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Recombinant Murine Growth Hormone Particles are More Immunogenic with Intravenous than Subcutaneous Administration

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Abstract

Evaluation and mitigation of the risk of immunogenicity to protein aggregates and particles in therapeutic protein products remains a primary concern for drug developers and regulatory agencies. In order to investigate how the presence of protein particles and the route of administration influence the immunogenicity of a model therapeutic protein, we measured the immune response in mice to injections of formulations of recombinant murine growth hormone (rmGH) that contained controlled levels of protein particles. Mice were injected twice over six weeks with rmGH formulations via the subcutaneous (SQ), intraperitoneal (IP) or intravenous (IV) routes. In addition to soluble, monomeric rmGH, the samples prepared contained either nanoparticles of rmGH or both nano- and micro-particles of rmGH. The appearance of anti-rmGH IgG1, IgG2a, IgG2b, IgG2c and IgG3 titers following the second injection of both preparations implies that multiple mechanisms contributed to the immune response. No dependence of the immune response on particle size and distribution was observed. The immune response measured after the second injection was most pronounced when IV administration was used. Despite producing high anti-rmGH titers mice appeared to retain the ability to properly regulate and use endogenous growth hormone.

Introduction

Therapeutic protein products are routinely prescribed for a number of indications—sometimes as the only treatment option. Proponents of protein therapeutics note their specificity and ease of modification.¹ However, it is now known that all protein therapeutics have the potential to cause an immune response in patients^{2,3} and reported incidences in patients range from <3–100%.⁴ Immunogenicity can give rise to clinical consequences such as loss of drug product efficacy or even production of cross-reactive antibodies that neutralize activity of endogenous protein.^{5–7} For example, in the 1990's, reports of patients on erythropoietin therapy emerged wherein patients diagnosed with pure red cell aplasia

were positive for anti-erythropoietin antibodies. The development of neutralizing antibodies (Nabs) to erythropoietin that cross reacted with endogenous protein resulted in patients with severe anemia, a dependence on transfusions and few treatment alternatives.⁸ Similarly, a Canadian study in which serum samples from 2,711 patients on Avonex®, Rebif® or Betaseron® were submitted over a 3-year period found a negative correlation between the magnitude of the anti-IFN β NAb response and therapeutic efficacy.⁹ Non-neutralizing antibodies also warrant monitoring as they may enhance clearance of the therapeutic, thus reducing efficacy¹⁰ and requiring dose adjustments.

Many factors might contribute to immunogenicity of therapeutic proteins, including the presence of aggregates and particles, origin of the product, dosing regimen, manufacturing and handling procedures, the disease state of the patient and route of administration.^{4,5,11–15} Conventional wisdom, based on studies with vaccine formulations, suggests that SQ administration is more immunogenic than IV administration.³ Human clinical studies to test such a hypothesis directly are unethical and *post hoc* conclusions drawn from various published studies are difficult to interpret. For example, one clinical study found that IFN β -1a had a higher incidence of immunogenicity in patients when injected SQ than IM. However, the products that were injected by the two routes were different (and presumably contained different protein particle and aggregate loads¹⁶) and were administered at different doses, thus making a direct comparison of injection routes difficult.¹⁷ In another example, following the discovery that SQ administration of the erythropoietin product—Eprex—contributed to immunogenicity, a mandate to switch exclusively to IV administration probably contributed to reduced worldwide cases of immunogenicity,¹⁸ although improvements in handling, storage and manufacturing most likely also contributed.¹⁹

Also, there are mixed results in published studies that directly tested the effect of route of administration on immunogenicity of protein aggregates in animal models. Braun et al. found that administration of 0.3 μ g of IFN- α 2a aggregates once weekly for 5 weeks produced increasing immune response in mice in the following order: SQ>IP>IM $\gg$ IV.¹¹ Another group also found higher immunogenicity for SQ administration as compared to IV administration of 4 weekly injections of rFVIII in Hemophilia A mice.²⁰ Interestingly, they later found that IV injections of PEGylated rFVIII were more immunogenic than SQ administration.²¹ Likewise, Kijanka et al. recently found that IV injections of Betaferon® (EU) were more immunogenic than SQ or IM injections.¹² Furthermore, none of these earlier studies report results for sample particle contents, because particle counters and size analyzers in the subvisible range (especially in the nanoparticle size range) have only recently become integrated into standard characterization protocols for therapeutic proteins.

In elucidating mechanisms of immunogenicity, protein aggregates can be compared to viruses, whose highly organized and repetitive protein surfaces crosslink the antigen receptor expressed by B lymphocytes to cause activation and differentiation to plasma cells.²² When these highly organized and repetitive protein surfaces are presented as a virus-like particle or in conjunction with particle adjuvants, macrophage uptake and the immune response are enhanced.^{23,24} In terms of the response to particles, others have hypothesized involvement of T cell independent (TI) or Th2 mechanisms.^{15,25,26} Studies done with TI

antigens show that a specific number of epitopes that engage the B cell receptor (BCR) presented in a repetitive spatial arrangement formed an “immunon,” which was hypothesized to be important for BCR crosslinking and signaling.²⁷ Involvement of a Th2 type response may also contribute to the development of antibodies to protein particles. Adsorption of antigen to aluminum adjuvants leads to a strong Th2 response.²⁸ Likewise, conversion of soluble protein to particles may result in a Th2 response.

