In Vivo Characterization of Sustained-Release Formulations of Human Growth Hormone

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ABSTRACT

Long-acting formulations of recombinant human growth hormone (rhGH) were prepared by stabilizing and encapsulating the protein into three different injectable, biodegradable microsphere formulations composed of polymers of lactic and glycolic acid. The formulations were compared in juvenile rhesus monkeys by measuring the serum levels of rhGH and two proteins induced by hGH, insulin-like growth factor-I and IGF binding protein-3 (IGFBP-3) after single s.c. administration. All three formulations, which differed principally in the composition of the polymer, provided sustained elevated levels of all three proteins for several weeks, and the rate of release of rhGH differed among the formulations consistent with the molecular weight of the polymer used. All three formulations induced a higher level of insulin-like growth factor-I and insulin-like growth factor binding protein than was induced by daily injections of the same amount of rhGH in solution. After three monthly injections of one of the formulations, both the rhGH and IGF-I levels remained elevated for nearly 90 days. Immunogenicity of the rhGH released from this formulation, as assessed by the incidence of seroconversion to hGH and the titer of anti-hGH antibody in both the rhesus monkeys and transgenic mice expressing rhGH, was no greater than that of the unencapsulated protein. In addition, the microsphere injection sites appeared normal by macroscopic evaluation between 1 to 2 mo after microsphere administration and by microscopic evaluation between 2 to 3 mo. These results show that serum levels of a therapeutic protein can be sustained for an extended period when encapsulated into different formulations of injectable, biodegradable microspheres.

rhGH, a 191 amino acid protein, is used to treat short stature caused by growth hormone deficiency, and promising clinical results have been obtained in the treatment of Turner's syndrome and growth failure due to chronic renal insufficiency (Daughaday, 1995). In addition, because of its anabolic effects, there is clinical evidence that hGH may be useful in treating trauma, clinical malnutrition, osteoporosis and to facilitate wound healing.

hGH is stored in the anterior pituitary and secreted in a pulsatile fashion mainly during sleep (Finkelstein et al., 1972). Because it is a protein, it is not absorbed orally to any significant extent (Lee, 1995). rhGH is currently administered by daily or thrice weekly injections over a period of several years. However, recent clinical studies have shown that continuous infusion of rhGH via a pump results in growth velocity and IGF-I levels comparable to those achieved with daily injections (Jorgensen et al., 1991, Laursen et al., 1994, Laursen et al., 1995, Tauber et al., 1993). This result demonstrates that continuous, as well as pulsatile, administration of rhGH is efficacious, although the full range of potential differences between these two regimens of growth hormone administration has not yet been fully investigated.

One method to produce injectable, sustained-release formulations of proteins, including rhGH, is to encapsulate the drug into injectable microspheres of biodegradable PLGA from which the drug is released slowly by diffusion and as the polymer degrades (Cleland, in press). Microspheres made from PLGA are biocompatible and biodegradable into lactic and glycolic acid and thus do not have to be removed. These polymers are also used to make sutures, bandages and bone plates (Austin et al., 1995, Pihlajamaki et al., 1992, Winde et al., 1993). Depending on different polymer

ABBREVIATIONS: rhGH, recombinant human growth hormone; PLGA, poly lactide co-glycolide; IGF-I, insulin-like growth factor I; IGFBP-3, insulin-like growth factor binding protein 3; LHRH, leutinizing hormone releasing hormone; CL, clearance; Vd, volume of distribution; AUC, area under the curve; F, bioavailability; iv., intravenous; sc., subcutaneous; HPLC, high performance liquid chromatography; Cmax, maximum concentration; Tmax, time to maximum concentration.
properties, such as polymer chain length, lactide:glycolide ratio, and the presence of polymer end-group modification, the degradation rate and hence the rate of drug release can be controlled.

The processes commonly used to produce PLGA microspheres, such as that used to make the currently marketed sustained-release formulations of LHRR (Ogawa et al., 1988a, 1988b), were developed to encapsulate relatively small and stable molecules. They use elevated temperatures, surfactants or aqueous/organic solvent interfaces, conditions that denature and inactivate many proteins. To accomodate the stability needs of proteins, we have developed a process that is carried out at cryogenic temperatures, uses no water and hence avoids oil-aqueous interfaces and requires no surfactants (Johnson et al., 1996). This process, specifically designed to encapsulate relatively labile macromolecules, results in PLGA microspheres that release protein with physical, chemical and biological properties essentially identical to those before encapsulation.

