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Recombinant Rat and Mouse Growth Hormones: Risk Assessment of Carcinogenic Potential in 2-Year Bioassays in Rats and Mice

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Recombinant rat growth hormone (rrGH) and recombinant mouse growth hormone (rmGH) were developed to evaluate the potential carcinogenicity of each biologically active growth hormone (GH) as assessed in the respective species. Biological activities of rrGH and rmGH were demonstrated by showing an increase in body weight gain and serum levels of insulin-like growth factor-1 (IGF-1) in hypophysectomized rats receiving daily sc injections for 6 days. With the exception of pharmacologically mediated weight gain, rrGH and rmGH had no adverse effects in 5-week oral toxicity studies and no production of anti-recombinant GH antibodies. The high doses selected for the carcinogenicity studies provided systemic exposures of GH up to approximately 10-fold over basal levels. In the 105-week mouse carcinogenicity study, daily sc injections of rmGH at 0.1, 0.2, or 0.5 mg/kg/day were well tolerated and had no effects on survival or incidence of tumors. In the 106-week rat carcinogenicity study, daily sc injections of rrGH at 0.2, 0.4, or 0.8 mg/kg/day had a favorable effect on longevity in female rats administered 0.4 or 0.8 mg/kg/day, an increased weight gain in females and males, and no increase in the incidence of tumors. The absence of carcinogenic effects of recombinant GH administered daily for 2 years to rodents was consistent with publications of clinical experience, indicating a lack of convincing evidence for an increased risk of cancer in children receiving human recombinant GH replacement therapy.

Key Words: **risk assessment; endocrine; pharmaceuticals.**

Growth hormone (GH) is a pituitary polypeptide hormone synthesized and secreted by somatotroph cells in the anterior pituitary gland. Subcutaneous injections of GH (three to seven times per week) have been used over the past 40 years to treat children with growth hormone deficiency (GHD), with the median duration of GH replacement in childhood being 3.5–4 years (Toogood *et al.*, 1996). Recombinant DNA–derived GH became available in 1985, shortly after human pituitary GH was discontinued due to the occurrence of Creutzfeldt-Jakob disease in several recipients. Recombinant human GH (rhGH) has been approved by regulatory agencies for treatment of GHD in adults with a history of hypothalamic pituitary disease, short stature associated with chronic renal insufficiency, short stature in patients with Turner's syndrome or Prader-Willi syndrome, infants born small of gestational age who do not reach sufficient height, and for use in human immunodeficiency virus–associated wasting in adults (Gharib *et al.*, 2003).

Potential concerns regarding the incidence of cancer in humans receiving long-term treatment with GH come from several sources. Patients with acromegaly have been reported to have an increase in premalignant adenomatous polyps in the colon from an expected 12–35% and prevalence of adenocarcinoma of the colon calculated as 6.9 per 100 cases of acromegaly (Toogood *et al.*, 1996). However, an association of acromegaly with increased colon cancer has not been consistently observed. As epidemiologists will agree, patients who have acromegaly are exposed to levels of GH far in excess to the levels attained during GH replacement therapy. Further, these high exposures to endogenous GH in acromegaly may extend as long as 20 years.

Longitudinal epidemiological studies have followed children or adults on human pituitary GH replacement therapy (prior to the introduction of rhGH). Initial reports from Japan suggested an increased incidence of leukemia in GH-treated patients (Watanabe *et al.*, 1993); however, subsequent studies did not confirm such an increase (Gharib *et al.*, 2003; Ogilvy-Stuart and Gleeson, 2004). A cohort study in the United Kingdom published preliminary findings that patients treated with human pituitary GH had significantly greater risks of mortality from colorectal cancer, Hodgkin's disease, and cancer overall (Swerdlow *et al.*, 2002). In agreement with several other cohort studies, the authors of this U.K. cohort concluded that the evidence suggested that leukemia risk was not substantially raised if high-risk groups such as those with

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chromosomal fragility were excluded. The Lawson Wilkens Pediatric Endocrine Society concluded that an increased risk of cancer has not been an issue in pediatric patients treated with GH, although they recommended the regular monitoring of insulin-like growth factor-1 (IGF-1) and IGF-1–binding protein-3 concentrations to ensure age-appropriate limits during therapy (Sperling *et al.*, 2002).

To determine if recombinant GH increased the risk of development of tumors, carcinogenicity studies in rodents were designed to rigorously test this hypothesis in well-controlled studies. Mouse recombinant growth hormone (rmGH) and rat recombinant growth hormone (rrGH) were produced in mouse myeloma cells in large quantities with stringent criteria to ensure high purity and stability. Biological activity of rrGH and rmGH was demonstrated in hypophysectomized rats. The high doses selected for the carcinogenicity studies provided systemic exposures of GH up to approximately 10-fold over basal levels. The high dose in the rat carcinogenicity study (0.8 mg/kg/day) was chosen based on doses that induced weight gain in a bioassay with hypophysectomized Wistar rats and in Sprague-Dawley (SD) rats in a 5-week study. The high dose in the mouse carcinogenicity study (0.5 mg/kg/day) provided maximum GH-induced weight gain in the bioassay with hypophysectomized Wistar rats, although it was lower than the dose needed to induce weight gain in mice in a 5-week study. There was a limited quantity of rmGH available for the 2-year bioassays. The rat carcinogenicity study was conducted for 106 weeks with daily sc injections at 0.2, 0.4, and 0.8 mg/kg/day of rrGH and the mouse carcinogenicity study was conducted for 105 weeks with daily sc injections at 0.1, 0.2, and 0.5 mg/kg/day of rmGH. For comparison with clinical therapeutic doses, children with GHD who have received 0.043 mg/kg/day of rhGH all achieved at least a normal growth velocity during the first year of treatment (with many having a high growth velocity) (Mandel *et al.*, 1995). The starting dose for GH therapy may be only 0.005–0.015 mg/kg/day for a 20-kg child and then the dose may be titrated upward slowly (Gharib *et al.*, 2003).

MATERIALS AND METHODS

Production of recombinant GH. The large-scale production of rmGH and rrGH at Merck & Co., Inc., was previously described (Zhou *et al.*, 1996). Briefly, either mouse or rat GH was expressed in NSO murine myeloma cells transfected with a vector containing the glutamine synthetase (Celltech, Ltd., Slough, UK) gene and two copies of mouse or rat GH cDNA. Mouse and rat GH genes were cloned from mouse and rat pituitary tissues. RNA was isolated using the guanidine isothiocyanate procedure. The GH genes were amplified using 25 cycles of reverse transcriptase–polymerase chain reaction on both mouse and rat RNA samples using published GH sequences to generate primers. This resulted in the expected size fragments of 660 base pairs. The fragments were ligated into a Bluescript vector, and DNA was prepared in ampicillin-resistant *Escherichia coli*. Colonies were screened for the presence of the correct size fragment (660 base pair). Positive colonies were selected at random, and the inserts were entirely sequenced in both directions. Both the DNA sequences agreed with literature reports and contained an open reading frame of 648 nucleotides.

For optimal expression, the mouse GH vector also contained sequences for targeting integration by homologous recombination. Fed-batch culture processes for such clones were developed using a serum-free, glutamine-free medium and scaled up to 250-l production reactors. The final rmGH and rrGH titers were approximately 580 and 240 mg/l, respectively. Both rmGH and rrGH consisted of a peptide chain of 190 amino acids (molecular weight ~22,000) with two disulfide bonds and were nonglycosylated. They differed in sequence by only three uncharged amino acids. However, their physical behaviors in solution, with regard to aggregation, differed significantly, and the development of the formulations has been presented (Sanyal *et al.*, 1997). The effects of varying pH, temperature, and formulation conditions on protein structure and intermolecular association were examined. Stabilizing buffer and pH conditions were identified that minimized aggregation and maintained the tertiary structure and *in vivo* bioactivity. Frozen (− 70°C) formulations in these solutions had excellent long-term stability, as judged by analytical and biological assays.

Recombinant GH was stored at approximately − 70°C and brought to approximately 20°C in a circulating water bath before preparing dilutions with the vehicle. Purity was assessed as 98% pure at initiation by SDS gel electrophoresis. The vehicle for rrGH was a sterile 0.01M potassium phosphate solution at pH 7.9 and the vehicle for rmGH was a sterile 10mM sodium/potassium phosphate solution at ph 7.8. All dilutions were kept on wet ice until completion of daily dosing.