In this study, we determined the immune response in mice to administration of formulations of recombinant murine growth hormone (rmGH). We characterized particle and aggregate dose loads within each formulation utilizing size exclusion chromatography and counting and sizing of nano- and microparticles. We then compared the immune response resulting in mice following injections administered via the SQ, IP or IV routes. Immune responses to rmGH were followed by measuring serum levels of anti-rmGH antibodies of various IgG isotypes, allowing us to propose mechanisms for how different routes of administration might affect immunogenicity. Finally, the ability of any anti-rmGH antibodies to neutralize endogenous growth hormone was inferred from serum levels of insulin-like growth factor-1 (IGF-1) measured throughout the study.

Materials and Methods

Materials

Serum-gel clotting tubes (41.1500.005) were obtained from Sarstedt (Nümbrecht, Germany). Goat anti-mouse F(ab')₂ (115-005-072), mouse IgG (015-000-003), peroxidase-goat anti-mouse IgG2a (115-035-206), peroxidase-goat anti-mouse IgG2b (115-035-207), peroxidase-goat anti-mouse IgG2c (115-035-208), peroxidase-goat anti-mouse IgG3 (115-035-209) and ChromPure mouse IgG (015-000-003) were obtained from Jackson ImmunoResearch (West Grove, PA). Peroxidase-goat anti-mouse IgG (H+L) (62-6520) and peroxidase-goat anti-mouse IgG1 (877586) were obtained from Invitrogen (Eugene, OR). TMB substrate, Tween 20® and Immulon 4HBX flat bottom plates were obtained from Thermo Scientific (Waltham, MA). Sulfuric acid, chloramphenicol, ampicillin, IPTG, citric acid monohydrate, trisodium citrate dehydrate, tris-HCl, EDTA, sodium deoxycholate monohydrate, reduced glutathione, oxidized glutathione, bis-tris, NaCl, urea and yeast extract were obtained from Fisher Chemical (Fair Lawn, NJ). Sterile 1 mL tuberculin syringes (309659) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Quantikine ELISA for mouse/rat IGF-1 was obtained from R&D Systems (Minneapolis, MN). Amicon Ultra centrifugal filter devices were obtained from Millipore (Cork, Ireland).

Expression and Purification of rmGH

rmGH was expressed and purified, following a slight modification of previously published protocols.^{29,30} A stock of *Escherichia coli* cloned with the rmGH plasmid was stored frozen. A 5mL culture was started in LB broth containing 50 µg/mL chloramphenicol and 50 µg/mL ampicillin and incubated overnight in a shaker at 225 rpm and 32°C. The next morning, the culture was transferred to 100 mL of growth media containing 100 mM MES (pH 7), 4% (w/v) yeast extract, 1% (w/v) NaCl, 1% glycerol, 50 µg/mL chloramphenicol, 50 µg/mL ampicillin and incubated in a shaker at 225 rpm and 32°C for 3 hours. Next, cells were

transferred to a flask containing 1 L of growth media and incubated in a shaker at 225 rpm and 32°C for 5 hours. rmGH production was induced with 0.75 mM IPTG and flasks were incubated in the shaker at 225 rpm and 32°C for another 3 hours. Cells were harvested by centrifugation at 6200 g and lysed with two passes through a French press. Next, an inclusion body washing step was performed in which inclusion bodies were homogenized in a buffer containing 50 mM Tris-HCl (pH 8.5) and 5 mM EDTA (inclusion body wash buffer). Then, the inclusion bodies were sonicated for 7 minutes total (alternating 20 seconds intervals of sonication and rest) on ice. Inclusion bodies were then centrifuged at 17,000 g for 30 minutes at 4°C and the supernatant discarded. Next, the inclusion bodies were homogenized in a buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM EDTA and 1% sodium deoxycholate and the cycle of sonication, centrifugation and supernatant removal was repeated 2–4 more times in inclusion body wash buffer until the supernatant became clear. Then, inclusion bodies were resuspended in inclusion body washing buffer using a homogenizer set at 15,000 rpm and centrifuged at 17,000 g for 30 minutes at 4°C and the supernatant was discarded. This step was repeated once more with inclusion body washing buffer then again with MilliQ water. Next, inclusion bodies were solubilized into a buffer containing 100 mM Tris-HCl (pH 9.0), 2 M urea, 1 mM reduced glutathione, and 0.1 mM oxidized glutathione and protein refolding was accomplished by pressurization to 200 MPa overnight at room temperature in BaroFold (Boulder, CO) PreEMT high-pressure technology. Pressure-treated rmGH was loaded onto a Toyopearl Super Q 650M preparative column equilibrated in 20 mM BisTris at pH 8.0 and elution was performed with a linear gradient of 5 column volumes of a buffer containing 40 mM BisTris (pH 8.0), 0.5 M NaCl and 0.4 M urea at a flow rate of 3 mL/min. Peak fractions were collected in 5 mL increments and analyzed with non-reducing SDS-PAGE, size exclusion chromatography and circular dichroism spectroscopy. Fractions containing correctly folded, monomeric rmGH were pooled and buffer exchanged into 10 mM citrate (pH 5.0) using centrifugal filters with a molecular weight cutoff of 10,000 kDa.