Our study assesses the pharmacokinetics, the biologic effect and the potential immunogenicity of rhGH released from three different PLGA microsphere formulations. We find that all exhibit sustained-release and induce sustained biological effect of rhGH and one of the formulations, chosen for more extensive investigation, demonstrates no greater immunogenicity than when the protein in solution is administered by frequent injections. In addition, there are no adverse effects either systemically or at the site of injection. Moreover, when an equivalent total dose is administered in a continuous fashion rather than by frequent injections, rhGH elicits a greater effect as measured by IGF-I levels. These results suggest that, as has been demonstrated with nonprotein drugs (Langer, 1990), sustained-release formulations of therapeutic proteins have the potential to improve the convenience of use and the safety and efficacy of this increasingly important class of drugs.

Methods

Preparation of microspheres. Microspheres were fabricated as described (Johnson et al., 1996). Briefly, to form the Zn-hGH complex, six molar equivalents of zinc acetate were added to rhGH (Nutropin, Genentech, Inc., South San Francisco, CA) in 4 mM sodium bicarbonate pH 7.2. After complex formation, the suspension was sprayed through a sonic nozzle into liquid nitrogen, placed at -80°C and lyophilized. After lyophilization the protein powder was suspended in a solution of polymer in methylene chloride. The polymer used for formulation I (table 1) was from Birmimgham Polymers (Birmingham, AL) (lot 115–56-1, internal viscosity 0.23 dl/g), and polymers RG502H (internal viscosity 0.17 dl/g) and RG503H (internal viscosity 0.4 dl/g) (Boehringer Ingelheim, Petersburg, VA) were used for formulations II and III, respectively. Zinc carbonate was added (6% w/v for formulation I and 1% w/v for formulations II and III) and the suspension was then sprayed through a sonic nozzle into liquid nitrogen overlaying frozen ethanol and placed at -80°C for 24 hr. at which time an equal volume of -50°C ethanol was added. After 48 hr the microspheres were recovered using a 0.65-μ filter and dried under vacuum. The microspheres have a mean diameter of approximately 50 μ and are suspended in an aqueous vehicle (3% w/v carboxymethyl cellulose, low viscosity; 1% v/v polysorbate 20 and 0.9% w/v NaCl) before injection.

Animals. Juvenile (prepubescent) male rhesus monkeys (Macaca mulatta) were housed at Corning Hazleton, Inc. (Madison, WI). At the initiation of treatment, they were between 11 and 27 mo old and weighed between 1.9 and 3.8 kg. Monkeys were maintained on a 14-hr light/10-hr dark cycle. Details of dose administration are given in the legend to table 2. To determine the local effects of the microspheres, the s.c. tissue surrounding the injection sites was isolated, fixed in 10% neutral buffered formalin, embedded in glycomethacrylate, sectioned at 2 to 3 μ and stained with H&E or with an immunohistochemical stain for hGH.

Mice (BalbC and rhGH transgenic BalbC) were housed at Charles River Laboratories (Wilmington, MA). Both sexes of mice were used and no animal was older than 10 mo at the initiation of the study. Sera from those expressing rhGH were screened by ELISA for endogenous rhGH levels and all animals included in the study had a level of more than 50 ng/ml.

Quantitation of serum protein concentrations. Serum concentrations of rhGH in the monkeys were determined using an immunoradiometric assay (RADIM Group, Rome, Italy). Endogenous monkey GH was detected by this assay. Total IGF-I was measured using a radioimmunoassay (Liberman et al., 1992) and IGFFBP-3 concentrations were determined by an immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX). All concentrations reported in the figures are means ± S.E.M.

