adsorption. Either aggregation or shielding of the tryptophans from the solvent due to the proximity of the glass surface could cause this effect. Heating of rhIFNβ-1a at 90°C resulted in a 5.4-nm blue shift, caused by extensive aggregation (Fig. 2a and Table 3), as was reported previously.[25,28] In contrast, unfolding of rhIFNβ-1a in 6 M guanidine hydrochloride resulted in a 9.1-nm red shift, indicating a more hydrophilic environment of the tryptophans.

ANS Fluorescence

8-Anilino-1-naphthalene-sulfonic acid becomes fluorescent upon binding to apolar sites of rhIFNβ-1a, thus providing information on the tertiary structure of the protein.[28,34,35] No interaction was detected between ANS and the glass particles in the absence of protein. The ANS fluorescence of rhIFNβ-1a adsorbed on glass showed a 2.7-fold increase in intensity as compared with the bulk protein (Fig. 2b and Table 3). This suggests an increase in the accessibility of hydrophobic patches of adsorbed rhIFNβ-1a, which might be due to partial unfolding of the protein. Heating of rhIFNβ-1a at 90°C resulted in a 20% decrease in ANS fluorescence intensity, which is explained by the lower accessibility of apolar sites upon aggregation. Unfolding of rhIFNβ-1a in guanidine hydrochloride greatly affected its conformation; the ANS fluorescence intensity decrease of 97% indicated that hardly any hydrophobic patches were preserved.

Tryptophan Fluorescence Quenching

The Stern–Volmer constant (K_{SV}) was obtained from the initial slope of the Stern–Volmer plot. Bulk and unfolded rhIFNβ-1a had similar K_{SV} (Fig. 2c and Table 3), indicating that the presence of guanidine hydrochloride did not induce major changes in the quenching level of the three tryptophans of interferon beta. Because Trp22 is located near the surface of rhIFNβ-1a, it is already relatively exposed to the solvent and remains exposed upon unfolding of the protein.[36,37] Although Trp79 and Trp143 are buried in the hydrophobic core of the protein, we speculate that the quenching of these amino acids was hardly affected by guanidine hydrochloride. The reason for this is probably the proximity of both tryptophans to the intramolecular disulfide bridge that stabilizes the hydrophobic core,[33,36] which may lead to strong internal quenching.[38] Both rhIFNβ-1a adsorbed on glass and rhIFNβ-1a heated at 90°C showed K_{SV} values that were considerably lower than those of bulk and unfolded protein, indicating reduced accessibility of the tryptophans to acrylamide.

