In Vivo Bioassay for the Potency Determination of Human Growth Hormone in Dwarf “Little” Mice*

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ABSTRACT

Homozygous 40- to 90-day-old “little” mice (lit/lit), derived from the C57BL/6J strain, have been used to set up an in vivo body weight gain bioassay for GH whose performance has been compared to the widely used hypophysectomized rat assay. A log dose-response curve has been analyzed in order to choose doses in the linear range that are suitable for setting up useful, precise, and economical 2 x 2 factorial assays. A comparison between the response in the two sexes has also been carried out, showing no significant difference between male and female little mice of this age.

Three assays have been carried out in these animals for the potency determination of a local human GH (hGH) reference preparation in terms of First International Standard of GH (human) for bioassay (WHO 80/505), comparing them to a classical assay in hypophysectomized rats performed with the same preparations. The calculated potency values were in good agreement, while the statistical parameters indicated a comparable assay precision, even in a practical and rapid 4-day assay.

We suggest the substitution of hypophysectomized rats with little mice for this in vivo test, still required for potency and bioidentity determination of clinical preparations of recombinant hGH. This avoids the highly invasive, costly, and time-consuming surgery, providing a faster, more flexible, and economical assay method, while still directly measuring parameters of in vivo linear growth in an animal model closely related to isolated hGH deficiency type I. (Endocrinology 132: 2051–2055, 1993)

Since the fundamental work of Marx et al. (1), the biological activity of GH preparations has been determined mostly by the body weight gain (BWG) assay (2, 3) or the tibia assay test (4), both in hypophysectomized rats.

In 1982, a report of the World Health Organization (WHO) Expert Committee on Biological Standardization, when setting up the First International Standard of GH (human) for bioassay (80/505), stated that the in vivo weight gain assay was probably the defining test for hGH determination (5, 6). Following this principle, Groesback and Parlow (7) carried out an extensive study on the principal factors influencing the precision of this assay, obtaining more reliable and consistent values for the index of precision (λ) of the order of 0.2, a value considered highly respectable. More recently, however, with the increasing utilization of recombinant human GH (hGH), there have been repeated calls to abandon this bioassay as a routine control procedure for GH due to the fact that the method is highly invasive, imprecise, and costly as well as to the increasing pressure against animal use (8). The assay, however, should remain as an unreplaceable bioidentity test (9, 10), especially for the characterization and validation of the manufacturing process (11, 12).

Having carried out in vivo hGH bioassays in our laboratory during the last 15 yr (13, 14), we were aware of the need for an alternative animal model that while still measuring parameters of linear growth, does not require such laborious, costly, and time-consuming surgery as hypophysectomy. The model we have investigated is the “little” mouse, an autosomal recessive mutant from the C57BL/6J strain, noted and produced by Jackson Laboratory (Bar Harbor, ME), in which an inherited growth defect appears to resemble human isolated GH deficiency type I (15–17).

These mutants (lit/lit), males and females, whose weight at 30 days of age is approximately 60% that of normal mice, were studied with regard to hGH dose-response relationship in a BWG assay to choose useful and economical doses within the linear range of the curve. Little mice were then used in different 2 x 2 factorial assay designs for the calibration of a secondary hGH standard compared with the classical BWG assay in hypophysectomized rats. Aspects related to sensitivity, precision, accuracy, and economy of this new bioassay were also studied.

Materials and Methods

Animals and housing conditions

Female Wistar rats, 45–60 days of age, were hypophysectomized in our laboratory by the parapharyngeal approach and, 2 weeks after surgery, maintained under weight surveillance for 15 days, eliminating all animals whose weight varied more than ±3% in 10 days or was higher than that on the day of surgery.

Little mice (C57BL/6J lit/lit) were purchased from Jackson Laboratory, and a breeding colony was set up in our animal house. Mutant mice were produced from matings of C57BL/6J lit/lit females to C57BL/6J lit/+ males. Offspring were weighed every 7 days and weaned at 4 weeks, separating male and female little mice whose weights were under 60% of their heterozygous (lit/+ ) littermates; both sexes, at 45–90 days of age, were used in the assay. Special care was taken when weaning lit/lit individuals to place extra long spouts on the water bottle and pellets.
of food on the cage floor to ensure that there would be no retardation of growth because of inability to obtain sufficient quantities of food and water.