Purified rmGH was tested with a Limulus Amebocyte Lysate gel clot assay (Lonza; Basel, Switzerland) with a cutoff of 0.125 EU/mL. The rmGH was stored frozen upright at –80°C in 15 mL polystyrene conical tubes in 2 mL aliquots. The rmGH exhibited produced CD spectra, fluorescence spectra and thermal melting transition temperature (T_m) comparable to previously published values for murine, ovine, bovine and human growth hormone^{29,31–33} and was >99% pure (determined by SDS-PAGE densitometry).

Sample Preparation—Stock solutions of rmGH in a formulation containing 10 mM citrate pH 5.0 and containing soluble, monomeric rmGH in addition to nanometer and micron-sized protein particles were prepared by thawing frozen aliquots of purified rmGH (at a concentration of approximately 0.2–0.3 mg/mL) at room temperature for 30 minutes. Ultra-centrifuged rmGH samples containing soluble, monomeric rmGH in addition to nano-sized protein particles were created by ultra-centrifuging a freeze-thawed rmGH sample at 110,000 g for 1 hour at 4°C. Prior to administration, the protein concentration of both sample types (freeze-thawed rmGH and ultracentrifuged rmGH) was adjusted to achieve a total dose of 20 µg rmGH comprised of soluble, monomeric and particulate protein

(determined by absorbance at 280 nm adjusted for light scattering in the 350–500 nm range) in 100 μ L for injection.

Size Exclusion Chromatography (SEC)—Analytical size exclusion chromatography was performed on a Superdex 75 10/300 GL column using an Agilent 1100 HPLC (Santa Clara, CA). A mobile phase of phosphate buffered saline pH 7.4 at a 0.4 mL/min flow rate was used and the eluate was monitored at 215 nm. Triplicate samples were analyzed for each rmGH preparation.

Particle Tracking Analysis (PTA)—Nanoparticle analysis was performed with particle tracking analysis with a NanoSight LM20 (NanoSight Ltd; Amesbury, UK) equipped with a 405-nm laser and NTA 2.3 software. Samples were loaded into the chamber until liquid reached the tip of the nozzle (500 μ L) before data acquisition. Video was captured at room temperature for 60 seconds at a setting recommended for low polydispersity samples and with manual shutter and gain settings. The size detection limit was automatically set by the software. Triplicate samples of a 10 mM citrate buffer (pH 5.0), rmGH formulations that had been freeze-thawed and rmGH formulations that had been ultra-centrifuged were analyzed.

Resonant Mass Measurement (RMM)—An Archimedes particle metrology system (Affinity Biosensors; Santa Barbara, CA) was used to count particles detected via resonant mass measurement. Each day, the sensor and detector were manually adjusted to maximize the amplitude and sum (above 6 V) and minimize the baseline (0.01–0.02 V). Hi-Q micro sensors were calibrated with NIST-traceable Scientific Duke Standard 4010A (1.019 μ m polystyrene) before sample measurements were performed. Triplicate samples were loaded for 30 seconds and particle counting was stopped after 200 particles had been counted or after 10 minutes.

MicroFlow Imaging (MFI)—A Brightwell (Ottawa, Canada) 4100 instrument was used for particle sizing and counting via micro-flow imaging. Instrument configuration was in “set point 3” mode and low magnification to allow for particle size detection in the 1–50 μ m size range. Triplicate samples were loaded at a sample volume of 550 μ L, with an analyzed volume of 500 μ L.

Estimation of Mass of Protein Particles per Dose—Protein mass estimates were calculated as previously reported³⁴ with a few modifications. Briefly, mass estimates were calculated using the particle counts per size bin and particle diameter information from each particle sizing technique and the following equation:

$$\text{Estimated protein mass per size bin} = (0.75) \times (\text{volume of a sphere}) \times (1.43 \text{ g/mL}) \times (\text{number of particles})$$

Mass estimates were then summed over the total particle size ranges for each instrument (PTA, RMM and MFI) to arrive at a mass per milliliter value and then adjusted to estimate the mass of protein particles in a 100 μ L dose of rmGH.