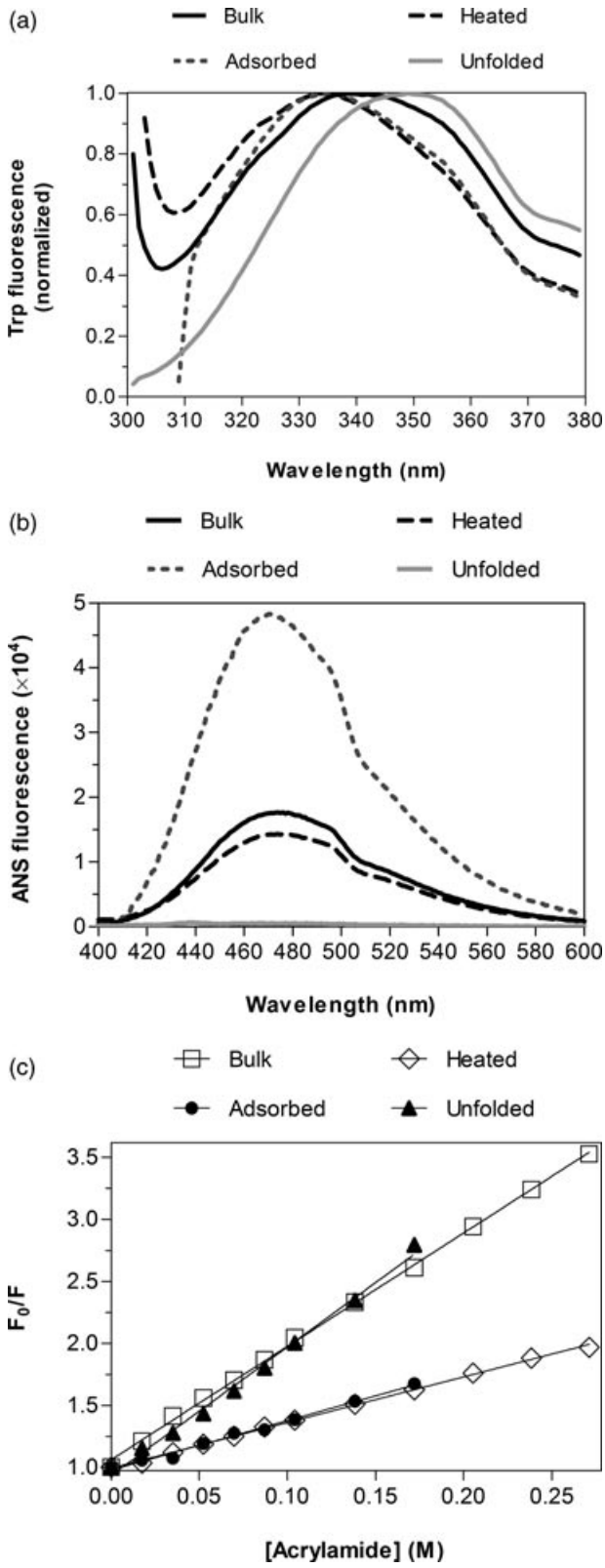

Figure 2. Fluorescence spectroscopy of bulk rhIFNβ-1a and rhIFNβ-1a adsorbed on glass, heated at 90°C and unfolded in 6 M guanidine hydrochloride, using (a) intrinsic tryptophan fluorescence (normalized curves), (b) ANS fluorescence, and (c) tryptophan fluorescence quenching (Stern–Volmer plot).

 DOI 10.1002/jps

Table 3. Front-Face Fluorescence Characteristics of Bulk rhIFNβ-1a and rhIFNβ-1a Adsorbed on Glass, Heated at 90°C and Unfolded in 6 M Guanidine Hydrochloride

	Bulk	Adsorbed on Glass	Heated at 90°C	Unfolded in 6 M Guanidine
Tryptophan fluorescence peak wavelength (nm ± SD)	339.7 ± 0.6	335.0 ± 1.0	334.3 ± 0.6	348.8 ± 0.5
ANS fluorescence relative intensity	1.0	2.7	0.8	0.03
Stern–Volmer constant, K_{SV} (with r^2) (M^{-1})	9 (1.0)	4 (0.99)	4 (1.0)	10 (0.99)

Immunogenicity in a Transgenic, Immune Tolerant Mouse Model

rhIFNβ-1a Dispersions with Glass, Metal, or Polystyrene Particles

The first BABs could be detected in responding mice after 7 days of treatment (not shown). At day 21, the wildtype mice had formed BABs (total IgG) (Fig. 3a) and NABs (Table 4) against all rhIFNβ injection samples, including all protein–particle dispersions and the reference samples of bulk rhIFNβ-1a and Betaferon®–rhIFNβ-1b. The transgenic mice, as opposed to wildtype mice, possessed the gene for hIFNβ, providing a level of immune tolerance for this protein.[12,25] The transgenic mice showed a different degree of immunogenicity for each of the rhIFNβ samples (Fig. 3b). The number of mice developing IgG was the lowest for bulk rhIFNβ-1a (3/7) and rhIFNβ-1a with polystyrene (3/8), whereas the immunogenicity of rhIFNβ-1a with glass was moderate (4/8) and that of rhIFNβ-1a with metal (7/8) and Betaferon®–rhIFNβ-1b (7/7), the highest. The number of transgenic mice with NABs against interferon beta was relatively low and hardly differed among the treatment groups (Table 4).

rhIFNβ-1a Adsorbed on Metal Versus Free in Solution

In order to determine whether the particularly high immunogenicity of the dispersion of rhIFNβ-1a with metal was due to the adsorbed or the free protein fraction, both fractions were separated and tested individually in mice. Again, all samples and references induced high IgG titers in wildtype mice (Fig. 3c), whereas their immunogenicity varied in transgenic mice (Fig. 3d). The protein fraction adsorbed on metal particles, containing 34% of the original protein concentration, was significantly more immunogenic in transgenic mice than the free protein fraction in solution, containing 66% of the original protein concentration. Hence, rhIFNβ-1a adsorbed on metal particles was responsible for the dramatic decrease in the immune tolerance for hIFNβ in transgenic mice.