The lots of rrGH and rmGH were the same for all studies described in this article.

Test species and animal husbandry. Studies were conducted at Merck Research Laboratories (West Point, PA) and were in compliance with the U.S. Food and Drug Administration Good Laboratory Practice regulations. Animal use was in accordance with the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Merck Research Laboratories reviewed and approved all procedures used in this study. Animal care and management in this investigation were in compliance with guidelines by the National Institutes of Health and animal welfare regulations of the U.S. Department of Agriculture.

Sprague-Dawley (Crl:CD[SD] BR) rats (SD rats) were purchased from Charles River Laboratories, Raleigh, NC. Rats were housed individually in stainless steel suspended cages in a balanced randomized column design in environmentally controlled rooms with generally a 12-h light cycle according to a balanced random allocation scheme. SD rats were fed an optimized amount of PMI Certified Rodent Diet daily (limit fed). Males received approximately 24 g and females were fed 17 g.

Male Wistar rats were obtained hypophysectomized from Charles River Laboratories, Portage, MI. Hypophysectomized rats were housed in individual stainless steel cages in a climate-controlled room, and PMI Certified Rodent Diet and deionized water with 5% dextrose were available *ad libitum*.

Mice (Crl:CD-1[ICR] BR) were purchased from Charles River Laboratories. The mice were housed in plastic cages with wire tops and hardwood bedding (two to three animals per cage) in an environmentally controlled room with a 12-h light cycle according to a balanced random allocation scheme. PMI Certified Rodent Diet and drinking water were available *ad libitum*.

Mice and rats were identified with tattoos instead of microchips to prevent complications with daily sc dosing.

Rat bioassay for pharmacologic activity. A bioassay was used to test the bioactivity of rmGH and rrGH and verify the long-term stability of the formulations under specific storage conditions. The bioassay consisted of treating hypophysectomized rats with daily sc injections of rmGH (0.0625, 0.125, 0.25, and 1.0 mg/kg/day) or rrGH (0.0625, 0.125, 0.25, 0.5, and 1.0 mg/kg/day) for 6 days. Hypophysectomized male Wistar rats were approximately 6 weeks old and weighed approximately 70–76 g at study start. Body weight gain was measured on day 6. Serum levels of GH and IGF-1 were evaluated on day 6 at 3 h after injection. Serum levels of GH were determined by radioimmunoassay (RIA) with a rat GH kit obtained from Dr A. F. Parlow, Pituitary

Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA. The procedure for RIA has been previously described (Leung *et al.*, 1986). Serum levels of IGF-1 were measured by RIA using a previously described method (Cahill *et al.*, 1995).

Rat 5-week study to measure serum GH and IGF-1 levels, determine rrGH tolerability, measure reactive antibody response, and evaluate volume density of pituitary cell populations. The rrGH was administered sc in the interscapular area to rats daily at doses of 0.1, 0.5, and 1.5 mg/kg/day for 5 weeks (35 days). The dosing volume was 1 ml/kg. The vehicle controls received a potassium phosphate solution. Twenty male and 20 female SD rats were used in each dosage group and in the control group. At the start of the study, the rats were 38 days old and weighed 114–214 g. Body weights and food consumption were monitored.

Serum levels of GH were determined by RIA on samples collected on day 6 at 0.5, 1, 2, 3, 4, 6, 8, and 24 h after dosing. Serum samples were also collected from rats in all dose groups at the end of the study on day 36 (24 h after the last injection) for measurement of serum levels of IGF-1 and for the presence of antibodies to rat GH.

An ELISA was used to detect immunoglobulins directed against rrGH. To establish the ELISA for anti-rat GH IgG assays, microtiter plates were coated with rrGH at 0, 1, and 10 μg/ml, 100 μl per well, overnight at 4°C. Detecting reagents were horseradish peroxidase–conjugated anti-rat IgG goat serum and horseradish peroxidase–conjugated anti-rabbit IgG rat serum. The 160 experimental serum samples were initially assayed (rep = 2) at 1:1000 against 1 μg/ml rrGH to screen for samples with specific immunoreactivity.

Ophthalmologic examinations were performed on rats in the control and the 1.5-mg/kg/day groups during week 4. Urinalysis, hematology, and serum biochemical determinations were conducted in week 4 for rats in all dose groups. Hematologic examination included erythrocyte parameters, platelet counts, leukocyte count, and differential leukocyte count. Serum chemistry determinations included glucose, urea nitrogen, creatinine, total protein, albumin, albumin/globulin ratio, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activity, total cholesterol, triglycerides, sodium, potassium, chloride, total calcium, and phosphorus. Urinalyses parameters included volume, pH, specific gravity, urobilinogen, ketones, protein, glucose, bilirubin, occult blood, and light microscopic examination of urine sediments.

At the termination of the study, all surviving rats were killed by exsanguination under ether anesthesia and examined grossly. Weights of heart, spleen, brain, pituitary, kidneys, testes, prostate, thyroid, liver, adrenals, and ovaries were collected for all animals at scheduled necropsy. A complete light microscopic examination of hematoxylin and eosin–stained sections of approximately 52 tissues per rat was performed for 10 rats per sex in the control and 1.5-mg/kg/day groups. In addition, sections of the pituitary gland from 10 rats per sex in the control and 1.5-mg rrGH/kg/day groups were processed for immunohistochemistry to identify specific pituitary cell types. Immunostaining was done with a rabbit polyclonal antibody directed against rat GH (Biogenesis, Poole, United Kingdom) to identify somatotrophs, rabbit polyclonal antibody directed against synthetic adrenocorticotropic hormone (ACTH, Biogenesis) to identify adrenocorticotrophs, and rabbit polyclonal antibody directed against rat prolactin (PRL, Biogenesis) to identify lactotrophs. The avidin-biotin-peroxidase technique was used to develop the immunohistochemical reaction. Quantitative analysis of the somatotroph, adrenocorticotroph, and lactotroph cell populations in immunostained sections was done by a point-counting method, and the volume density of each cell population was determined, in both sexes. The volume density of the somatotroph and lactotroph cell populations was expressed as a percentage of the pars distalis volume. The volume of the adrenocorticotroph cell population was expressed as a percentage of the pars distalis volume, pars intermedia volume, and the total gland volume. A one-way analysis of variance was used for statistical analysis of volume density data.

Mouse 5-week study to determine rmGH tolerability, measure reactive antibody response, and evaluate micronuclei. The design of the 5-week tolerability and antibody response study in mice was similar to the study described above for rats. Twenty male and 20 female CD-1 mice were used in each dosage group and in the control group. The mice were approximately 6 weeks old at study start and weighed between 16.7 and 36.1 g. The doses were 0.1, 0.5, and 1.5 mg/kg/day, and the dosing volume for mice was 7 ml/kg. This 5-week study did not include the evaluation serum IGF-1 or GH levels due to the limitation of blood samples. Hematology and serum biochemical determinations were done for all dose groups for blood collected at study termination. At the termination of the study, all surviving mice were euthanized by exsanguination under anesthesia and examined for any gross changes. Weights of brain, pituitary, heart, kidneys, liver, spleen, testes, and prostate were collected, and a complete light microscopic examination of hematoxylin and eosin–stained sections of approximately 53 tissues per mouse was performed for 10 mice per sex in the control and 1.5-mg/kg/day groups.

An ELISA was used to detect immunoglobulins directed against rmGH. To establish the ELISA for anti-mouse GH IgG assays, microtiter plates were coated with rmGH at 0 and 1 μg/ml, 100 μl per well, overnight at 4°C. Detecting reagents were horseradish peroxidase–conjugated anti-mouse IgG goat serum and horseradish peroxidase–conjugated anti-rabbit IgG rat serum. The 77 experimental serum samples and 16 nonimmune control sera were initially assayed at 1:100 against 1 μg/ml rmGH to screen for samples with specific immunoreactivity (absorbance units over threefold that of the mean reagent control). The samples were then assayed at 1:1000.

In addition, blood and bone marrow samples were collected at necropsy for evaluation of micronuclei. Bone marrow and blood from five mice per sex per dose group were stained with acridine orange and then examined using a fluorescent microscope. Bone marrow was evaluated for the presence of micronuclei in 2000 polychromatic erythrocytes (PCEs) and the number of PCEs per 1000 erythrocytes. Blood was evaluated for the presence of micronuclei in 2000 normochromatic erythrocytes (NCEs) and the number of PCEs per 1000 erythrocytes. The assay included a separate set of animals dosed with 0.35 mg/kg mitomycin C as a positive control for analysis of acridine orange–stained micronuclei in bone marrow.