Detection of anti-hGH antibodies. The presence of anti-hGH antibodies in the serum was determined using a radioimmunoprecipitation assay. Nonimmune serum was used as a negative control. Serum samples were incubated with ^125I-rhGH (INC Pharmaceuticals, Inc., Costa Mesa, CA) and the antibody-bound ^125I-rhGH precipitated with 16% PEG 8000. Serum samples at a dilution of 1:10 having a value less than twice the negative control value were scored as negative.

Pharmacokinetic analysis. Noncompartmental pharmacokinetic analysis was performed on the rhGH concentration vs. time profile, using RSTRIP (Micromath Scientific Software, Salt Lake City, UT), a program designed for exponential stripping and parameter estimation. All predose concentrations and all postdose concentrations lower than the lowest standard (1.5 ng/ml) were considered to be zero. The following pharmacokinetic parameters were determined: area under the curve (by trapezoidal integral) from time zero to day 2 (AUC0–2); AUC from time zero to the last day of sampling (AUC0–t); maximum blood concentration (Cmax), time to the maximum concentration (Tmax), cumulative release and absolute bioavailability (F).

Results

Preparation of rhGH containing microspheres. The PLGA microspheres were made by precipitating rhGH from solution by the addition of zinc acetate and encapsulating the Zn-protein powder using a nonaqueous, cryogenic process (see “Methods”). Zinc, which reversibly complexes with hGH and causes the dimerization of the protein (Cunningham et al., 1991), is present at high concentrations in the secretory granules of the anterior pituitary (Thorlacius-Ussing, 1987). A zinc complex is believed to be the form in which the protein is naturally stored. The three microsphere formulations used in this study, which were selected from a panel of 11 formulat
exchange chromatography (Johnson et al., 1997), in press), differed in the content of zinc carbonate and polymer composition (table 1). The lactide:glycolide ratio of all three polymers was 50:50. The rhGH extracted from all three microsphere formulations was shown to be identical to that before encapsulation by size exclusion chromatography, reverse phase HPLC, and anion exchange chromatography (Johnson et al., in press). This indicates that the encapsulation process caused no detectable change in the protein.

Pharmacokinetics of rhGH release. To evaluate the pharmacokinetics and pharmacodynamics of rhGH release, juvenile rhesus monkeys were used because of their low level of endogenous growth hormone. This experiment included three groups of animals receiving the microsphere formulations and three additional groups receiving rhGH solution by different means; all animals in each of these six groups received a total of 24 mg of rhGH (table 2). The animals receiving the microspheres (groups 1–3) received a single s.c. injection of 160 mg of microsphere formulations I to III (24 mg rhGH), respectively; group 4 received the entire amount of rhGH as a single s.c. bolus of protein in solution and group 5 received rhGH in solution administered daily for 28 days (0.86 mg/day). To mimic the expected release rate from the microspheres, group 6 received 15% of the 24 mg (3.6 mg) as a s.c. bolus (to mimic the initial release) and the remaining 20.8 mg were delivered continuously via a 28-day osmotic pump surgically implanted s.c. A seventh group received only the microsphere vehicle, which resulted in no change in the growth hormone. IGF-I or IGFBP-3 levels (data not shown).

rhGH serum profiles for the first 48 hr are shown in figure 1 and the parameters describing the pharmacokinetics of the protein are given in table 2. As expected, all animals receiving the microspheres exhibited a peak in the serum level within the first day caused by the initial release of protein. However, in each case the T_{max} values were significantly greater than those groups receiving protein solution (0.4 vs. 0.1 days) indicating that the microspheres delayed the peak serum concentration. In addition, the serum profiles of rhGH induced by the microspheres were flatter and more extended than those induced by the protein solution and the maximum serum concentrations reached at the same dose were also lower (cf. C_{max} values of groups 1–3 vs. group 6).