DISCUSSION

Adsorption of rhIFN*β*-1a

The interaction of therapeutic proteins with foreign materials encountered during manufacture and fill and finish operations can severely compromise the stability of the protein.[40] The protein can adsorb to subvisible particles shed by the surface of such materials.[18,41] Thus, heterogeneous aggregates that increase the immunogenicity of the protein might form. This study showed that rhIFNβ-1a in phosphate buffers at physiological pH readily adsorbs to particles of glass, metal, and polystyrene. The adsorption to these materials was partly driven by electrostatic interactions. As long as the particle concentration and ionic strength were not too high, the isotherms of rhIFNβ-1a adsorption to glass and metal approached the maximum surface packing levels derived from the RSA model.[31] At higher particle concentrations or ionic strength, the measured isotherms, including those of polystyrene, showed considerable curvature, indicating more reversible adsorption with lower affinity. Thus, increasing the particle concentration (above the theoretical adsorption footprints of 120 mg glass, 4100 mg metal, and 23 mg polystyrene per milligram protein) or ionic strength (using 300 mM PBS instead of 150 mM PBS) led to much lower packing levels than calculated from the RSA model. This implies that the actual particle surface covered by protein molecules under these conditions is less than the 54.7% proposed by RSA. At high protein-to-particle ratios, it is more likely that the protein–particle assemblies fulfill the criterion of a multimeric epitope presentation breaking B-cell tolerance, for which a spacing of 5–10 nm is required between at least 10 repeating epitopes.[42–44] For this reason, we used relatively low particle amounts and a buffer of 150 mM PBS at pH 7.2 in our immunogenicity studies. Hence, incubation conditions were similar to *in vivo* conditions of pH and ionic strength, and the protein coverage likely approximated 55%. However, more work would be needed to gain insight into the orientation of the adsorbed proteins on the particles and how this affects immunogenicity.

Glass Microparticles

Glass-adsorbed rhIFNβ-1a was generally more susceptible to desorption than rhIFNβ-1a adsorbed on metal or polystyrene beads. In fact, acid desorption from glass beads has been applied to purify human interferon.[45,46] Braude et al.[47] proposed that the binding of murine interferon beta to glass was mediated by the interaction of the amino group of lysines and the guanidinium group of arginines with the silanol

