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. 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 6 weeks with rmGH formulations via the subcutaneous, intraperitoneal, or intravenous (i.v.) routes. In addition to soluble, monomeric rmGH, the samples prepared contained either nanoparticles of rmGH or both nano- and microparticles 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 i.v. administration was used. Despite producing high anti-rmGH titers mice appeared to retain the ability to properly regulate and use endogenous growth hormone. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:128–139, 2014
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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% to 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 1990s, 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 2711 patients on Avonex®, Rebif®, or Betaseron® were submitted over a 3-year period found a negative correlation between the magnitude of the anti-INFβ NAb response and therapeutic efficacy.[9] Non-Nabs also warrant monitoring as they may enhance clearance of the therapeutic, thus reducing efficacy[10] 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 subcutaneous (s.q.) administration is more immunogenic than i.v. 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 IFNβ-1a had a higher incidence of immunogenicity in patients when injected s.q. than intramascular (i.m.). However, the products that were injected by the two routes were different (and presumably contained different protein particle and aggregate loads[16]) and were administered at different doses, thus making a direct comparison of injection routes difficult.[17] In another example, following the discovery that s.q. administration of the erythropoietin product—Eprex—contributed to immunogenicity, a mandate to switch exclusively to i.v. 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.[11] found that administration of 0.3 μg of IFN-α2a aggregates once weekly for 5 weeks produced increasing immune response in mice in the following order: s.q. > i.p. > i.m. ≫ i.v.. Another group also found higher immunogenicity for s.q. administration as compared with i.v. administration of 4 weekly injections of rFVIII in Hemophilia A mice.[20] Interestingly, they later found that i.v. injections of PEGylated rFVIII were more immunogenic than s.q. administration.[21] Likewise, Kijanka et al.[12] recently found that i.v. injections of Betaferon® (EU) were more immunogenic than s.q. or i.m. injections. Furthermore, none of these earlier studies report results for sample particle contents

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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 with 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 performed 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 cross-linking 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 (SEC) and counting and sizing of nano- and microparticles. We then compared the immune response resulting in mice following injections administered via the s.q., i.p. or i.v. 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, Pennsylvania). Peroxidase-goat anti-mouse IgG (H+L) (62-6520) and peroxidase-goat anti-mouse IgG1 (877586) were obtained from Invitrogen (Eugene, Oregon). TMB substrate, Tween 20®, and Immulon 4HBX flat bottom plates were obtained from Thermo Scientific (Waltham, Massachusetts). Sulfuric acid, chloramphenicol, ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), citric acid monohydrate, trisodium citrate dehydrate, Tris–HCl, ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate monohydrate, reduced glutathione, oxidized glutathione, bis–tris, NaCl, urea and yeast extract were obtained from Fisher Chemical (Fair Lawn, New Jersey). Sterile 1 mL tuberculin syringes (309659) were purchased from Becton, Dickinson and Company (Franklin Lakes, New Jersey). Quantikine ELISA for mouse/rat IGF-1 was obtained from R&D Systems (Minneapolis, Minnesota). 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 5 mL 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 h. 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 h. 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 h. 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 min total (alternating 20 s intervals of sonication and rest) on ice. Inclusion bodies were then centrifuged at 17,000*g* for 30 min 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 two to four 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 min 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, Colorado) 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 five 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 nonreducing SDS-PAGE, SEC 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