The complete serum profiles are shown in figure 2. Relative to the predose levels of growth hormone, which were equal to or less than 1 ng/ml for all groups, each of the microsphere formulations gave sustained elevated levels of rhGH. In contrast, in the animals receiving the entire dose as a single injection, the rhGH levels returned to predose levels within 24 hr. The levels induced by the microsphere formulations differed somewhat from one another. In contrast to formulations I and II, formulation III showed a lag phase in which the levels dipped from days 10 to 17 after which they increased. The profile induced by formulation II most closely paralleled that induced by the constant release of protein from the osmotic pump. The pump induced levels of 16 ng/ml until about day 25 and thereafter, the levels fell to base-line levels (the pump was surgically removed on day 31). Formulation II gave a level of approximately 10 ng/ml until day 17 at which time it fell to about 5 ng/ml. The GH levels induced by all three formulations were slightly elevated after day 30. The reason for this may be cross-reactivity with endogenous monkey GH (which would be expected to increase as the animals age) or to a low level of release of rhGH from the microspheres.

The cumulative amount released from the microspheres, calculated from the serum levels, indicated that, by day 61, 105, 83 and 87% of the protein had been released from for-

### Table 2: Pharmacokinetic parameters of microencapsulated rhGH

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (kg)</th>
<th>Dose (mg rhGH)</th>
<th>Form of protein</th>
<th>AUC_{0–24h} (ng·h/ml)</th>
<th>C_{max} (ng/ml)</th>
<th>T_{max} (days)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.1 ± 0.3</td>
<td>24.0</td>
<td>Form. I</td>
<td>710 ± 110</td>
<td>320 ± 100</td>
<td>0.38 ± 0.12</td>
<td>66 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>3.3 ± 0.2</td>
<td>24.0</td>
<td>Form. II</td>
<td>290 ± 90</td>
<td>270 ± 40</td>
<td>0.44 ± 0.02</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>3</td>
<td>3.2 ± 0.2</td>
<td>24.0</td>
<td>Form. III</td>
<td>230 ± 30</td>
<td>340 ± 20</td>
<td>0.40 ± 0.03</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>3.3 ± 0.2</td>
<td>25.9</td>
<td>Solution</td>
<td>660 ± 50</td>
<td>NA</td>
<td>0.11 ± .02</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>5</td>
<td>3.0 ± 0.2</td>
<td>24.0 (0.86/day)</td>
<td>Solution</td>
<td>NA</td>
<td>180 ± 30</td>
<td>0.07 ± 0.01</td>
<td>85 ± 13</td>
</tr>
<tr>
<td>6</td>
<td>3.2 ± 0.2</td>
<td>24.0 (3.6 ± 20.8)</td>
<td>Solution</td>
<td>NA</td>
<td>550 ± 50</td>
<td>0.01 ± 0.02</td>
<td>50 ± 8</td>
</tr>
</tbody>
</table>

Microsphere formulations are described in table 1. All values are means ± S.E.; NA, not applicable. There were four animals in each group with the exception of group 1 which contained three. All protein was administered s.c. in the dorsal cervical region. Microspheres (160 mg) were suspended in 1.2 ml of vehicle and injected using a 21-gauge needle. The rhGH in solution (13.2 mg/ml) was in 8.8 mg/ml NaCl, 2.5 mg/ml phenol, 2.0 mg/ml Tween 20 and 10 mM sodium citrate, pH 6.0.

In vitro analysis, in which microspheres were suspended in aqueous buffer and the rate of protein release was measured, showed that approximately 15% of the protein was released in the first 24 hr with the remainder released over approximately 1 mo.
mulations I to III, respectively. In addition, the subcutaneous bioavailability (F) of the protein was determined by comparison of the AUC values of groups 1 to 6 to those determined from animals that received rhGH solution via a single i.v. infusion over 5 min (table 2, footnotes). The bioavailability of the protein released from the three microsphere formulations (66, 51 and 61% from formulations I-III, respectively) were comparable to that of the protein in solution (groups 4–6). These results indicate that the majority of the protein released from the microspheres was bioavailable to an extent similar to rhGH formulated as a solution and administered s.c.

Biological effect of hGH. The IGF-I and IGFBP-3 serum levels induced by the three microsphere formulations, the osmotic pump and daily injections of rhGH solution are shown in figures 3 and 4, respectively. Whereas the single injection of the entire amount of rhGH (group 4) resulted in no change in the levels of either of these proteins (not shown), each of the microsphere formulations and the osmotic pump induced sustained elevated levels of both proteins. Consistent with the shorter sustained level of rhGH from formulation I, the IGF-I and IGFBP-3 levels were elevated for a shorter duration relative to the other formulations. In addition, as with the rhGH levels in the animals receiving formulation III, there was an initial elevation followed by a dip in the levels of these two proteins. Notably, continuous administration of rhGH by either the osmotic pump or the microsphere formulations resulted in significantly higher serum concentrations of both of these proteins than when an equivalent overall dose of protein as solution was administered by daily injections. The continuous concentration of rhGH that appeared necessary to sustain an elevation in IGF-I was approximately 5 ng/ml.