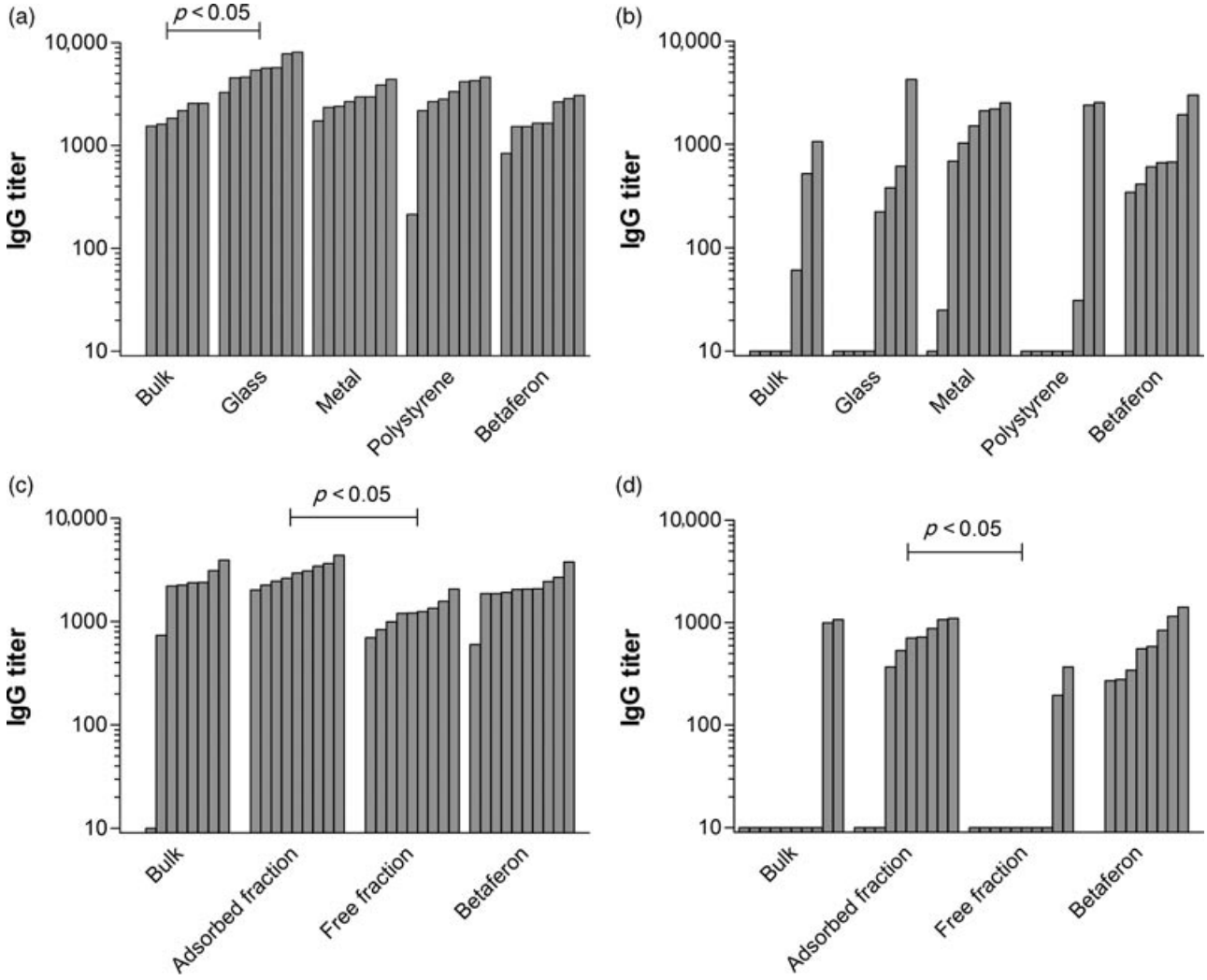

Figure 3. Immunogenicity of rhIFNβ-1a incubated with glass, metal, and polystyrene particles in (a) wildtype and (b) transgenic mice, and immunogenicity of the adsorbed protein fraction (34%) and free protein fraction (66%) of rhIFNβ-1a incubated with metal in (c) wildtype and (d) transgenic mice. The immunogenicity results of bulk rhIFNβ-1a and Betaferon®–rhIFNβ-1b are presented as controls (a–d). Bars show titers of total IgG against rhIFNβ at day 21 for each individual mouse injected daily, from Monday to Friday, i.p. for 3 weeks. Nonresponders were given a titer of 10. Statistical analyses (Kruskal–Wallis nonparametric one-way analysis of variance with Dunn's multiple comparison test) were performed, comparing the titers of the treatment groups. Groups that differ significantly ($p < 0.05$) from each other are indicated.

(hydroxyl) groups of glass. In our study, intrinsic tryptophan fluorescence and acrylamide quenching showed that the tryptophans of rhIFNβ-1a became more buried upon adsorption to glass. This was, most probably, caused by shielding of Trp22 from the solvent due to its proximity to the glass surface. Moreover, adsorption to glass caused an increase in the accessibility of hydrophobic patches according to ANS fluorescence, which suggested partial unfolding of the protein. Thus, the conformation of rhIFNβ-1a adsorbed on glass was slightly perturbed, but not to the same extent as aggregated rhIFNβ-1a (induced by heating at 90°C) or guanidine-treated rhIFNβ-1a. Several monoclonal antibodies also demonstrated only minimal changes in the tertiary structure after adsorption to glass microparticles.[48]

Metal Microparticles

Incubation with metal considerably altered the composition of the free protein fraction, raising the percentage of rhIFNβ-1a oligomers from 15 ± 1% to 26 ± 1%. This may be explained by preferred adsorption of the monomer, or aggregation induced by interaction of the protein with the metal surface, which has also been observed for a monoclonal antibody.[24] Like the monoclonal antibody, rhIFNβ-1a demonstrated

Table 4. Number of Wildtype and Transgenic Mice out of the Total Number of Mice per Treatment Group, Showing NABs Against rhIFNβ on Day 21