Mouse 7-day study to measure serum levels of GH. The serum levels of GH were evaluated in a separate study where 48 male and 48 female mice, at 72–75 days of age, received daily sc injections of rmGH at 0.5 mg/kg/day for 7 days. Control mice (72 males and 72 females) received deionized water by gavage for 7 days. Serum GH levels were evaluated on study day 7 at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min after dosing. Blood samples were collected from six mice per sex per time point in the control group and four mice per sex per time point in the rmGH group. Serum GH levels were determined by RIA with a rat GH kit obtained from Dr A. F. Parlow.

Two-year carcinogenicity study in rats. A total of 500 SD rats were used in the study. Fifty male and 50 female rats at 37 days of age were included in each of the five dose groups. Female rats weighed 102–149 g at study start, and males weighed 129–195 g. Rats received daily sc injections of vehicle, 0.2, 0.4, or 0.8 mg/kg/day of rrGH in vehicle. There were two sets of control animals for a total of 100 males and 100 females receiving vehicle alone (aqueous buffer with 0.12M sodium chloride in 0.01 potassium phosphate, pH 7.8). The dosing volume was 1 ml/kg. All rats were examined daily for mortality and weekly for clinical signs and were palpated for masses every 4 weeks beginning in week 26. Body weights were recorded before the test, once in week 1, twice a week through week 13, and once a week thereafter. Ophthalmic examinations were conducted for all rats before the test and in study week 52 and for surviving rats of a vehicle-treated group and the 0.8-mg/kg/day group in study week 102.

Complete necropsies, including examination and collection of tissue samples from an extensive list, were done on all rats. Following fixation in buffered formalin or Bouins (testes only), 57 tissues from each rat were processed, sectioned, stained with hematoxylin and eosin, and examined microscopically. Any grossly noted masses and other tissues with grossly noted changes were also sampled and examined microscopically.

Two-year carcinogenicity study in mice. A total of 500 CD-1 mice were used in the study. Fifty male and 50 female mice at 39 days of age were included in each of the five dose groups. Female mice weighed 18.5–27.6 g at

study start, and males weighed 20.2–32.8 g. Mice received daily sc injections of vehicle, 0.1, 0.2, or 0.5 mg/kg/day of rmGH. There were two sets of control animals for a total of 100 males and 100 females receiving vehicle alone (10mM sodium phosphate solution). The dosing volume was 7 ml/kg.

The mice were observed daily for mortality and weekly for physical signs. All surviving mice were palpated for the presence of masses once every 4 weeks beginning in study week 26. Body weights were recorded before the test, once in study week 1, twice weekly in study weeks 2–13, and once weekly thereafter. Ophthalmic examinations were performed on all mice before the test, on all surviving mice in study week 51, and on the surviving mice in one set of controls and surviving mice of the 0.5-mg/kg/day group in study week 101.

Mice sacrificed prior to or at the termination of the study were anesthetized and then euthanized by exsanguination prior to necropsy. Complete necropsies, including examination and collection of tissues from an extensive list, were done on all mice. Pituitary gland weights were collected for all mice at terminal necropsy with the exclusion of pituitary glands with tumors identified grossly. Terminal body weights were taken for calculation of pituitary weight changes. Following fixation and processing, sections of tissue samples from all mice were stained with hematoxylin and eosin, and a total of 58 tissues per mouse were examined microscopically. Any grossly noted masses and other tissues with grossly noted changes were also sampled and examined microscopically.

Statistical methods. Males and females were analyzed separately.

Body weight data were analyzed by trend analysis (Tukey *et al.*, 1985) in four groups (drug weeks 14–52, 53–78, 79–104, and 14–104). Trend is defined as a progressive, either increasing or decreasing, response with increasing dose of the test compound. Animals surviving at the end of each time interval were used in the analysis.

For each organ and tumor site for each sex, the response variable of interest was the incidence of animals with tumor. The statistical analysis took into account the context of observation of the tumor as well as survival differences among the groups. Tumor incidence patterns were compared over the length of the entire study. Palpable and nonpalpable tumor results were summarized at and summed over each week that a tumor was detected. Nonpalpable nonlethal tumors were cumulated for animals that died during prespecified time intervals. The Peto *et al.* (1980) method was used to analyze the palpable and nonpalpable tumor data. For the nonpalpable nonlethal tumors, the Peto *et al.* method of analysis requires the specification of age strata. These were specified prior to the study to be the first year followed by 90-day intervals (i.e., days 1–364, 365–455, 456–546, 547–637, and 638–728). There was a separate stratum for rats and mice that survived to day 729, the start of terminal sacrifice.

Trend was assessed using an arithmetic dose scale for an increase or decrease in tumor incidence with increasing dose of the test article. Both unadjusted and adjusted (for multiplicity of tests) p values were calculated. When applying a trend test simultaneously to many tumor sites, it was important to keep the observed statistically significant results in proper perspective. Therefore, the multiplicity adjustments for the test of Peto *et al.* (1980) were conducted, which generally followed the recommendations made by Mantel (1980), Heyse and Rom (1988), and Harter (1957).

The analysis of mortality data was done by a statistical comparison of survival curves. It was of interest to determine if the survival pattern for each dose group had arisen from identical survivor functions. The estimate of the survivor function was conducted by the product-limit method. This method used the censored observations as well as the uncensored observations.

The significance level for all statistical tests was set at 0.05.

RESULTS

Rat Bioassay for Pharmacologic Activity

Removal of the pituitary gland in the Wistar rats resulted in low serum levels of IGF-1, which ranged from 37–62 ng/ml in groups of vehicle-treated hypophysectomized rats in our laboratory. This low baseline level of IGF-1 allowed the detection of increased IGF-1 serum levels as a biological response to injected GH. For reference, the mean serum level of IGF-1 in male SD rats with intact pituitaries at 11 weeks of age was 1217 ng/ml as described in the 5-week tolerability study.

The biological activity of the rrGH was confirmed in hypophysectomized male rats after six daily sc injections of rrGH. On day 6, the mean serum levels of GH at 3 h after injection in rats dosed with rrGH at 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/kg/day were 22, 37, 102, 232, and 401 ng/ml, respectively (supplementary Fig. 1). The serum level of GH in vehicle-treated rats was below the limit of detection (1.9). A dose-dependent increase in body weight gain as well as increased serum levels of IGF-1 were observed. The rrGH-treated rats had increased mean body weight gains as compared to pretest values, with increases of 9, 9, 11, 15, and 18% for the dose groups 0.0625, 0.125, 0.25, 0.5, and 1.0 gm/kg/day, respectively. The vehicle-treated hypophysectomized rats had no significant increase in body weight gain. Rats treated with rrGH showed a dose-dependent increase in IGF-1 on study day 6 (supplementary Fig. 1). The serum levels of IGF-1 at 3 h after injection in rats dosed with 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/kg/day of rrGH were 153, 186, 211, 271, and 323 ng/ml, respectively, as compared to 60 ng/ml in vehicle-treated rats.

Serum levels of GH and IGF-1 and body weights are also evaluated for hypophysectomized rats after six daily sc injections of rmGH. On day 6, the mean serum levels of GH at 3 h after injection in rats dosed with rmGH at 0.0625, 0.125, 0.25, and 1.0 mg/kg/day were 13.7, 26.2, 81.6, and 457 ng/ml, respectively (supplementary Fig. 2). The rmGH-treated rats had increased mean body weight gains as compared to pretest values, with increases of 10, 15, 18, and 18% for the dose groups 0.0625, 0.125, 0.25, and 1.0 gm/kg/day, respectively. The vehicle-treated hypophysectomized rats had no significant increase in body weight gain. Rats treated with rmGH showed a dose-dependent increase in IGF-1 on study day 6 (supplementary Fig. 2). The serum levels of IGF-1 at 3 h after injection in rats dosed with 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/kg/day of rmGH were 103, 126, 153, and 205 ng/ml, as compared to 46 ng/ml in vehicle-treated rats.

These studies showed that the rrGH and rmGH formulations were biologically active.