Effect of sequential administration of microencapsulated rhGH. Because formulation II gave the most consistent and longest lasting levels of rhGH, IGF-I and IGFBP-3, the effect of three monthly doses of this formulation was evaluated. Juvenile monkeys were given the same dose per body weight as in the previous experiment, and the levels of rhGH (Figure 5A) and IGF-I (Figure 5B) were measured. (Because changes in IGFBP-3 levels paralleled those

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2 The osmotic pumps delivered protein at a rate of 0.62 mg/day and the average serum concentration induced by this rate of infusion was 16 ng/ml (between days 2 and 25) (fig. 2). The rate of release and the cumulative percent release (as a percentage of the total amount delivered) from microspheres was calculated from the serum concentration.
of IGF-I in the first experiment, IGFBP-3 levels were not measured in this experiment). Levels of rhGH after each of the three doses were similar to each other and were maintained between 10–20 ng/mL throughout most of the three month period and above the pre-dose level throughout the entire period. As predicted from the results of the previous experiment, thrice weekly doses of an equivalent amount of rhGH in solution resulted in little, if any, elevation of IGF-I levels.

**Analysis of microsphere injection sites.** To determine the local effect of the microspheres, the sites of injection of the animals receiving each of the three formulations were examined histologically. The injection site from the animals receiving formulations I and II were surgically recovered at day 61 after injection whereas the site from animals receiving formulation III, because of its delayed release pattern, were recovered at day 84. Injection sites of all four animals receiving formulation I were approximately 12 mm in diameter and up to 4-mm thick (not shown). These contained a small amount of polymer and rhGH immunoreactive material and were surrounded by a slight foreign body inflammation. In contrast, the reaction at injection sites of animals receiving formulation II was less pronounced; the injection site could be identified in only one of the four animals and those in the other three animals had completely resolved. This site was smaller (approximately $4 \times 0.1$ mm) and contained less polymer than the sites of animals receiving formulation I. A few small cystic spaces, less than 100 μm in diameter, were surrounded by a mild foreign body inflammatory reaction and a small amount of rhGH immunoreactive material was present. Injection sites of two of the four animals receiving formulation III could not be identified. The other two showed traces of immunoreactive rhGH and minimal inflammation. The size of the immunoreactive area was approximately $5 \times 0.2$ mm.

In the monkeys receiving three monthly injections of formulation II, there was no clinical evidence of local irritation. The injection sites were palpable until approximately 4 wk. Figure 6 shows both macroscopic (A-D) and microscopic (E-F) views of the three sites at which microspheres were injected approximately 1 month apart. One month after microsphere administration, there was an area of discoloration at the injection site (D) and evidence of a typical foreign body reaction of macrophages and multinucleated giant cells (H). Two months after administration of microspheres, the injection site was macroscopically normal (C) and microscopically, there was evidence of macrophages (G); no polymer could be detected. Three months after injection (B and F) the site appeared both macroscopically and microscopically normal. There was no evidence of fibrosis.
Immunogenicity of microsphere formulations. The immunogenicity of the rhGH solution and rhGH released from the microspheres was evaluated by measurement of anti-hGH antibodies. There is potential for anti-hGH antibody formation in rhesus monkeys because the monkey and human growth hormone sequences differ by four amino acids (Li et al., 1986). Seroconversion to rhGH was determined by incubating sera with radiolabeled rhGH followed by precipitation with polyethylene glycol. The results, as well as the endpoint titers of the positive sera, are shown in table 3.