First *In Vivo* Study ($n = 8$)[a]	Wildtype	Transgenic
Bulk	6/6	1/7
Metal	8/8	2/8
Glass	5/7[b]	2/8
Polystyrene	7/8	2/8
Betaferon®	5/8	3/7
Second *In Vivo* Study ($n = 10$)[a]	**Wildtype**	**Transgenic**
Bulk	7/8	0/10
Metal-adsorbed fraction	9/9	1/10
Metal-free fraction	9/9	1/10
Betaferon®	7/10	2/8

[a] Ten out of 160 mice in total died before day 21. The unusually high death rate was due to unknown causes, but common for this particular mouse strain.[25,39]

[b] NAB result of one mouse is missing because the sample volume was too low.

a stronger interaction with metal than with glass during desorption studies. It was speculated by Bee et al.[24] that the strong interaction with metal is related to the surface chemistry of stainless steel, which is rich in chromium oxide. Oxidation and/or unfolding of the protein on the stainless steel surface can cause aggregation of the adsorbed protein as well as the free protein. Structural changes of rhIFNβ-1a adsorbed on metal could not be assessed because the large size of the metal particles caused high background scatter, hampering detection of the protein with front-face fluorescence. A preliminary test with liquid chromatography–mass spectrometry (LC–MS) suggested that incubation of rhIFNβ-1a with metal particles induced oxidation of the free protein, affecting, in particular, the Trp22 (results not shown). Likewise, Bee et al.[21] described an increase in carbonyl groups due to the oxidation of a monoclonal antibody in the presence of metal.

Polystyrene Nanoparticles

Besides a higher porosity and smaller size, polystyrene nanoparticles had different adsorption characteristics than glass and metal microparticles. That is, the adsorption of protein to polystyrene showed minimal response to high salt, and rhIFNβ-1a oligomers were more prone to adsorption than monomers. Also, the interaction of the protein with polystyrene was stronger than with metal and glass, which may explain why polystyrene beads are successfully used to adsorb protein in immunoassays and drug delivery.[49]

Immunogenicity of rhIFNβ-1a Interacting with Particles

All protein–particle dispersions elicited high titers of BABs and NABs against hIFNβ in wildtype mice, indicating that native epitopes were still intact. rhIFNβ is a foreign protein for wildtype mice and, consequently, elicits a classical immune response.[12,25] The transgenic mice, possessing the gene for human protein, were relatively immune tolerant for bulk rhIFNβ-1a and rhIFNβ-1a incubated with polystyrene, while being moderately tolerant for rhIFNβ-1a incubated with glass. rhIFNβ-1a incubated with metal particles was nearly as immunogenic as Betaferon® –rhIFNβ-1b. Only few transgenic mice showed interferon beta NABs, consistent with previous observations.[12,25]

The protein fraction adsorbed to metal appeared to be responsible for high IgG titers. Seven out of 10 mice showed BABs against the adsorbed fraction, whereas only two out of 10 mice showed BABs against the free protein fraction despite its high protein and protein aggregate content. Maybe, heterogeneous aggregates of rhIFNβ-1a adsorbed on metal particles met the criteria of multimeric epitope assemblies that are capable of breaking B-cell tolerance,[42–44] whereas heterogeneous aggregates with glass or polystyrene particles did not. This study suggests that the Trp22 residue in rhIFNβ-1a became oxidized possibly due to molecular properties of the stainless steel surface.[21,24] It is possible that rhIFNβ-1a molecules adsorbed on metal particles have a specific orientation and/or different epitope presentation, perhaps because of oxidation or chemical modification of the protein, thereby mimicking a T-cell independent antigen.[50]

Moreover, particle size and packing density and conformation of the adsorbed protein may be important. To further investigate the influence of particle size, it would be interesting to test whether metal nanoparticles with adsorbed rhIFNβ-1a are able to break immune tolerance. Particle-to-protein ratios and buffer conditions were chosen such that similar high packing efficiencies of rhIFNβ-1a on glass, metal, and polystyrene were achieved. The conformation of the protein slightly altered upon adsorption to glass, according to fluorescence measurements, but only partially because wildtype mice still recognized native epitopes. There is a possibility that the protein desorbed from glass, but not from metal particles after injection in mice because the interaction with glass is weaker than with metal. Furthermore, it is possible that proteins need to be covalently coupled to polystyrene particles instead of being adsorbed to achieve immunogenicity.[49]