Rat 5-Week Study to Measure Serum GH and IGF-1 Levels, Determine rrGH Tolerability, Measure Reactive Antibody Response, and Evaluate Volume Density of Pituitary Cell Populations

In this 5-week study, the rrGH formulation was well tolerated by sc administration in rats, with no evidence of systemic toxicity or local irritation. Increased levels of circulating GH were observed at all doses, confirming that the animals were exposed to high systemic levels of rat GH. Absence of circulating antibody to GH showed that the injected hormone was not recognized as a foreign protein.

TABLE 1
Mean Systemic Exposure of GH in Rats after 6 Days of sc Administration of rrGH and Mice after 7 Days of sc Administration of rmGH

	AUC[a] (ng h/ml)		Mean C_{max} (ng/ml)	
Species and dose	Male	Female	Male	Female
Systemic exposures 0.5–8 h				
Rat vehicle	107 ± 33	93 ± 16	66	23
Rat 0.1 mg/kg/day rrGH	198 ± 27	187 ± 21	107	79
Rat 0.5 mg/kg/day rrGH	625 ± 27	591 ± 21	332	307
Rat 1.5 mg/kg/day rrGH	1905 ± 56	1737 ± 69	692	701
Systemic exposures 0–3 h				
Mouse vehicle	47 ± 10	21 ± 3	28	42
Mouse 0.5 mg/kg/day rmGH	432 ± 19	321 ± 20	326	194
Rat 0.5 mg/kg/day rrGH[b]	607 ± 35	568 ± 24	332	307

[a]Mean ± SE.
[b]AUC 0–3 h for rat systemic exposure of rrGH was calculated to provide a comparison with mouse systemic exposure of rmGH.

Serum levels of GH were evaluated on day 6. The mean systemic exposures, expressed as area under the curve (AUC) for 0.5–8 h, and the maximum serum concentrations of GH expressed by C_{max} (maximum concentration) values in nanograms per milliliter are shown in Table 1. As shown in Figure 1, C_{max} was achieved at approximately 0.5–1 h after injection of rrGH. The serum levels of GH in the 0.1-, 0.5-, and 1.5-mg/kg/day groups were increased dose proportionately in both sexes (Figs. 1 and 2). The systemic exposure is expressed as AUC for 0.5–8 h and C_{max} in Table 1. Evaluation of serum levels of IGF-1 at approximately 24 h after the last injection showed no significant difference in the mean IGF-1 serum levels in rrGH-treated rats as compared to controls. The mean and SE of IGF-1 levels in females dosed with vehicle, 0.1, 0.5, and 1.5 mg/kg/day were 790 ± 32, 804 ± 31, 796 ± 26, and 858 ± 32 ng/ml, respectively. The mean and SE of IGF-1 levels in males dosed with vehicle, 0.1, 0.5, and 1.5 mg/kg/day were 1217 ± 43, 1197 ± 38, 1179 ± 30, and 1147 ± 41 ng/ml, respectively.

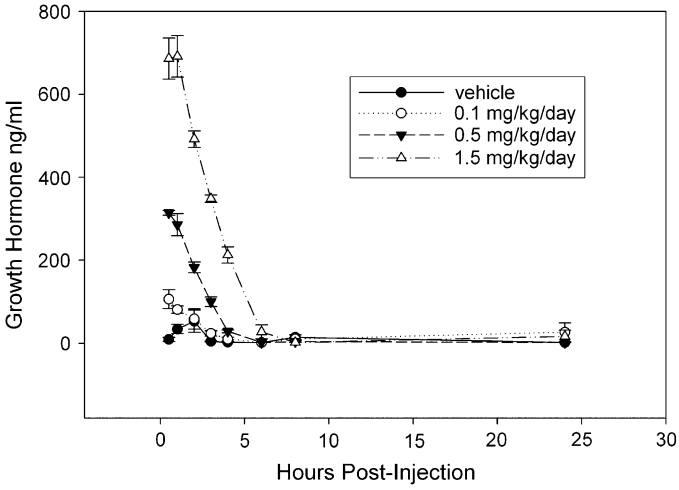

FIG. 1. Systemic exposure of GH in male SD rats dosed with sc injections of rrGH for 6 days. The C_{max} was achieved at approximately 0.5–1 h after injection of rrGH, and the serum levels of GH in the 0.1-, 0.5-, and 1.5-mg/kg/day groups were increased dose proportionately.

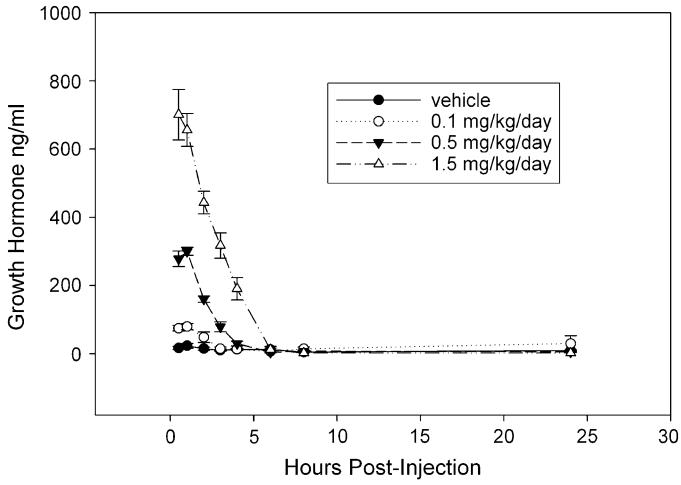

FIG. 2. Systemic exposure of GH in female SD rats dosed with sc injections of rrGH for 6 days. The C_{max} was achieved at approximately 0.5–1 h after injection of rrGH, and the serum levels of GH in the 0.1-, 0.5-, and 1.5-mg/kg/day groups were increased dose proportionately.

The sc administration of rrGH to rats for 5 weeks had no effect on survival and no effects on physical signs, food consumption, ophthalmologic examinations, urinalysis, or hematologic parameters. Body weight gains in female rats treated with 0.5 or 1.5 mg/kg/day were greater (65 and 70 g, respectively) as compared to vehicle-treated female rats that gained 53 g during the 5-week study. Female rats receiving 0.5 or 1.5 mg/kg/day were associated with a very slight increase in serum alkaline phosphatase activity (213 and 221 U/l, respectively), as compared to controls (172 U/l). There were no alterations in organ weights at study termination including no change in pituitary gland weight and no gross or light microscopic changes that were attributed to rrGH. Changes at the sc injection site were similar between control and treated animals and were considered of minimal severity and most likely due to the physical trauma of repeated daily injection.

Serum samples collected from rats in all dose groups at the end of the 5-week study were evaluated for the presence of immunoglobulins directed against rrGH in an ELISA. When diluted at 1:1000, 15 of the 160 samples showed specific reactivity. The incidence of the immunoreactivity was equivalent in the various groups, including controls, and there was no obvious correlation of the intensity of the ELISA signal with the dose levels of rrGH. The conclusions from these data were that the incidence of serum immunoreactivity against rat GH was 10% in the rat population due to presence of endogenous antibody to GH and there was no effect by the rrGH injections. Absence of circulating antibody to GH showed that the injected hormone was not recognized as foreign.

Quantitative analysis of somatotrophs (containing GH), lactotrophs (containing PRL), and adrenocorticotrophs (containing ACTH), identified by immunostaining, showed no changes in the volume density of each population following the daily sc injection of 1.5 mg rrGH/kg/day for 5 weeks (supplementary Table 1). The volume density of ACTH cell populations, when expressed as a percentage of the total gland, was similar in the control and rrGH-treated groups, therefore the slight increase in the ACTH cell populations observed in the pars distalis was considered incidental to rrGH administration.

Mouse 5-Week Study to Determine rmGH Tolerability, Measure Reactive Antibody Response, and Evaluate Micronuclei

The rmGH formulation was well tolerated with no evidence of systemic toxicity or local irritation, and the absence of circulating antibody to GH showed that the injected hormone did not elicit an immunologic response.

The sc administration of rmGH for 5 weeks had no effect on survival and no effects on physical signs, food consumption, ophthalmologic findings, or hematologic or serum chemistry parameters. There was a slight increased in body weight gain in male mice only at the high dose. Over the 5-week study, the mean body weight gain was 9.1 g in male mice treated with 1.5 mg/kg/day and 8.0 g in vehicle-treated male mice. This increased weight gain was an expected pharmacologic response. There was no effect of rmGH on body weight gain in the females. Injection of rmGH did not cause irritation at the injection sites. There were no effects on pituitary weight or other organ weights at study termination and no gross or light microscopic changes in treated mice that were attributed to rmGH.