All of the animals receiving formulation I formed circulating antibodies to hGH whereas only one and two of the four animals receiving formulations II and III, respectively, seroconverted (table 3, experiment A). The maximum titers reached in each of the seropositive animals were relatively low, with the titers induced by formulation I being the highest. In contrast, in this experiment none of the animals receiving rhGH solution seroconverted. The animals receiving the three monthly injections of formulation II (table 3, experiment B) showed an incidence of seroconversion (two of seven seropositive animals) comparable to the experiment in which only one dose was given. In this experiment the animals that received the same total amount of rhGH as solution (36 thrice weekly injections) showed a similar incidence of seroconversion (one of four). Maximum titers of antibodies in the seropositive animals in both groups were also comparable.

The immunogenicity of the rhGH released from formulation II was further evaluated using transgenic BalbC mice expressing rhGH. Although these animals do not develop...
antibodies to native hGH, nonnative protein (e.g., aggregated rhGH) is immunogenic (J. Cleland, E. Duenas and A. Jones, unpublished data). Both nontransgenic and transgenic mice received either one dose or three monthly doses of microspheres, or were dosed for either 4 or 12 wk with thrice weekly s.c. injections of rhGH in solution (table 4). Comparable groups received an equivalent amount of protein.

In the nontransgenic animals receiving one dose of microspheres (group 2) about half of the animals seroconverted by day 30 whereas, in those receiving 1 mo of thrice weekly injections of rhGH solution (group 1), nearly all of the animals seroconverted. In both groups the percentage that were seropositive dropped by day 100. In contrast, none of the transgenic animals receiving the protein administered in either form seroconverted (groups 3 and 4). To investigate sequential administration of formulation II, three monthly doses of microspheres or 3 mo of thrice weekly injections of rhGH solution were given to wild-type (groups 5 and 6) or transgenic (groups 7 and 8) mice. Essentially all of the wild-type animals seroconverted by day 51 and were still positive at day 100. The average titer was comparable for the animals receiving the microspheres or the protein solution. In contrast, none of the transgenic animals receiving the microspheres or the protein solution seroconverted.

### Table 3

<table>
<thead>
<tr>
<th>Experiment A—treatment</th>
<th>No. Seroconverting</th>
<th>Maximum Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsphere formulation I</td>
<td>3/3</td>
<td>2.5, 2.5, 2.6</td>
</tr>
<tr>
<td>Microsphere formulation II</td>
<td>1/8</td>
<td>2.0</td>
</tr>
<tr>
<td>Microsphere formulation III</td>
<td>2/4</td>
<td>1.2, 1.3</td>
</tr>
<tr>
<td>1 injection, rhGH solution</td>
<td>0/4</td>
<td>NA</td>
</tr>
<tr>
<td>Daily injections, rhGH solution</td>
<td>0/8</td>
<td>NA</td>
</tr>
<tr>
<td>Osmotic pump, rhGH solution</td>
<td>0/4</td>
<td>NA</td>
</tr>
<tr>
<td>Experiment B—treatment</td>
<td>No. Mice Seroconverting</td>
<td>Maximum Titer</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>1×/mo, 3 mo, formulation II</td>
<td>2/7</td>
<td>1.3, 1.5</td>
</tr>
<tr>
<td>3×/wk, 12 wk, rhGH solution</td>
<td>1/4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Sera were analyzed for the presence of anti-hGH antibodies from samples taken between days 23 and 56 (experiment A) and on days 28, 56 and 76 (experiment B). For the animals for which any of the serum samples tested positive, the titer was determined and the maximum titer reached is listed. Titters are expressed as the log of the dilution that gave a value that was twice the value of the negative control, NA. Not applicable.