More research is needed to determine the characteristics responsible for immunogenicity, but it is unmistakable that adsorbed proteins bear a high risk of breaking immune tolerance. Manufacturers should be aware of possible adsorption of bulk rhIFNβ to hydrophilic surfaces encountered during production of the protein or storage in prefilled syringes, as well as the high risk for immunogenicity if the protein adsorbs on metal particles shed during, for example, fill

and finish. Adsorption of rhIFNβ-1a on micro- and nanoparticles in the presence of free protein, as described in this work, is representative of what could happen during manufacturing, although actual particle concentrations will be much lower in "real life". In our animal studies, the estimated particle amounts (based on mean particle size and specific surface area) were 8×10^8 glass particles, 3×10^6 metal particles, and 5×10^9 polystyrene particles per injection. For comparison, the amount of metal particles we used per injection was about 500 times more than the compendial requirements, specifying a maximum of 6000 particles per container for particulates larger than 10 μm.[41] Further studies should focus on defining the levels of particles, mass of adsorbed protein, and number of injections needed to elicit an immune response against the protein.

CONCLUSIONS

Recombinant human interferon beta-1a adsorbs readily to common surface materials such as glass, metal, and polystyrene. Moreover, rhIFNβ-1a adsorbed on metal particles was shown to enhance the immunogenicity of the protein in our transgenic immune tolerant mouse model. This underlines the importance of measuring and characterizing subvisible particles in protein formulations.

ACKNOWLEDGMENTS

Biogen Idec, Inc. is acknowledged for kindly providing rhIFNβ. Thanks to Jared S. Bee for his help with the adsorption experiments and fluorescence measurements, as well as for scientific discussion and suggestions. Christian Schöneich and Victor S. Sharov are acknowledged for providing support with LC–MS. This research was financially supported by the European Community under its 6th Framework (project NABINMS, contract number 018926), the Colorado University at Boulder, Utrecht Institute for Pharmaceutical Sciences, and the Dutch Foundation for Pharmacological Sciences.

REFERENCES

1. Knobler RL, Greenstein JI, Johnson KP, Lublin FD, Panitch HS, Conway K, Grant-Gorsen SV, Muldoon J, Marcus SG, Wallenberg JC, Williams GJ, Yoshizawa CN. 1993. Systemic recombinant human interferon-beta treatment of relapsing–remitting multiple sclerosis: Pilot study analysis and six-year follow-up. J Interferon Res 13(5):333–340.
2. Antonelli G, Dianzani F. 1999. Development of antibodies to interferon beta in patients: Technical and biological aspects. Eur Cytokine Netw 10(3):413–422.
3. van Baarsen LGM, Vosslamber S, Tijssen M, Baggen JMC, van der Voort LF, Killestein J, van der Pouw Kraan TCTM, Polman CH, Verweij CL. 2008. Pharmacogenomics of interferon-β therapy in multiple sclerosis: Baseline IFN signature determines pharmacological differences between patients. PLoS One 3(4):e1927.