Animal Experiments—Experiments involving mice were approved by the institutional animal care and use committee in protocol 77412(05)1D. Mice were housed five to a cage in sterile, air-filtered cages and allowed access to food and water *ad libitum*. Eight CB6F1 (Balb/c x C57BL/6 F1) adult (<6 weeks of ages) female mice were used per experimental group. On days 2 and 23 of the study, mice were administered 10 mM Citrate buffer, pH 5 or rmGH formulations by SQ, IP or IV (via tail vein) routes. Samples containing buffer alone, 20 µg freeze-thawed rmGH or 20 µg ultra-centrifuged rmGH were administered by injection with 1 mL sterile tuberculin syringes. Submandibular blood draws were performed on day 1 to obtain baseline, pre-treatment serum. Subsequent blood draws were performed on days 22 and 36. Blood was collected directly into serum gel clotting tubes, incubated at room temperature for 30 minutes, then centrifuged at 1600 g for 6 minutes at 4°C. The serum was stored frozen in aliquots at -80°C until analysis. At the end of the study, mice were euthanized via CO₂ asphyxiation and cervical dislocation.

Anti-rmGH ELISA—rmGH was coated onto Immulon 4HBX ELISA plates at 5 µg/mL (100 µL/well) overnight at room temperature. Blocking was performed with 300 µL/well of a solution containing PBS, 0.05% Tween 20®, 2% BSA pH 7.4 for 1 hour at room temperature. Wells were then washed twice with PBS, 0.05% Tween 20®, pH 7.4 and filled with 50 µL/well of diluted sera in triplicate for 1 hour at room temperature. Wells were washed five times with PBS/Tween 20® and then incubated with 50 µL/well of peroxidase-goat anti-mouse IgG1, peroxidase-goat anti-mouse IgG2a, peroxidase-goat anti-mouse IgG2b, peroxidase-goat anti-mouse IgG2c or peroxidase-goat anti-mouse IgG3 at the appropriate dilution (1:3000 for peroxidase-goat anti-mouse IgG3 and 1:1000 for all others) for 1 hour at room temperature. Wells were then washed five times with PBS/Tween 20® and incubated with 50 µL of TMB for 25 min. The color reaction was stopped with 30 µL of 0.5 M sulfuric acid and plates were read at 450 nm. Pre-treatment serum obtained from blood drawn at day 1 was used to determine cutoffs for endpoint titer determination as previously published.³⁵ We defined titer as the highest dilution with a detectable signal above the cutoff. Titer averages for each isotype were computed with only data from responders in each group.

Total IgG ELISA—Twelve 6 week old mice (naïve mice) and three 10 and 16 week old mice per experimental group were selected randomly for total IgG monitoring. Goat anti-mouse IgG F(ab')₂ was coated onto Immulon 4HBX ELISA plates at 10 µg/mL (100 µL/well) overnight at 4°C in carbonate/bicarbonate buffer, pH 9.6. Wells were then washed twice with PBS, 0.05% Tween 20®, pH 7.4 and blocking was performed with 200 µL/well PBS, 0.05% Tween 20®, 2% BSA pH 7.4 for 1 hour at room temperature on a plate shaker. Wells were then washed twice and incubated with 100 µL/well of mouse IgG standard dilutions (0.1–1000 ng) or diluted sera for 1 hour at room temperature on a plate shaker. Wells were washed six-times with PBS/Tween 20® and then incubated with 100 µL/well of peroxidase-goat anti-mouse IgG (H+L) at a dilution of 1:100,000 for 1 hour at room temperature on a plate shaker. Wells were then washed six-times and incubated with 50 µL of TMB for 10 min on a plate shaker. The color reaction was stopped with 30 µL of 0.5 M sulfuric acid and plates were read at 450 nm. Standard curves were fit with a sigmoidal-dose

response model and unknown concentrations interpolated in GraphPad Prism software version 5.02 for Windows (San Diego, CA).

Anti-IGF-1 ELISA—Three mice per group were selected randomly for IGF-1 serum level monitoring. Mouse IGF-1 was determined with a mouse/rat IGF-1 Quantikine ELISA kit according to manufacturer's specifications (R&D Systems, Minneapolis, MN). Standard curves were fit with a sigmoidal-dose response model and unknown concentrations interpolated in GraphPad Prism software version 5.02 for Windows (San Diego, CA).

Statistical Analysis—All statistical analyses were performed using SPSS version 21 software. Each experimental group consisted of a sample size of 8 and non-responders (serum samples with undetectable anti-rmGH titers) were entered as 0 for statistical significance determinations. Non-parametric Kruskal-Wallis tests were used to determine statistical difference between experimental groups with p values ≤ 0.05 considered significant.

Results

Characterization of Quantity and Size Distributions of Protein Aggregates

Size Exclusion Chromatography (SEC)—No soluble high molecular weight species (HMWS) were detected via SEC in either the freeze-thawed or ultra-centrifuged rmGH samples (Fig. 1). This observation was consistent with previous studies wherein HMWS were not detected in rmGH formulations after application of other stresses (heat, agitation, pH shift).¹⁵

Particle Tracking Analysis (PTA), Resonant Mass Measurement (RMM) and MicroFlow Imaging (MFI)—When analyzed by PTA, the freeze-thawed rmGH formulations prepared from both day 2 and day 23 injections showed higher nanoparticle concentrations than corresponding samples that had been ultra-centrifuged rmGH. However, the particle concentrations for both freeze-thawed and ultra-centrifuged rmGH preparations were higher in samples prepared on day 2 than in those prepared for injections on day 23 (Fig. 2). We later determined that our centrifugation step needed to proceed longer in order to reproducibly remove nano-sized particles (data not shown). Presumably, slight differences in initial sample and/or sample handling accounted for the different nanoparticle concentrations for the ultra-centrifuged samples prepared on day 2 and day 23.