All animals were kept in clear plastic cages with a metal grill top in an air-conditioned room; the temperature was maintained at 24 ± 1°C, and lighting was regulated on a 12-h light, 12-h dark schedule.

hGH preparations
hGH (First International Standard for Bioassay; 80/505), with an assigned potency of 4.4 IU/ampule (2.59 IU/mg), was kindly provided by the WHO International Laboratory for Biological Standards (NIBSC, South Mimms, Hertfordshire, United Kingdom). Pituitary hGH (Second Brazilian Secondary Standard) was extracted, purified, characterized, and ampouled in our laboratory (IPEN-CNEN/SF); the protein content of each ampule was 1.71 ± 0.06 mg, as determined by the method of Lowry et al. (18).

Bioassay procedures
Two × 2 factorial assay designs were carried out as described in previous work (14), using each time a total of 40 animals, sex administration, and changing (as detailed in Results) assay duration, dosage, injection frequency, and, obviously, animal model. The animals' body weights were measured daily throughout the entire period of assay and used to calculate individual growth curves, whose slopes were then used for the calculation of potency and related precision parameters, according to Bliss's statistics (19). The index of precision was determined in units of log10 dose, following Gaddum's terminology, as λ = s/b, where b and s are composite values, respectively, of the slope and the so about the slope. They are determined from the dose-response lines for the standard and the unknown when shown to agree within the sampling error. The fiducial limits, also in logarithms of assumed limits, were calculated as X' = C' M' ± t C S M, where M' is the log10 of determined relative potency of the unknown, t is the Student's t for P = 0.05, S M is the SE of M', and C is calculated according to C² = R² - s/b², where B² is the variance related to the combined slope of the dose-response curve. The convention adopted (3) was to define an x-day assay as the design in which the animals are injected for x-1 days, without weekend interruption, weighing them for the last time on the xth day.

Sensitivity determination
The sensitivity calculation was based on the response and sd at zero and the minimal dose administered, according to Rodbard's definition (20).

Results
The growth curves of male and female little mice (lit/lit) compared with those of their heterozygous litter mates (lit/+) in our laboratory are presented in Fig. 1. The data are in good agreement with literature values (15); the body weight of little mice was about 60% that of their heterozygous littermates at the age of 4 weeks and about 45% after 10–11 weeks of age. It is interesting to note that there was a significant difference between males and females (P = 0.05) in both heterozygous and little mouse growth curves after 4 weeks of age.

The difference in response between male and female little mice at 45–90 days of age treated with the same dose of hGH (24 µg/day) was tested in separate assays (Table 1) and found to be nonsignificant in both single and cumulative t tests (P = 0.05), confirming the data presented by Beamer and Eichler (21) with higher doses of GH1 (ovine) and for a much longer treatment period (6 weeks). A dose-response curve was, therefore, carried out at the dosage levels of 5, 10, 20, and 50 µg/day, using a mixed population of males and females of the same age and comparing their weight increases to values previously obtained with hypophysectomized rats using the same hGH preparation (Second Brazilian Standard) in approximately the same dose range (Fig. 2A).

Both curves exhibited good linearity in the range usually used in our assays; the correlation coefficients for rats and mice were, respectively, r = 0.9862 and r = 0.9996. To represent rat and mice dose-response curves in the same figure, two different ordinate scales were necessary, one 10-fold more expanded than the other. Since the little mice used in our assays normally weigh about one tenth of our hypophysectomized rats, we considered this artificial presentation to be proper and quite practical for the purpose of a comparison between the two different animal models. In Fig. 2B, the same dose-response relationship is presented, not as the direct weight increase, but as its percentage, calculated based on the animal weights on assay day 1. There was a significantly higher percent response in little mice and a good parallelism between the two curves; the slopes for the rat and mice curves were, respectively, 3.6% and 3.3% weight.
FIG. 2. Dose-response curve determined at the dosage levels of 5, 10, 20, and 50 µg/mouse-day and 12, 24, and 50 µg/hypophysectomized rat-day, always using the same hGH preparation (Second Brazilian Standard). A, Direct weight increase; B, percent weight increase.