4. Comabella M, Lunemann JD, Rio J, Sanchez A, Lopez C, Julia E, Fernandez M, Nonell L, Camina-Tato M, Deisenhammer F, Caballero E, Tortola MT, Prinz M, Montalban X, Martin R. 2009. A type I interferon signature in monocytes is associated with poor response to interferon-beta in multiple sclerosis. Brain 132(12):3353–3365.
5. Sorensen PS. 2008. Review: Neutralizing antibodies against interferon-beta. Ther Adv Neurol Disord 1(2):125–141.
6. Bertolotto A, Deisenhammer F, Gallo P, Sorensen PS. 2004. Immunogenicity of interferon beta: Differences among products. J Neurol 251(Suppl 2):II15–II24.
7. Schellekens H. 2002. Bioequivalence and the immunogenicity of biopharmaceuticals. Nat Rev Drug Discov 1(6):457–462.
8. Hermeling S, Aranha L, Damen JMA, Slijper M, Schellekens H, Crommelin DJA, Jiskoot W. 2005. Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b. Pharm Res 22(12):1997–2006.
9. Fradkin AH, Carpenter JF, Randolph TW. 2009. Immunogenicity of aggregates of recombinant human growth hormone in mouse models. J Pharm Sci 98(9):3247–3264.
10. van Beers MMC, Jiskoot W, Schellekens H. 2010. On the role of aggregates in the immunogenicity of recombinant human interferon beta in patients with multiple sclerosis. J Interferon Cytokine Res 30(10):767–775.
11. Hermeling S, Jiskoot W, Crommelin DJA, Bornæs C, Schellekens H. 2005. Development of a transgenic mouse model immune tolerant for human interferon beta. Pharm Res 22(6):847–851.
12. van Beers MMC, Sauerborn M, Gilli F, Hermeling S, Schellekens H, Jiskoot W. 2010. Hybrid transgenic immune tolerant mouse model for assessing the breaking of B cell tolerance by human interferon beta. J Immunol Methods 352(1–2):32–37.
13. Seefeldt MB, Rosendahl MS, Cleland JL, Hesterberg LK. 2009. Application of high hydrostatic pressure to dissociate aggregates and refold proteins. Curr Pharm Biotechnol 10(4):447–455.
14. Braun A, Kwee L, Labow MA, Alsenz J. 1997. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. Pharm Res 14(10):1472–1478.
15. Tzannis ST, Hrushesky WJM, Wood PA, Przybycien TM. 1997. Adsorption of a formulated protein on a drug delivery device surface. J Colloid Interface Sci 189(2):216–228.
16. McLeod AG, Walker IR, Zheng S, Hayward CP. 2000. Loss of factor VIII activity during storage in PVC containers due to adsorption. Haemophilia 6(2):89–92.
17. Jones LS, Kaufmann A, Middaugh CR. 2005. Silicone oil induced aggregation of proteins. J Pharm Sci 94(4):918–927.
18. Chi EY, Weickmann J, Carpenter JF, Manning MC, Randolph TW. 2005. Heterogeneous nucleation-controlled particulate formation of recombinant human platelet-activating factor acetylhydrolase in pharmaceutical formulation. J Pharm Sci 94(2):256–274.
19. Bee JS, Nelson SA, Freund E, Carpenter JF, Randolph TW. 2009. Precipitation of a monoclonal antibody by soluble tungsten. J Pharm Sci 98(9):3290–3301.
20. Kueltzo LA, Wang W, Randolph TW, Carpenter JF. 2008. Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze–thawing. J Pharm Sci 97(5):1801–1812.
21. Bee JS, Davis M, Freund E, Carpenter JF, Randolph TW. 2010. Aggregation of a monoclonal antibody induced by adsorption to stainless steel. Biotechnol Bioeng 105(1):121–129.