Serum samples collected at the end of the 5-week study were assayed for the presence of immunoglobulins directed against rmGH. When diluted at 1:100, nine of the 77 samples showed specific reactivity. Since five samples were from control mice and four from rmGH-treated mice, there was no effect by the rrGH injections and the serum reactivity was considered to be due to the presence of endogenous antibody to GH. When diluted at 1:1000, all samples were negative. It was concluded from these data that there was no effect of the rmGH injections on eliciting specific immunologic responses.

There were no significant increases in micronucleated cells in bone marrow PCEs or blood NCEs in male and female mice treated with rmGH when compared with concurrent vehicle control (data not shown). The frequencies of micronuclei in PCEs for each treatment group were within the control range for CD-1 mice. These negative results for rmGH in the micronucleus test showed lack of clastogenic action and effects on cell division, which lead to chromosome loss. In addition, the frequencies of PCEs in bone marrow and blood were not affected by treatment with rmGH, indicating no effect of rmGH on erythrocyte production and maturation.

Mouse 7-Day Study to Measure Serum Levels of GH

The serum levels of GH were evaluated in CD-1 mice given a sc injection of rmGH at 0.5 mg/kg/day in a dedicated study. Serum GH levels were determined on study day 7 at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min after dosing. The mean serum levels are shown in Figure 3, and the AUC 0–3 h and C_{max} are in Table 1. In computing the means and AUC values, individual raw data values below the limit of detection were set at 0.245 ng/ml. The C_{max} was achieved at approximately 15 min after the injection of rmGH in males and at approximately 60 min in females. The systemic exposure (AUC 0–3 h) for mice injected with 0.5 mg/kg/day was 8.4-fold over basal levels in males (vehicle treated) and 15.2-fold over basal levels in females.

Two-Year Carcinogenicity Study in Rats

Daily sc administration of rrGH during the natural life span of SD rats elicited a treatment-related decrease in mortality in the female rats and no effect on mortality in the male rats (supplementary Figs. 3 and 4). Eighty-two percentage of female rats treated with 0.4 mg/kg/day ($p = 0.01$) and 80% of rats treated with 0.8 mg/kg/day ($p = 0.024$) survived to study termination, as compared to 62 and 64% in the control groups. The increased survival was due, at least in part, to the decrease in deaths due to pituitary tumors in females in the 0.4- and 0.8-mg/kg dose groups. The incidences of pituitary tumor as the cause of death in female rats were 12 and 8 in the control groups, 10 in the 0.2-mg/kg/day group, 4 in the 0.4-mg/kg/day group, and 2 in the 0.8-mg/kg/day group.

No treatment-related physical signs or ocular signs were observed in either gender. Female rats treated with rrGH had a greater average body weight at all dose levels ($p < 0.001$) (supplementary Fig. 5). At study week 52, the mean body

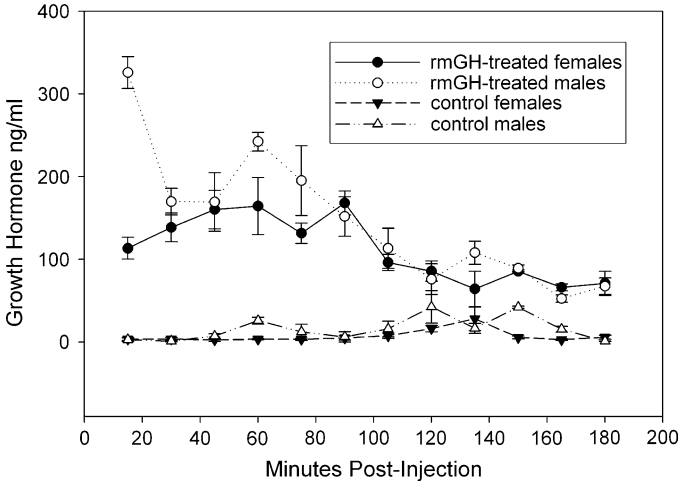

FIG. 3. Systemic exposure of GH in CD-1 mice dosed with sc injections of rmGH at 0.5 mg/kg/day for 7 days. The C_{max} was achieved at approximately 15 min after injection of rmGH in males and at approximately 60 min in females.

weight for females in control, 0.2-, 0.4-, and 0.8-mg/kg/day groups were 305, 322, 339, and 352 g, respectively. At the end of the study, the mean body weight for females in control, 0.2-, 0.4-, and 0.8-mg/kg/day groups were 324, 343, 361, and 381 g, respectively. In males, there was a greater body weight at 0.8 mg/kg/day ($p < 0.001$) and 0.4 mg/kg/day ($p < 0.05$) (supplementary Fig. 6). At study week 52, the mean body weight for males in control, 0.2-, 0.4-, and 0.8-mg/kg/day groups were 567, 569, 584, and 592 g, respectively. At the end of the study, the mean body weight for males in control, 0.2-, 0.4-, and 0.8-mg/kg/day groups were 627, 630, 647, and 650 g, respectively. The increased body weight was attributed to known pharmacologic effects of GH in rats.

By the completion of the 106-week study in rats, there were no treatment-related increases in incidence or spectrum of neoplastic changes (Table 2). No statistically significant increasing trends in tumor incidence were detected through the top dose tested in female and male rats. There was a decreasing trend in pituitary adenoma in female rats through the top-dose group of 0.8 mg/kg/day after adjustment for multiplicity of statistical tests ($p < 0.001$ *N*).

Although trend analysis determined that there were no significant increases in tumor incidence, the incidence for a few tumors in Table 2 could be misinterpreted as being treatment related when viewed without knowledge of the spontaneous or background incidence in aging SD rats. The ranges of the incidence of selected tumors in 44 groups of control rats that were fed an optimized diet in carcinogenicity studies in our laboratory are shown in Table 3. In addition to the incidence of schwannoma in the heart and osteosarcoma in the bone, the incidence of schwannoma and osteosarcoma as a primary tumor in any location was included for this rrGH study in Table 2 and the historical control ranges in Table 3. In addition, the incidence of tumors of similar lineage were also shown as combined for the liver and the mammary gland in Tables 2 and 3. With the exception of a treatment-related decrease in pituitary tumors in female rats, there were no effects of rrGH on tumor incidence by statistical analysis and when compared to ranges of the incidence in groups of control rats from historical data.

Trauma by the injection, introduction of foreign material into the subcutis during daily injections, and/or irritation by the vehicle and rrGH induced focal hemorrhage, very slight to slight chronic inflammation, and very slight to marked fibrosis at the injection site in the intrascapular region of the rats. These changes were observed in both female and male rats at all doses. There was a higher incidence in rrGH-treated rats as compared to vehicle-treated rats. In females, the incidence of inflammation and/or fibrosis was 5 and 6 in control groups and 11, 19, and 33 in the 0.2-, 0.4-, and 0.8-mg/kg/day dose groups, respectively. In males, the incidence of inflammation and/or fibrosis was 8 and 8 in control groups and 21, 34, 35 in the 0.2-, 0.4-, and 0.8-mg/kg/day dose groups, respectively. The fibrosis and inflammation were not associated with an increased incidence of neoplastic changes at the site of injection.

Two-Year Carcinogenicity Study in Mice

Daily sc administration of rmGH over the natural life span of CD-1 mice elicited no treatment-related effects on mortality. The overall survival at scheduled termination was 42.6%. A statistically significant increase in survival was noted in female mice only of the 0.2-mg/kg/day group (62% survival, $p = 0.012$, negative trend).

No treatment-related physical signs or ocular signs were observed in either gender. Although increased body weight was shown in the bioassay for activity of rmGH, there was no significant increase in body weight in males or females by trend analysis in the mouse carcinogenicity study (supplementary Figs. 7 and 8). At study week 52, the mean body weight for females in control, 0.1-, 0.2-, and 0.5-mg/kg/day groups were 36.8, 37.5, 37.1, and 38.2 g, respectively. At the end of the study, the mean body weight for females in control, 0.1-, 0.2-, and 0.5-mg/kg/day groups were 40.6, 40.8, 40.8, and 41.7 g, respectively. At study week 52, the mean body weight for males in control, 0.1-, 0.2-, and 0.5-mg/kg/day groups were 46.1, 48.3, 49.3, 47.6 g, respectively. At the end of the study, the mean body weight for males in control, 0.1-, 0.2-, and 0.5-mg/kg/day groups were 48.1, 49.8, 51.3, and 48.9 g, respectively. There were no gross changes at necropsy and no effects on pituitary gland weight that were attributed to treatment with rmGH. There were no treatment-related neoplastic or nonneoplastic changes.