When analyzed on RMM, the day 2 ultra-centrifuged sample showed a nanoparticle population distribution that was skewed to the lower size range (0.2–0.4 μm). In contrast, in the freeze-thawed sample, particle size distributions were fairly even across a larger size range (0.2–1 μm) (Fig. 3A). Day 23 samples run on RMM showed almost no detectable particles in the ultra-centrifuged sample. And as with the day 2 sample, the day 23 freeze-thawed sample had fairly even concentrations of particles spread over a large size range (0.2–1 μm) (Fig. 3B).

The rmGH formulations that had been stressed by freeze-thawing (both those produced on days 2 and 23) contained markedly higher levels of micron-sized particles compared to the

ultra-centrifuged samples (Fig. 4). Thus the freeze-thawed rmGH formulations contained both nano- and micron-sized particles, whereas the particle fraction in ultra-centrifuged rmGH formulations contained a larger proportion of nano-sized particles (Figs. 2–4).

Estimation of Mass of Protein Particles per Dose—The total mass of protein aggregate injected per dose ranged from 0.8–2.6 μg (Fig. 5). On both preparation days, the particle mass in the freeze-thawed rmGH doses was much higher than those in the ultra-centrifuged doses. In both preparations nano-sized protein particles contributed very little to the total protein rmGH mass in the formulations.

Immunogenicity

Anti-rmGH IgG Isotype Antibody Titers—No anti-rmGH antibody was detected in the sera of mice injected with buffer by the SQ, IP or IV routes at day 22 or day 36 (data not shown). In all treatment groups, one injection of rmGH was sufficient for detectable titers of anti-rmGH antibodies to appear (Fig. 6A–C and 7A–C). Mice injected by SQ and IP routes with freeze-thawed rmGH and mice injected by all routes of administration with ultra-centrifuged rmGH maintained detectable IgG1 and IgG2a titers throughout the study (Fig. 6A, 6B, 6D, 6E, and 7). However, mice injected with freeze-thawed protein by the IV route produced detectable levels of IgG2a without IgG1 at day 22 (Fig. 6C) and later developed a mixed IgG1/IgG2a response at day 36 (Fig. 6F). Titers of IgG2c and/or IgG3 were detected in all groups after two injections, except for the ultra-centrifuged rmGH injected IP (Fig 6D–F, 7D and 7F). More interestingly, after the second injection both preparations of rmGH injected by the IV route elicited an immune response so high that it exceeded the upper detection limit of our assay, an anti-rmGH titer of 5×10^5 (Fig. 6F and 7F). Attempts to raise the upper limit of detection with an extended dilution series were unsuccessful as our assay already works at the limit resulting from the noise of the instrument and detection antibodies (data not shown).

Total IgG Concentration—Injection site inflammation or possible contaminants could lead to increased total IgG concentrations and thus an increase in the background in our ELISA assay. This increased background might be incorrectly observed as increasing anti-rmGH titers and result in the report of false positives. However, the total IgG concentrations, within error, did not increase over the course of the study (Fig. 8) and fell within the published IgG concentration ranges for mice of similar strain and ages.³⁶

IGF-1 Concentration and Weight Gain—In order to investigate whether the appearance of anti-rmGH antibodies were causing a neutralizing response to endogenous growth hormone, we monitored serum IGF-1 concentrations and mouse weight gain. Growth hormone is released episodically by the pituitary gland and cleared quickly, and thus it is not useful to track its levels directly.³⁷ For this reason we tracked levels of IGF-1, which is stimulated by GH and maintained in circulation at predictable levels. Over the course of our study, IGF-1 levels did not vary significantly (Fig. 9) and align well with reports of expected IGF-1 ranges for mice of corresponding age.^{38,39} Additionally, the average weights of mice injected with buffer alone versus mice injected with rmGH preparations containing particles did not vary significantly (Fig. 10) and align well with the expected weight gain as posted by

the distributor.⁴⁰ These observations are not a consequence of use of a non-biologically active rmGH since previous studies have shown that hypophysectomized rats that did not gain weight in the absence of rmGH, regained normal weight gain when administered similar rmGH preparations.²⁹

Discussion

We found our attempt to create specific protein particle size distributions reproducibly in rmGH samples a difficult endeavor. The freeze-thawed formulation prepared on day 2 contained more protein nanoparticles than did the corresponding sample prepared on day 23 (Fig. 2 and 3). Controlling particle types and levels in protein samples is difficult because aggregation pathways and responses of protein samples to stress are varied, numerous and difficult to control—even commercial grade pharmaceutical products display lot-to-lot variability in particle loads.^{16,41}

Also, it is important to note that although PTA (0.03–1 μm), RMM (0.2–4 μm) and MFI (1–50 μm) measure particle distributions in overlapping size ranges, particle counts detected by each technique in overlapping size ranges do not match. PTA utilizes light scattering analysis of Brownian motion, RMM detects buoyant mass and MFI performs image analysis of flowing samples to determine particle size and concentration information. The use of all three techniques on the same sample demonstrates that apparent particle size distributions may depend on the measurement technique. However, the qualitative agreement between the methods allows us to provide interpretations of the relative effects of particle size distributions and load on the immune response.