22. Tyagi AK, Randolph TW, Dong A, Maloney KM, Hitscherich C, Carpenter JF. 2009. IgG particle formation during filling pump operation: A case study of heterogeneous nucleation on stainless steel nanoparticles. J Pharm Sci 98(1):94–104.
23. Ennis RD, Pritchard R, Nakamura C, Coulon M, Yang T, Visor GC, Lee WA. 2001. Glass vials for small volume parenterals: Influence of drug and manufacturing processes on glass delamination. Pharm Dev Technol 6(3):393–405.
24. Bee JS, Chiu D, Sawicki S, Stevenson JL, Chatterjee K, Freund E, Carpenter JF, Randolph TW. 2009. Monoclonal antibody interactions with micro- and nanoparticles: Adsorption, aggregation, and accelerated stress studies. J Pharm Sci 98(9):3218–3238.
25. van Beers MMC, Sauerborn M, Gilli F, Brinks V, Schellekens H, Jiskoot W. 2010. Aggregated recombinant human interferon beta induces antibodies but no memory in immune-tolerant transgenic mice. Pharm Res 27(9):1812–1824.
26. Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, McKenzie IFC, Plebanski M. 2004. Size-dependent immunogenicity: Therapeutic and protective properties of nano-vaccines against tumors. J Immunol 173(5):3148–3154.
27. Scheerlinck JP, Gloster S, Gamvrellis A, Mottram PL, Plebanski M. 2006. Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. Vaccine 24(8):1124–1131.
28. Fan H, Ralston J, Dibiase M, Faulkner E, Middaugh CR. 2005. Solution behavior of IFN-beta-1a: An empirical phase diagram based approach. J Pharm Sci 94(9):1893–1911.
29. Lakowicz JR. 1999. Principles of fluorescence spectroscopy. 2nd ed. New York: Springer.
30. Gilli F, van Beers M, Marnetto F, Jiskoot W, Bertolotto A, Schellekens H. 2008. Development of a bioassay for quantification of neutralising antibodies against human interferon-beta in mouse sera. J Immunol Methods 336(2):119–126.
31. Evans JW. 1993. Random and cooperative sequential adsorption. Rev Mod Phys 65(4):1281–1329.
32. Utsumi J, Mizuno Y, Hosoi K, Okano K, Sawada R, Kajitani M, Sakai I, Naruto M, Shimizu H. 1989. Characterization of four different mammalian-cell-derived recombinant human interferon-β1s. Eur J Biochem 181(3):545–553.
33. Karpusas M, Nolte M, Benton CB, Meier W, Lipscomb WN, Goelz S. 1997. The crystal structure of human interferon-beta at 2.2-Å resolution. Proc Natl Acad Sci U S A 94(22):11813–11818.
34. Jiskoot W, Visser AJWG, Herron JN, Sutter M. 2005. Fluorescence spectroscopy. In Methods for structural analysis of protein pharmaceuticals; Jiskoot W, Crommelin DJA, Eds. Arlington: AAPS Press, pp 27–82.
35. Hawe A, Sutter M, Jiskoot W. 2008. Extrinsic fluorescent dyes as tools for protein characterization. Pharm Res 25(7):1487–1499.
36. Karpusas M, Whitty A, Runkel L, Hochman P. 1998. The structure of human interferon-beta: Implications for activity. Cell Mol Life Sci 54(11):1203–1216.
37. Runkel L, deDios C, Karpusas M, Betzenhauser M, Muldowney C, Zafari M, Benjamin CD, Miller S, Hochman PS, Whitty A. 2000. Systematic mutational mapping of sites on human interferon-beta-1a that are important for receptor binding and functional activity. Biochemistry 39(10):2538–2551.
38. Qiu W, Li T, Zhang L, Yang Y, Kao YT, Wang L, Zhong D. 2008. Ultrafast quenching of tryptophan fluorescence in proteins: interresidue and intrahelical electron transfer. Chem Phys 350(1–3):154–164.
39. Van Beers MMC, Sauerborn M, Gilli F, Brinks V, Schellekens H, Jiskoot W. 2011. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. Pharm Res doi:10.1007/s11095-011-0451-4 (Epub ahead of print).
40. Randolph TW, Carpenter JF. 2007. Engineering challenges of protein formulations. AIChE J 53(8):1902–1907.
41. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJA, Middaugh CR, Winter G, Fan YX, Kirshner S, Verthelyi D, Kozlowski S, Clouse KA, Swann PG, Rosenberg A, Cherney B. 2009. Overlooking subvisible particles in therapeutic protein products: Gaps that may compromise product quality. J Pharm Sci 98(4):1201–1205.
42. Dintzis RZ, Okajima M, Middleton MH, Greene G, Dintzis HM. 1989. The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence. J Immunol 143(4):1239–1244.
43. Chackerian B, Lenz P, Lowy DR, Schiller JT. 2002. Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. J Immunol 169(11):6120–6126.
44. Bachmann MF, Zinkernagel RM. 1997. Neutralizing antiviral B cell responses. Annu Rev Immunol 15(1):235–270.
45. Billiau A, van Damme J, van Leuven F, Edy VG, de Ley M, Cassiman JJ, van den Berghe H, de Somer P. 1979. Human fibroblast interferon for clinical trials: Production, partial purification, and characterization. Antimicrob Agents Chemother 16(1):49–55.
46. Braude IA, de Clercq E. 1979. Purification of mouse interferon by sequential chromatography. J Chromatogr A 172(1):207–219.
47. Braude IA, Edy VG, de Clercq E. 1979. Mechanism of binding of mouse interferon to controlled pore glass. Biochim Biophys Acta 580(1):15–23.
48. Hoehne M, Samuel F, Dong A, Wurth C, Mahler HC, Carpenter JF, Randolph TW. 2011. Adsorption of monoclonal antibodies to glass microparticles. J Pharm Sci 100(1):123–132.
49. Kalkanidis M, Pietersz GA, Xiang SD, Mottram PL, Crimeen-Irwin B, Ardipradja K, Plebanski M. 2006. Methods for nanoparticle based vaccine formulation and evaluation of their immunogenicity. Methods 40(1):20–29.
50. Sauerborn M, Brinks V, Jiskoot W, Schellekens H. 2010. Immunological mechanism underlying the immune response to recombinant human protein therapeutics. Trends Pharmacol Sci 31(2):53–59.