There were no specific organ or tumor types in either the male or female mice that had statistically significant ($p \leq 0.05$) increasing or decreasing trends after adjustment for multiplicity. The incidences of primary neoplasms were summarized in Table 4.

There were three tumor types (hemangiosarcoma in the skin, osteosarcoma in the bone, and lymphoma) that could be misinterpreted as being treatment related when viewed without knowledge of the spontaneous or background incidence in aging CD-1 mice. The ranges of the incidence of selected tumors in 58 groups of control mice from carcinogenicity studies in our laboratory are shown in Table 3. The incidence of spontaneous lymphomas in female CD-1 mice is relatively high. Data from 30 female control groups showed a mean of 10 lymphomas per group, and for 9 of these groups the incidence was above 12 per group. Comparing the incidence of hemangiosarcoma in the skin, osteosarcoma in the bone, and lymphoma with historical control ranges of incidence showed no increase attributed to rmGH. Although the incidence of a papilloma in the nonglandular mucosa of the stomach in female mice treated with 0.2 mg/kg/day was three, which is slightly higher than our historical incidence of zero to two in female control groups, the incidence of papilloma in the stomach of female mice treated with 0.5 mg/kg/day of rmGH was zero, indicating lack of a dose-dependent response or trend. There were no effects of rmGH on tumor incidence by statistical analysis and when compared to ranges of the incidence in groups of control rats from historical data.

TABLE 2
Primary Neoplasms in the 106-Week sc Carcinogenicity Study in Rats with rrGH ($n = 50$ per group)

	Males					Females				
Dose	C1	C2	0.2 mkd	0.4 mkd	0.8 mkd	C1	C2	0.2 mkd	0.4 mkd	0.8 mkd
Cardiovascular										
Heart										
Endocardium, benign schwannoma	0	0	1	1	0	0	0	0	2	1
Digestive										
Salivary gland										
Fibroma	1	0	0	0	0	0	0	0	0	0
Stomach										
Nonglandular mucosa, squamous cell carcinoma	0	0	0	0	0	0	1	0	0	0
Small intestine										
Mucinous adenocarcinoma	0	0	1	0	0	0	0	0	0	0
Polypoid adenoma	0	0	1	0	0	0	0	0	0	0
Leiomyoma	1	0	0	2	0	1	1	0	0	0
Large intestine										
Hemangioma	0	0	0	0	0	0	1	0	0	0
Hemangiosarcoma	0	0	1	0	0	0	0	0	0	0
Leiomyosarcoma	0	0	0	0	0	0	0	1	1	1
Liver										
Hepatocellular adenoma	0	0	2	3	1	0	0	3	2	3
Hepatocellular carcinoma	2	3	3	1	1	0	1	0	0	0
Total adenomas and carcinomas	2	3	5	4	2	0	1	3	2	3
Pancreas										
Islet, adenoma	2	4	5	7	6	1	0	0	2	2
Islet, carcinoma	0	1	0	0	1	0	0	0	0	0
Peritoneum										
Malignant mesothelioma	0	0	1	2	0	0	0	0	0	0
Benign paraganglioma	1	0	0	1	0	0	0	0	0	0
Malignant schwannoma	1	0	0	0	0	0	0	0	0	0
Endocrine										
Adrenal										
Benign pheochromocytoma	5	3	5	2	6	0	1	3	5	1
Malignant pheochromocytoma	0	2	2	0	2	0	0	1	0	0
Cortex, adenoma	2	1	1	3	1	0	1	0	2	0
Cortex, carcinoma	0	0	0	1	1	1	0	0	0	0
Parathyroid										
Adenoma	6	3	0	2	5	0	0	1	0	0
Pituitary										
Adenoma	29	20	18	22	22	37	34	28	27	14
Thyroid										
Parafollicular cell, adenoma	6	5	8	10	8	7	4	2	4	4
Parafollicular cell, carcinoma	2	0	2	0	3	2	0	0	1	0
Follicular cell, adenoma	1	0	0	2	0	0	0	0	0	0
Follicular cell, carcinoma	1	0	1	0	0	0	0	0	0	0
Hematopoietic and lymphoid										
Bone marrow										
Hemangioma	0	0	0	1	0	0	0	0	0	0
Spleen										
Hemangiosarcoma	0	1	0	0	0	0	0	0	0	0
Lymph node										
Hemangioma	0	0	1	0	0	0	0	0	0	0
Hemangiosarcoma	0	0	1	1	1	0	0	0	0	0
Thymus										
Malignant thymoma	0	0	0	0	0	0	0	0	0	1
Site unspecified										
Histiocytic sarcoma	1	0	2	1	0	0	1	1	0	0
Leukemia	2	0	0	0	0	0	0	0	0	0
Lymphoma	0	3	1	1	1	2	2	2	2	1

TABLE 2—*Continued*

	Males					Females				
Dose	C1	C2	0.2 mkd	0.4 mkd	0.8 mkd	C1	C2	0.2 mkd	0.4 mkd	0.8 mkd
Integument and mammary										
Skin										
Benign basal cell tumor	0	0	0	2	1	0	0	0	0	0
Malignant basal cell tumor	0	0	0	0	0	1	0	0	0	0
Fibroma	3	2	2	1	4	1	0	0	1	2
Fibrosarcoma	0	0	1	0	0	0	0	1	1	0
Keratoacanthoma	5	0	2	1	2	0	0	0	0	1
Lipoma	1	0	2	4	1	0	1	0	0	0
Osteosarcoma	0	1	0	0	0	0	0	1	0	0
Papilloma	0	1	1	0	0	0	0	0	1	0
Malignant schwannoma	0	0	0	1	0	0	1	0	0	0
Sebaceous adenoma	0	0	0	0	0	1	0	0	0	0
Trichoepithelioma	0	0	1	0	1	0	0	0	0	0
Mammary gland										
Adenocarcinoma	1	0	0	0	0	1	4	3	0	5
Adenoma	0	0	0	0	0	3	7	4	5	0
Carcinosarcoma	0	0	0	0	0	0	0	1	0	0
Total adenocarcinomas, adenomas, and carcinosarcomas	1	0	0	0	0	4	11	8	5	5
Fibroadenoma	3	1	2	1	4	13	10	15	15	13
Injection site										
Fibroma	0	0	0	0	0	0	0	0	0	1
Musculoskeletal										
Bone										
Osteosarcoma	1	0	0	0	1	0	0	0	1	0
Skeletal muscle										
Fibroma	0	1	0	0	0	0	0	0	0	0
Hemangioma	1	0	0	0	0	0	0	0	0	0
Rhabdomyosarcoma	0	0	0	0	0	0	0	1	0	0
Nervous										
Brain										
Malignant astrocytoma	1	0	1	0	0	0	0	1	0	1
Malignant glioma	1	0	0	0	0	0	0	0	0	0
Benign granular cell tumor	0	1	0	0	2	0	0	0	0	0
Benign oligodendroglioma	0	0	1	0	0	0	0	0	0	0
Spinal cord										
Malignant astrocytoma	0	0	0	0	1	0	0	0	0	0
Nerve										
Benign neuroma	0	0	0	0	0	0	0	0	1	0
Malignant schwannoma	0	0	0	0	0	1	0	1	0	0
Reproductive										
Ovary										
Malignant granulosa cell tumor						0	0	0	0	1
Benign sertoli cell tumor						0	0	0	1	0
Benign theca cell tumor						0	0	0	0	1
Malignant theca cell tumor						1	1	1	0	0
Uterus										
Fibroma						0	1	1	0	0
Hemangioma						0	0	0	1	0
Stromal polyp						4	3	3	0	4
Stromal sarcoma						0	0	1	1	0
Cervix										
Cervix, lipoma						0	1	0	0	0
Cervix, stromal polyp						1	0	0	1	0
Vagina										
Stromal sarcoma						0	0	1	0	0
Clitoral gland, squamous cell papilloma						0	0	0	1	0
Testis										
Leydig cell, adenoma	1	1	2	0	3					

TABLE 2—*Continued*

	Males					Females				
Dose	C1	C2	0.2 mkd	0.4 mkd	0.8 mkd	C1	C2	0.2 mkd	0.4 mkd	0.8 mkd
Prostate										
Adenoma	0	0	0	1	0					
Respiratory										
Lung										
Alveolar adenoma	0	0	1	0	0					
Special senses										
Eye										
Choroid, malignant amelanotic melanoma	0	0	0	0	1	0	0	0	0	0
Ear										
Zymbal's gland, carcinoma	1	1	0	1	0	0	1	0	0	0
Urinary										
Kidney										
Tubular adenoma	0	1	3	0	1	0	0	0	0	0
Lipoma	0	1	0	0	1	0	0	0	0	0
Total primary schwannomas, benign or malignant, any location	1	0	1	2	0	1	1	1	2	1
Total primary osteosarcomas, any location	1	1	0	0	1	0	0	1	1	0

C1 = control group 1; C2 = control group 2; mkd, mg/kg/day.