Overall, the higher quantities of microparticles (Fig 4) and resulting higher dose of protein particles (Fig. 5) in our freeze-thawed preparation compared to the ultra-centrifuged preparation did not result in the development of a statistically significant increase in anti-rmGH antibodies (Table 1). This result is in contrast to reports showing that increasing the dose of a highly aggregated protein therapeutic increases resultant immunogenicity.^{12,15} It is possible that the differences in microparticle quantity and aggregate dose were not great enough in our study to produce resolvable differences in immunogenicity. In all rmGH samples, the protein particles constitute only a small percentage of the total protein mass injected per dose (5.0% $\pm$ 0.8% for ultra-centrifuged rmGH prepared on day 2, 14.7% $\pm$ 1.2% for freeze-thawed rmGH prepared on day 2, 5.7% $\pm$ 1.3% for ultra-centrifuged rmGH prepared on day 23 and 13.5% $\pm$ 1.6% for freeze-thawed rmGH prepared on day 23). It is also possible that immunogenicity is more heavily influenced by nanoparticle populations and the impact of microparticles is negligible in comparison.

In an earlier study, it was documented that injection of 2 μg control, unfrozen rmGH for 5 days a week over 3 weeks resulted in a few instances of only low-level immune response in mice.¹⁵ Furthermore, treatment of mice with rmGH, which had been subjected to a high hydrostatic pressure treatment to reduce protein aggregates and particles to essentially undetectable levels, resulted in no measurable anti-rmGH antibodies in any of the treated mice.¹⁵ However, our data show that a single injection of even small amounts of rmGH protein particles is adequate to break tolerance and create anti-rmGH antibodies. Mice that

were injected by SQ and IP routes with freeze-thawed rmGH and by all routes with ultra-centrifuged rmGH displayed measureable levels of both IgG1 and IgG2a (Fig. 6A, 6B, 6D, 6E, and 7), indicating involvement of a T cell dependent mechanism.⁴² Speculations as to whether the responses are Th1 or Th2 biased in these groups cannot be made as the relative affinities of the antibodies used in the ELISA assays are not known. Mice injected IV with the freeze-thawed rmGH initially had levels of IgG2a at day 22, indicating a Th1 mediated mechanism, that later developed into a more Th1/Th2 mixed response at day 36 (Fig 6C and 6F). However, it is important to note that no conclusions on the response before day 22 can be made as blood was not drawn at this time. The appearance of IgG2c and/or IgG3 can be attributed to a T cell independent type 2 (TI-2).^{43,44} which is observed in groups injected twice with freeze-thawed protein by all routes (Fig. 6D, 6E and 6F) and groups injected twice with ultra-centrifuged protein SQ and IV (Fig. 7D and 7F). Interestingly, despite the production of anti-rmGH antibodies, the mice retained the ability to regulate and use growth hormone as evidenced by their normal weight gain (Fig. 10) and normal IGF-1 levels (Fig. 9). Although this finding is not entirely surprising as it is uncommon to find a neutralizing effect in patients with anti-recombinant human growth hormone antibodies.^{45–48}

Traditionally, Th1 immunity is characterized by phagocytic activity and described as cell-mediated with production of opsonizing antibodies for protection against intracellular pathogens and prevention of infection.⁴⁹ Meanwhile, Th2 responses are associated with protection against extracellular pathogens utilizing high antibody titers and humoral immunity for inhibition of inflammation.⁴⁹ It is unclear why the freeze-thawed rmGH IV-injected mice produced a Th1-like response initially, especially as infection or endotoxin contamination can be ruled out since total IgG titers did not change significantly overtime (Fig. 8). However, the Th2 response observed from the other mouse treatment groups may be explained by the particulate nature of the injections. Adsorption to aluminum adjuvant is the prototypical method for creation of a Th2 response to antigen.⁵⁰ One proposed explanation is the presence of particulate aluminum enhances phagocytosis by antigen presenting cells.⁵¹ Additionally, Babiuk et al. found that increased aggregate content in influenza vaccines is associated with polarization to a Th2 response.⁵² This may also explain our observation that injection of rmGH protein particles resulted in the production of Th2-like isotypes. TI-2 responses are typically initiated towards pathogenic surface antigens that are ordered in a highly repetitive form and cross-link receptors on B cells to activate them without T cell help.⁵³ Work by Dintzis et al. found that this mechanism requires formation of an immunon, which contains a minimum of 10–20 repetitive antigenic receptors with a specific spacing.²⁷ It is possible that protein aggregates are structured such that they are presented as immunons, thus explaining our data that suggest TI-2-mediated pathways are involved in the response to rmGH aggregates. Overall, the evidence of multiple immunological mechanisms reveals that the immunogenicity to protein particles and aggregates is dictated by a multi-layer antigen-specific and non-specific immune response.