Discussion

The little mice proved to be a valid alternative model to the BWG assay in hypophysectomized rats. They provide a faster, cheaper, and noninvasive in vivo assay for the potency and bioidentity determination of hGH extracts, allowing the utilization of male and female mice, 45–90 days of age, in 10-day or, better still, 5- or 4-day assays. These animals, after hGH administration, exhibit a higher percent weight increase than hypophysectomized rats, equal or better linear growth, and a useful range of the dose-response curve. Precision, with a perfectly acceptable index (λ) of 0.20–0.25, was also at least as good as that presented by the classical assay; the fiducial limits were all acceptable according to the criteria established by the European Pharmacopoeia. Moreover, considering all of the data obtained in our laboratory with the hypophysectomized rat BWG assay using the same assay design and International Standard for GH, human (WHO 80/505), the statistical data obtained with the little mice assay confirm the quality and good performance of this new bioassay in a much wider comparison.

The potency of a Secondary Standard preparation determined against the International Standard provided comparable values in the two animal models, indicating a similar accuracy for the two in vivo tests. The assay sensitivity also
TABLE 2. Comparison between the performance of hypophysectomized rat and little mouse assays in the calibration of a Secondary Brazilian Standard of hGH against WHO 80/505

| Assay design                  | Potency (IU/mg) | Fiducial limits (%) | Combined slope | SD  | λ    | Correlation coefficient
|-------------------------------|-----------------|--------------------|----------------|-----|------|------------------------
| Hypox rats, 10 days, 10-20 µg/| 2.46            | 72-147             | 1.349          | 0.280 | 0.208 | 0.930 ± 0.053          |
| day, 1 injection/day          |                 |                    |                |      |      |                        |
| Little mice, 10 days, 10-20 µg| 2.07            | 69-142             | 0.193          | 0.042 | 0.216 | 0.949 ± 0.043          |
| day, 1 injection/day          |                 |                    |                |      |      |                        |
| Little mice, 5 days, 20-40 µg/| 2.63            | 72-156             | 0.291          | 0.064 | 0.219 | 0.983 ± 0.072          |
| day, 2 injections/day         |                 |                    |                |      |      |                        |
| Little mice, 5 days, 10-50 µg/| 2.60            | 68-150             | 0.255          | 0.065 | 0.254 | 0.937 ± 0.059          |
| day, 2 injections/day         |                 |                    |                |      |      |                        |

* The acceptable fiducial limits (P = 0.95) are not less than 64% and not more than 156% of the stated potency, as recommended by the European Pharmacopœia (22).

^ Mean ± SD.

TABLE 3. Statistical parameters related to the last 10 assays in hypophysectomized rats, carried out at IPEN-CNEN laboratory

| Assay design | Potency (IU/mg) | Fiducial limits (%) | Combined slope | SD  | λ    | Correlation coefficient |
|--------------|-----------------|--------------------|----------------|-----|------|------------------------
| 1            | 2.10            | 75-157*            | 2.000          | 0.352 | 0.176 |                        |
| 2            | 3.09            | 76-128             | 1.356          | 0.222 | 0.164 |                        |
| 3            | 3.16            | 59*-149            | 1.411          | 0.358 | 0.204 |                        |
| 4            | 3.34            | 86-116             | 1.282          | 0.128 | 0.100 |                        |
| 5            | 2.04            | 7.5*-218*          | 1.329          | 0.396 | 0.298 |                        |
| 6            | 2.41            | 68-141             | 1.290          | 0.327 | 0.253 |                        |
| 7            | 1.89            | 75-134             | 1.557          | 0.324 | 0.208 |                        |
| 8            | 2.46            | 79-147             | 1.349          | 0.280 | 0.208 |                        |
| 9            | 4.19            | 78-144             | 1.484          | 0.203 | 0.137 |                        |
| 10           | 3.68            | 83-126             | 1.607          | 0.167 | 0.104 |                        |

* Out of the acceptable fiducial limits.

References

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