Although the injection sites in several mice injected with rmGH had focal hemorrhage, chronic inflammation, and fibrosis in all dose groups, the incidence did not increase with dose. The incidence in control groups for mice with chronic inflammation and/or fibrosis was 6–15 and the incidence in rmGH treated groups was 7–13. Although the total volume injected in the mouse subcutis was similar in mouse and rat, the dosing volume of the injection was 7 ml/kg in mice, providing a decreased concentration of GH in vehicle for mice as compared to the dosing volume of 1 ml/kg used in the rat carcinogenicity study.

DISCUSSION

Recombinant human GH is used chronically for the treatment of well-defined clinical conditions in children and adults. Therefore, a clear understanding of potential long-term adverse effects of exogenous GH administration or use of GH secretagogues, which increase endogenous GH levels, is important for risk assessment. Unlike evaluations of small molecules in laboratory animals for determining potential toxicity and carcinogenicity, proteins such as GH require species-specific proteins to elicit biological effects, as well as to avoid formation of antibodies. Thus, in order to evaluate the potential carcinogenic effects of recombinant GH, rrGH and rmGH genes were cloned, expressed, and shown to translate proteins with similar primary structure to the native counterparts derived from the pituitary in rats and mice. The physical behaviors of rrGH and rmGH in solution, with regard to aggregation, differed significantly. Stabilizing buffer and pH conditions were identified for each product and frozen formulations in solution provided excellent long-term stability as judged by analytical as well as biological assays (Sanyal *et al.*, 1997). Fed-batch culture processes for such clones were developed using a serum-free, glutamine-free medium and scaled up to 250 l production reactors to provide the rrGH and rmGH needed to conduct 2-year carcinogenicity studies in rats and mice, respectively (Zhou *et al.*, 1996).

Pharmacologic activities of the rrGH and rmGH were demonstrated in a hypophysectomized rat assay (body weight gain and induction of increased IGF-1 serum levels) and in 5-week tolerability studies (body weight gain). Increased body weight gain was also shown in the rats treated with rrGH in the rat carcinogenicity study. There was not a statistically significant increase in the body weight of mice treated with rmGH in the mouse carcinogenicity study by trend analysis. Given that the rmGH was shown to be pharmacologically active, we suspect that over a 2-year period of *ad libitum* feed, that body weight was not a sensitive indicator in mice.

Tolerability studies were performed in mice and rats to determine the doses for the carcinogenicity studies. Doses up to 1.5 mg/kg/day for rrGH and rmGH were well tolerated in the respective species. Assays to detect specific antibody production demonstrated that chronic administration of rrGH and rmGH did not induce host antibodies in either rats or mice. In the 5-week study with rats, daily dosing of 1.5 mg/kg/day of rrGH did not affect pituitary weights and did not change the proportion of cell populations within the pars distalis of the pituitary. By comparison, dogs that received daily sc doses of recombinant porcine GH for 14 weeks demonstrated an increased absolute volume of all cell populations within the pars distalis (GH-, PRL-, and ACTH-positive cells), using immunohistochemistry and morphometric analyses (Laroque *et al.*, 1998).

TABLE 3
Historical Ranges for Incidence of Tumors in Control Groups ($n = 50$ rodents per group) from Carcinogenicity Studies in Merck Research Laboratory for SD Rats and for CD-1 Mice

Species and tumor	Historical range	
	Males	Females
Rats[a]		
Heart, benign schwannoma	0	0–1
Heart, malignant schwannoma	0–1	0–1
Total primary schwannomas, benign or malignant, any location	0–4	0–3
Liver, hepatocellular adenoma	0–4	0–3
Liver, hepatocellular adenoma and carcinoma	0–7	0–4
Pancreas, islet adenoma	0–8	0–4
Adrenal gland, benign pheochromocytoma	0–8	0–4
Pituitary, adenoma	20–31	28–45
Mammary, adenoma, adenocarcinoma, and carcinosarcoma	0–1	3–20
Bone, osteosarcoma	0–1	0
Total primary osteosarcomas, any location	0–1	0–1
Mice[b]		
Stomach, papilloma, nonglandular mucosa	0–1	0–2
Lymphoma	0–8	3–17
Skin, hemangiosarcoma	0–1	0–1
Total primary hemangiosarcomas, any location	1–9	0–8
Bone, osteosarcoma	0	0–1
Total primary osteosarcomas, any location	0–2	0–3

[a]Historical ranges were based on incidence data from 44 rat control groups that were diet optimized (limit fed).

[b]Historical ranges were based on incidence data from 58 mouse control groups that were fed *ad libitum*.

Evaluation of serum levels of GH following daily sc injections of rrGH (for 6 days) in rats or rmGH (for 7 days) in mice demonstrated a slow absorption of GH from the injection site with systemic exposures lasting 5–8 h (Fig. 3). This slow absorption was important to provide an extended systemic exposure because recombinant and endogenous GH have a very short half-life in animals and humans (19 min) (Gharib *et al.*, 2003).

For comparison with clinical therapeutic doses, children with GHD who have received 0.043 mg/kg/day of rhGH all achieved at least a normal growth velocity during the first year of treatment (with many having a high growth velocity) (Mandel *et al.*, 1995). A 30-kg child receiving 0.043 mg/kg/day would get an injection of approximately 1.3 mg. By comparison, a 0.50-kg rat in the 0.8-mg/kg/day group received an injection of 0.4 mg which is about 30% of the daily injection for a human.

Estimates of the systemic exposures in the rat 2-year bioassay can be made using the exposures measured on study day 6 in the 5-week study in rats. Since there was approximately linear dose proportionality, simple linear regression analysis provided estimates of AUC 0.5–8 h values (sexes combined) for dosing at 0.2, 0.4, and 0.8 mg/kg/day at 288, 522, and 991 ng h/ml, respectively. Estimations of C_{max} (sexes combined) for dosing at 0.2, 0.4, and 0.8 mg/kg/day were 154, 239, and 408 ng/ml, respectively. Basal levels in rats (sexes combined) were approximately 45 ng/ml. The endogenous serum levels of GH in SD rats have been evaluated for age-related changes when comparing young (4–5 months old) and older (18–20 months old) male rats (Sonntag *et al.*, 1980). The periodicity and trough GH serum levels did not changed with age, but the peak levels of the pulsatile release was diminished which corresponded with a 60% decrease in the mean GH concentration in the older rats as compared to the younger rats. This decrease in mean GH serum concentrations in older rats would suggest an increase in the margin of serum levels of GH in the rrGH-treated rats, and potentially the rmGH-treated mice, in the second year of the 2-year bioassay as compared to control rats. In humans, the basal level of GH is typically < 3 ng/ml, although there are several pulses during the day (each lasting 10–30 min), and the pulses may reach 5–30 ng/ml. Although it is difficult to make a direct comparison between the exposures from a sc injection with the multiple short pulses occurring in healthy persons, the AUC (0–8 h) and the C_{max} following the sc injection of 0.2, 0.4, or 0.8 mg rrGH/kg/day were substantially higher than the basal or peak levels in humans.