The production of antibodies that recognize an antigen requires recognition by an antigen presenting cell (e.g. dendritic cells, macrophages, B cells), processing and presentation to a T cell with co-stimulatory molecules and activation of B cells. However, many other factors play a role in the type of immune response that is stimulated. Route of administration affects immunogenicity because injections into different tissues expose the antigen to different

transport kinetics and cell subsets. Particles injected SQ or IP must be transported passively by diffusion or actively by cells to the lymphatics for an immune response to occur. Manolova et al. has shown that SQ administration of 20–200 nm sized nanoparticles results in association with lymph node-resident dendritic cells (DCs) and macrophages within 2–3 hrs post-injection, suggesting free drainage of these smaller particles. On the other hand, larger particles (500–2000 nm) were mostly associated with injection site DC and hardly detectable quantities were at lymph nodes 8–24 hrs post-injection, with the antigen transported there by phagocytic monocytes.⁵⁴ Furthermore, even when antigens are within lymphatic vessels it has been shown that relatively small molecular weight (e.g. 14 kDa) antigens are delivered by conduits deep within the follicle whereas larger (e.g. 250 kDa) antigens are restricted to the lymph node sinus.⁵⁵ Conversely, with an IV injection, particles are directly introduced into the blood stream and filtered by various organs. Biodistribution studies with antigen-pulsed DCs have shown that IV injected DCs accumulate in the spleen, lungs and liver with little accumulation in lymph nodes. In contrast, half of DCs injected SQ and IP remained at the injection site while the rest migrated to lymph nodes (some of the IP injected DCs were also found in the spleen).⁵⁶ In addition to the differing transport kinetics, the route of administration also determines the cells that are most likely to interact with administered particles. Different subpopulations of DCs have been found in the thymus, spleen, Peyer's patches, lymph nodes and skin.⁵⁷ Moreover, different DC types have been found to have differing ability to polarize Th1 or Th2 subsets.^{58,59}

Our most interesting finding is that both preparations of rmGH are extremely immunogenic when administered IV. Anti-rmGH titers from serum collected on day 36 indicate that IV administration results in statistically significantly higher titers of all IgG isotypes (*p* value 0.027), except for IgG1, compared to SQ and IP (Table 1). An explanation for the observed robust immune response to an IV administration of rmGH protein particles may be attributed to the route's direct presentation of the rmGH aggregates to the B cells, DCs and T cells of the spleen. A study on IV injections of human γ -globulin in mice found that protein aggregates—but not protein monomers—localized to macrophages in the red pulp and marginal zones of the spleen within 30 minutes; 2 hours later the protein aggregates were found in macrophages of the white pulp.⁶⁰ The splenic marginal zone contains many macrophages, DCs and non-recirculating B cells with an IgM^{hi}IgD^{low}CD21^{hi}CD23^{low} phenotype.⁶¹ Compared to recirculating or immature B cells, marginal-zone B (MZ B) cells seem to have a lower threshold for activation, proliferation and differentiation^{62,63} and are thought to be involved in the immune response to blood-borne antigens through both T-cell dependent (TD) and independent (TI) mechanisms.^{44,61,64,65} However, they are better known for their response to TI antigens.^{62,66} Previous publications investigating the involvement of MZ B cells in the immune response to Betaferon® protein aggregates and hen egg lysozyme found that both MZ B and CD4+ T cells (such as Th1 or Th2 cells) were involved, however the response did not align with a classical TD or TI mechanism.^{25,63} Additionally, Mongini et al. reported that TI-2 antigens in the presence of T cells can cause production of all IgG isotypes.⁶⁷ These observations are similar to our own, and perhaps it is the quicker presentation to pertinent cell subsets that resulted in our finding that IV administration is much more efficient at stimulating production of rmGH antibodies than SQ or IP administration.

Conclusions

The immunogenicity of protein therapeutics is an issue that the field is just beginning to understand. Here we show that IV injection of rmGH protein aggregates greatly enhances immunogenicity compared to SQ or IP administration, a finding contrary to the popular viewpoint that SQ is the most immunogenic route of administration. Even though two preparations were tested, a preparation with particles only in the nanometer size range and a preparation containing both nano- and microparticles, no significant difference in resulting immunogenicity was found. We identified multiple potential mechanisms responsible for the immune response, including a mixed Th1/Th2 response and a TI-2 response, but no neutralizing response was observed. Additional work investigating which cell subsets in the spleen are responsible for the greatly enhanced immune response associated with IV injection would help us better understand the mechanisms involved.

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Figure 1.

SEC chromatograms of injections prepared on day 2 (A) and day 23 (B) for ultra-centrifuged (dotted line) and freeze-thawed (solid line) rmGH preparations. The rmGH monomer peak appears at 28 min and, since detection was done at 215nm, the citrate buffer peak appears at 40 min.

Figure 2.