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal</th>
<th>Form of rhGH</th>
<th>Treatment Duration (wk)</th>
<th>No. Mice Seroconverting on Day 30</th>
<th>Day 51</th>
<th>Day 100</th>
<th>Mean titer (day 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>wt</td>
<td>Solution</td>
<td>4</td>
<td>12/13</td>
<td>11/12</td>
<td>6/12</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>wt</td>
<td>Microspheres</td>
<td>4</td>
<td>7/13</td>
<td>6/12</td>
<td>3/13</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>hGH tg</td>
<td>Solution</td>
<td>4</td>
<td>0/12</td>
<td>0/10</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>hGH tg</td>
<td>Microspheres</td>
<td>4</td>
<td>0/9</td>
<td>0/9</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>wt</td>
<td>Solution</td>
<td>12</td>
<td>9/12</td>
<td>10/12</td>
<td>11/12</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>wt</td>
<td>Microspheres</td>
<td>12</td>
<td>0/13</td>
<td>11/11</td>
<td>12/12</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>hGH tg</td>
<td>Solution</td>
<td>12</td>
<td>ND</td>
<td>0/11</td>
<td>0/8</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>hGH tg</td>
<td>Microspheres</td>
<td>12</td>
<td>ND</td>
<td>0/12</td>
<td>0/10</td>
<td>NA</td>
</tr>
</tbody>
</table>

There were a total of 10 groups of BalbC mice, 5 nontransgenic (wt, wild-type) (groups 1, 2, 5, 6 and 9) and 5 rhGH transgenic (tg) groups (3, 4, 7, 8 and 10). Both transgenic and nontransgenic animals (groups 9 and 10) received no treatment and none of these animals seroconverted (data not shown). Groups 1 and 3 received a total of 12 s.c. injections of rhGH solution (10 μg dose) delivered thrice weekly (a total dose of 120 μg protein) and groups 5 and 7 received 36 s.c. injections, thrice weekly, of protein solution for 12 wk (a total amount of 360 μg protein). Groups 2 and 4 received a single s.c. injection of microspheres formulation II (120 μg of rhGH, 800 μg microspheres) and groups 6 and 8 received three injections of the same dose of microspheres (days 0, 28, and 56) for a total dose of 360 μg rhGH (2.4 mg microspheres). Statistical analysis (using the Fisher exact test) showed that the percent mice in groups 1 and 2 seroconverting on days 30 and 51 were significantly different (P = 0.036 and 0.034, respectively) whereas the difference on day 100 was not. The difference in percent seroconversion between groups 5 and 6 on day 30 was very significant (P = 0.0001). ND; Not done. NA, not applicable.

**Discussion**

Our data demonstrate that each of the rhGH microsphere formulations provided sustained release and a sustained biological effect. This occurred with all three formulations, each of which elevated IGF-I levels for 3 wk or more. In contrast, even daily injections of the same total amount of rhGH in solution gave a lower increase in the level of IGF-I. However, there were differences in the profile of rhGH release from the three different microsphere formulations and these differences are consistent with the differences in the polymer comprising the three formulations. For example, although both formulations I and II show a spike in rhGH concentration followed by 3 or more wk of sustained serum levels, formulation III, after the initial release, showed a dip in the rhGH serum levels and a concomitant dip in the IGF-I and IGFBP-3 levels. This was followed by a period of an increased release rate and elevated IGF-I and IGFBP-3 levels. Release of encapsulated molecules from PLGA microspheres occurs by two principal mechanisms: 1) release of the drug by diffusion through pores formed in the polymer matrix after hydration and 2) release of the drug as the polymer hydrolyzes and the microsphere degrades (Cleland, in press). The initial spike in rhGH serum levels (0–48 hr after administration), observed with all three formulations, is principally due to protein release by diffusion whereas the remaining release is due to both diffusion and polymer degradation. In general, the higher the molecular weight of the polymer the longer it takes to degrade and release the protein. The delay in the release of rhGH observed after treatment with formulation III can be explained by the higher molecular weight of the polymer used in this formulation.

Our results in the rhesus monkeys showed that rhGH has similar immunogenicity when delivered from microsphere formulation II or when delivered by multiple injections as a protein solution. This was confirmed in the rhGH transgenic mice, which did not develop antibodies after administration of either microspheres or protein solution. In addition, the immunogenicity was not increased when three monthly injections were given. This is significant because the probability of an immune response is enhanced when an antigen is formulated as a depot formulation and given multiple times over a several week period, e.g., as in vaccination (Cleland,
IGF-I is achieved and that undesirably high levels (e.g., those with the potential to cause acromegaly) are not maintained. Although sustained-release formulations will not be appropriate for all therapeutic proteins, our results with rhGH gives encouragement that formulations of proteins can be developed that induce a sustained biological effect.

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