Rats and mice were considered juvenile (5 weeks of age) at the start of the daily sc injections, and the dosing continued until the surviving rodents were approximately 110–111 weeks of age. Feeding of an optimized portion of food daily to SD rats in our laboratory has resulted in lower background tumor incidence in carcinogenicity studies (Keenan *et al.*, 1995). The 2-year carcinogenicity studies in rodents demonstrated no increased incidence of tumors in rats administered rrGH or mice administered rmGH. In addition, the mouse micronucleus test in bone marrow and blood erythrocytes, which can detect clastogenic agents and compounds that cause chromosome loss, was negative in mice administered daily sc injections of rmGH.

Previous publications from 1950 reported that ip administration of relatively high doses of an extract from ox anterior pituitary glands into male and female rats induced an increased incidence of pheochromocytomas, lymphosarcomas of the lung, fibroadenomata of the mammary gland, and adenomatous lesions in the anterior pituitary (Koneff *et al.*, 1951; Moon *et al.*, 1950a,b). However, the design of those studies had little similarity or resemblance to our studies or to clinical practice in use of rhGH from 1985 to the present. In addition, no information to confirm the purity or composition of the ox anterior pituitary abstract used in those studies was available, calling into question the usefulness of those older reports.

In conclusion, the lack of carcinogenic effects following chronic administration of recombinant GH in these 2-year bioassays lends greater weight of evidence that high circulating

TABLE 4
Primary Neoplasms in the 105-Week sc Carcinogenicity Study in Mice with rmGH ($n = 50$ per group)

	Males					Females				
Dose	C1	C2	0.1 mkd	0.2 mkd	0.5 mkd	C1	C2	0.1 mkd	0.2 mkd	0.5 mkd
Cardiovascular										
Heart										
Hemangiosarcoma	0	1	0	0	0	0	0	0	0	0
Malignant schwannoma	0	0	1	0	0	0	0	0	0	0
Digestive										
Stomach										
Benign carcinoid tumor	0	0	0	0	0	0	0	0	0	1
Squamous cell carcinoma	1	0	0	0	1	0	0	0	0	0
Glandular mucosa, polyp	0	0	0	1	0	0	1	0	0	0
Nonglandular mucosa, papilloma	0	0	0	0	0	0	0	0	3	0
Small intestine										
Adenoma	2	0	0	0	0	0	0	0	0	1
Hemangiosarcoma	0	0	0	0	0	2	0	0	0	0
Large intestine										
Leiomyosarcoma	0	0	0	0	0	0	0	0	0	1
Liver										
Hemangioma	0	0	0	0	1	1	0	0	1	2
Hemangiosarcoma	8	3	4	3	3	6	0	1	2	1
Hepatocellular adenoma	10	8	13	14	3	1	1	1	1	2
Hepatocellular carcinoma	5	4	3	1	3	0	0	1	2	0
Gallbladder										
Adenoma	0	0	0	0	0	0	1	0	0	0
Pancreas										
Islet, adenoma	0	1	1	0	2	1	0	1	2	2
Peritoneum										
Osteosarcoma	0	0	0	0	0	0	1	0	0	0
Endocrine										
Adrenal										
Benign pheochromocytoma	0	1	0	0	0	0	1	0	0	0
Malignant pheochromocytoma	0	0	0	1	1	0	0	0	0	0
Benign spindle cell tumor	0	0	0	1	1	3	0	0	2	0
Cortex, adenoma	1	0	0	0	0	0	0	0	0	0
Parathyroid										
Adenoma	0	0	0	0	0	1	0	0	0	1
Pituitary										
Pars distalis, adenoma	0	3	1	0	0	3	2	2	1	3
Thyroid										
Follicular cell, cystic adenocarcinoma	0	0	0	1	0	0	0	0	0	0
Follicular cell, adenoma	0	0	2	0	1	0	0	1	0	0
Hematopoietic and lymphoid										
Spleen										
Hemangioma	0	0	0	0	0	1	0	0	0	0
Hemangiosarcoma	1	3	0	0	1	0	2	1	2	1
Lymph node										
Hemangioma	0	0	0	0	0	0	1	0	0	0
Hemangiosarcoma	0	0	0	1	0	0	0	0	0	0
Thymus										
Undifferentiated sarcoma	0	0	0	0	0	0	1	0	0	0
Site unspecified										
Histiocytic sarcoma	0	0	1	1	0	1	1	1	3	0
Leukemia	0	1	0	0	2	1	0	0	0	1
Lymphoma	4	1	4	2	5	5	4	14	6	12
Integument and mammary										
Skin										
Fibroma	0	1	0	0	0	0	0	0	0	0
Fibrosarcoma	1	0	1	0	0	0	1	0	0	1
Hemangiosarcoma	0	0	0	0	2	0	0	0	0	0
Osteosarcoma	0	1	0	0	0	0	0	0	0	0

TABLE 4—*Continued*

	Males					Females				
Dose	C1	C2	0.1 mkd	0.2 mkd	0.5 mkd	C1	C2	0.1 mkd	0.2 mkd	0.5 mkd
Undifferentiated sarcoma	0	0	0	0	0	0	0	2	0	0
Malignant schwannoma	0	0	0	1	0	0	0	0	0	0
Mammary gland										
Adenoacanthoma	0	0	0	0	0	2	0	0	0	2
Adenocarcinoma	0	0	0	0	0	1	3	1	1	4
Musculoskeletal										
Bone										
Osteoma	0	0	0	0	1	0	0	0	0	0
Osteosarcoma	0	0	0	0	0	0	0	0	0	2
Skeletal muscle										
Malignant schwannoma	0	0	0	0	0	0	0	1	0	0
Nervous										
Brain										
Malignant glioma	0	0	0	0	0	0	1	0	0	0
Spinal cord										
Benign meningioma	0	0	0	0	0	0	0	0	1	0
Reproductive										
Ovary										
Cystadenocarcinoma						1	0	0	0	0
Cystadenoma						1	2	0	0	1
Benign granulosa cell tumor						0	0	0	2	1
Malignant granulosa cell tumor						1	0	0	0	0
Hemangioma						1	0	0	1	0
Hemangiosarcoma						0	0	1	0	0
Leiomyoma						0	0	0	1	0
Uterus										
Hemangiosarcoma						0	0	1	0	0
Leiomyoma						2	1	0	2	2
Leiomyosarcoma						2	0	0	0	0
Polyp						0	3	1	0	0
Stromal sarcoma						0	0	0	1	0
Cervix										
Cervix, polyp						0	1	0	0	2
Vagina										
Leiomyosarcoma						0	0	1	0	0
Bulbourethral gland										
Adenoma	0	0	0	1	0					
Testis										
Benign interstitial cell tumor	3	3	3	1	2					
Seminal vesicle										
Adenoma	0	0	0	0	1					
Benign granular cell tumor	0	1	0	0	0					
Respiratory										
Lung										
Adenocarcinoma	11	14	14	9	11	4	10	8	7	8
Adenoma	8	10	8	9	7	8	3	5	5	2
Special senses										
Eye										
Carcinosarcoma	0	1	0	0	0	0	0	0	0	0
Harderian gland, adenoma	6	4	3	3	7	3	3	1	2	0
Ear										
Hemangioma	0	0	1	0	0	0	0	0	0	0
Urinary										
Urinary bladder										
Benign mesenchymal tumor	0	0	0	0	0	0	1	0	0	1
Total primary hemangiosarcomas, any location	9	7	4	4	6	8	2	4	4	2
Total primary osteosarcomas, any location	0	1	0	0	0	0	1	0	0	2

C1, control group 1; C2, control group 2; mkd, mg/kg/day.

levels of GH would not be associated with greater risk of tumors in subjects receiving GH replacement therapy.

SUPPLEMENTARY DATA

The (1) volume density (%) of GH, PRL, and ACTH cell populations in the rat pituitary glands (supplementary Table 1); (2) mean serum IGF-1 and GH levels at 3 h after injection of rrGH on drug day 6 in hypophysectomized Wistar rats with SE bars (supplementary Fig. 1); (3) mean serum IGF-1 and GH levels at 3 h after injection of rmGH on drug day 6 in hypophysectomized Wistar rats with SE bars (supplementary Fig. 2); (4) percent mortality in female rats (supplementary Fig. 3); (5) percent mortality in male rats (supplementary Fig. 4); (6) mean body weights for female rats (supplementary Fig. 5); (7) mean body weights for male rats (supplementary Fig. 6); (8) mean body weights for female mice (supplementary Fig. 7); and (9) mean body weights for male mice (supplementary Fig. 8) as supplementary data are available online at http://toxsci.oxfordjournals.org/.

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