Particle size distributions for nano-sized populations collected via particle tracking analysis for injections prepared on day 2 (A) and day 23 (B) of ultra-centrifuged (gray) and freeze-thawed (black) rmGH preparations. Size bins of 0.001 μ m were used for representation of particle concentrations and error bars shown are standard deviation.

Figure 3. Particle size distributions for nano-sized populations collected via resonant mass measurement for injections prepared on day 2 (A) and day 23 (B) of ultra-centrifuged (gray) and freeze-thawed (black) rmGH preparations. Size bins of 0.017 μ m were used for representation of particle concentrations and error bars shown are standard deviation.

Figure 4.

Particle size distributions for micron-sized populations collected via flow imaging for injections prepared on day 2 (A) and day 23 (B) of ultra-centrifuged (gray) and freeze-thawed (black) rmGH preparations. Size bins of 0.25 μ m were used for representation of particle concentrations and error bars shown are standard deviation.

Figure 5.

Estimates of total mass of protein particles per dose of ultra-centrifuged (white bars) and freeze thawed (gray bars) rmGH for day 2 sample preparation (A) and day 23 sample preparation (B). Insets represent an expanded y-axis for PTA and RMM data and error bars shown are standard deviation.

Figure 6.

Anti-rmGH IgG isotype antibody titers for mice injected with freeze-thawed rmGH via the SQ route on day 22 (A), IP route on day 22 (B), IV route on day 22 (C), SQ route on day 36 (D), IP route on day 36 (E) and IV route on day 36 (F). No anti-rmGH antibody was detected in the sera of mice injected with buffer by the SQ, IP or IV routes at day 22 or day 36 (data not shown). Each data point represents the average anti-rmGH titer of one serum sample from one mouse, assayed in triplicate. Bars represent the average titer of responders and the fraction above each titer signifies the number of responders out of 8 mice.

Figure 7.

Anti-rmGH IgG isotype antibody titers for mice injected with ultra-centrifuged rmGH via the SQ route on day 22 (A), IP route on day 22 (B), IV route on day 22 (C), SQ route on day 36 (D), IP route on day 36 (E) and IV route on day 36 (F). No anti-rmGH antibody was detected in the sera of mice injected with buffer by the SQ, IP or IV routes at day 22 or day 36 (data not shown). Each data point represents the average anti-rmGH titer of one serum sample from one mouse, assayed in triplicate. Bars represent the average titer of responders and the fraction above each titer signifies the number of responders out of 8 mice.

Figure 8.

Total IgG serum concentrations of pre-treatment (closed circles), SQ injected (open circles), IP injected (open squares) and IV injected (open triangles) mice throughout the study. Bars represent the average of each age group.

Figure 9.
Average IGF-1 serum concentrations for mice throughout the study. Error bars shown are standard deviation.

Figure 10.

Weights of mice treated with rmGH-containing preparations are shown in panel A: freeze-thawed rmGH injected SQ (circles), freeze-thawed rmGH injected IP (triangles), freeze-thawed rmGH injected IV (squares), ultra-centrifuged rmGH injected SQ (inverted triangles), ultra-centrifuged rmGH injected IP (hexagons) and ultra-centrifuged rmGH injected IV (diamonds). Average weight of mice in groups injected with buffer only via the SQ route (circles), IP route (squares) and IV route (triangle) are shown in panel B. Error bars shown are standard deviation.

Table 1

Calculated *p* values for anti-rmGH titers from serum collected day 36 between experimental groups

Isotype	Preparation	Route	Freeze-thawed			Ultra-centrifuged		
			SQ	IP	IV	SQ	IP	IV
IgG1	Freeze-thawed	SQ		0.008	0.333	0.001	0.018	0.144
		IP			0.082	0.044	0.634	0.001
		IV				0.004	0.053	0.027
	Ultra-centrifuged	SQ					0.365	<0.001
		IP						0.001
		IV						
IgG2a	Freeze-thawed	SQ		0.153	<0.001	0.01	NS	<0.001
		IP			0.001	0.224	0.745	0.001
		IV				0.001	0.027	NS
	Ultra-centrifuged	SQ					0.628	0.001
		IP						0.027
		IV						
IgG2b	Freeze-thawed	SQ		0.747	<0.001	0.001	<0.001	<0.001
		IP			<0.001	0.007	0.004	<0.001
		IV				<0.001	<0.001	NS
	Ultra-centrifuged	SQ					0.239	<0.001
		IP						<0.001
		IV						
IgG2c	Freeze-thawed	SQ		0.144	<0.001	NS	NS	<0.001
		IP			<0.001	0.144	0.144	<0.001
		IV				<0.001	<0.001	NS
	Ultra-centrifuged	SQ					NS	<0.001
		IP						<0.001
		IV						
IgG3	Freeze-thawed	SQ		0.001	<0.001	0.602	0.143	<0.001
		IP			<0.001	0.001	<0.001	<0.001
		IV				<0.001	<0.001	NS

Isotype	Preparation	Route	Freeze-thawed				Ultra-centrifuged			
			SQ	IP	IV		SQ	IP	IV	
	Ultra-centrifuged	SQ						0.063	<0.001	
		IP							<0.001	
		IV								
NS, not significant										