Radioimmunoassay of canine growth hormone

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Abstract. A sensitive radioimmunoassay (RIA) for canine growth hormone (GH) was developed. Antibodies were elicited in rhesus monkeys. One antiserum exhibited a working titer at a dilution of 1:500 000. Radioiodination was performed enzymatically employing lactoperoxidase. Logit-log transformation and least squares fitting resulted in straight line fitting of the standard curve between 0.39 and 50 ng/ml. Formation of large-molecular [125I]GH during storage caused diminished assay sensitivity. Therefore [125I]GH was re-purified by gel chromatography. Using this procedure, high and reproducible assay sensitivity was obtained. Tracer preparations were used for as long as 3 months after iodination. Diluted plasma from normal and acromegalic dogs resulted in a dose-response curve parallel to the standard curve. Canine prolactin exhibited a cross-reactivity of 2%. The within-assay coefficient of variation (CV) was 3.8 and the between-assay CV was 7.2%. Mean plasma GH concentration in normal dogs was 1.92 ± 0.14 ng/ml (mean ± SEM). GH levels in acromegalic dogs were appreciably higher. Insulin-induced hypoglycemia, arginine and ornithine administration resulted in inconsistent and sluggish GH increment. A better response was obtained by injecting a low dose of clonidine. Clonidine administration to hypopituitary dogs resulted in absent or poor GH increment.

GH measurements by RIA have been described for several species and results obtained under experimental as well as clinical conditions have also been reported. Only a few reports concerned with the measurements of canine GH are available (Tsuchima et al. 1971; Coccola et al. 1976; Hampshire et al. 1975; Lovinger et al. 1974) and measurements have been almost exclusively restricted to studies of the regulation of GH secretion in normal dogs. Recent evidence that dogs treated with progestagens may develop acromegalic signs and/or disturbed glucose tolerance suggest that GH may play a major role in the development of the signs (Sloan & Oliver 1975).

For this reason, the development of a specific and sensitive homologous RIA for canine GH was undertaken. Here we report on the technical aspects of the GH RIA and on plasma GH levels in normal and acromegalic dogs. Results from several stimulation tests are reported.

Materials and Methods

1. Hormones and chemicals

Highly purified canine GH (cGH), essentially homogenous in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Lot D 1080 A), was a generous gift of Dr. A. E. Wilhelmi (Atlanta, GA, USA). Purified canine prolactin was kindly provided by Dr. M. Bevers (Department of Veterinary Gynaecology and Obstetrics, State University of Utrecht, The Netherlands).

For iodination procedures and standards to be used in assays, a GH aliquot was weighed and dissolved in 5 mM HCl, pH 4.0. The stock solution was then diluted with phosphate buffer (20 mM Na2HPO4, 20 mM NaH2PO4, pH 7.4) to a concentration of 0.25 mg/ml. Aliquots of 5 µg for iodination procedures and diluted aliquots for standards were maintained at −25°C until used. Aliquots kept in this way were thawed only once and used promptly. Lactoperoxidase was obtained from Sigma and Na[125I] (IMS. 30) from Amersham. H2O2 (Perhydrol)
was purchased from Merck. Bovine serum albumin (BSA, Sigma) was of RIA grade. Tris-barbital, Na-barbital buffer was prepared with High Resolution Buffer®, Gelman. Sodium azide (NaN₃) was purchased from Merck and antimonkey IgG antiserum from Antibodies Incorporated, Davis, CA, USA. Activated charcoal was obtained from Sigma. Regular insulin (Actrapid, Monocomponent) was purchased from Novo Industries, Copenhagen, Denmark. L-arginine and L-ornithine were obtained from Sigma. Clonidine (Catapresan) was purchased from Boehringer, Ingelheim, Germany.

2. Immunization procedure

Antibodies against cGH were produced in 3 rhesus monkeys. Immunization was performed by injecting a total volume of 2 ml of an emulsion of 50% Freund's adjuvant and 50% normal saline, pH 8, in which GH was dissolved. Injections were carried out at monthly intervals. The first three immunizations were performed by injecting the animals sc with incomplete Freund's adjuvant. Each monkey received 0.4 mg GH as primary and secondary injection. The third injection contained 0.2 mg GH. A fourth injection was given in an emulsion containing complete Freund's adjuvant and 0.7 mg GH. A final injection was given with 0.2 mg GH in incomplete Freund's adjuvant. The last two injections were given as dorsal, multiple, intradermal injections.

The animals were bled 10–14 days after each injection but the first. Blood was allowed to clot, centrifuged and sera were stored at −80°C. The presence of cGH antibodies was screened using the Ouchterlony immunodiffusion technique.

3. Iodination procedure

Iodination was performed enzymatically according to Thorell & Johansson (1971). Reagents were mixed at ambient temperature in the following order: 5 μg GH in 20 μl sodium phosphate buffer (40 mM, pH 7.4), 4 μl lactoperoxidase (0.5 mg/ml in sodium phosphate buffer) and 10 μl NaI (1 μCi). Then 5 μl H₂O₂ (Perhydroil 30%, diluted with double distilled H₂O, 1:15 000) was added and the tube was gently shaken for 2–5 s. The reaction was stopped by adding 0.5 ml column buffer to the reaction mixture. The reaction mixture was passed over a Sephadex G-100 column and the peak representing GH was pooled, well mixed and aliquots of 0.3 ml were kept frozen at −20 to −30°C. Iodination yield (specific activity) was calculated from the radioactivity present in the GH peak after gel chromatography.

4. Gel filtration

Iodination reaction mixtures were passed over a Sephadex G-100 column. [¹²⁵I]GH, stored frozen, was repurified on the same column immediately before being used in assay procedures. Sephadex columns of 1.5 x 28 cm with a total bed volume of 45–50 ml were used. Chromatography was developed in the cold room at 4°C with a buffer containing 9 g High Resolution Buffer®, 5 g BSA and 0.2 g NaN₃/l. The pH of the elution buffer was adjusted to 8.6. The flow rate was kept at 7.5 ml/h. Fractions of 0.95 ml were collected.

5. Assay procedure

All assay dilutions were performed with a buffer containing Na₂HPO₄ 5 mM, NaH₂PO₄ 5 mM, 2.5% BSA and 0.2 g NaN₃/l. The pH was adjusted to 7.5.

'Growth hormone free' plasma was obtained from hypophysectomized dogs, allowed to mix with a trace amount of [¹²⁵I]GH (~ 10⁴ cpm/ml) and extracted overnight with activated charcoal (0.1 g charcoal/ml plasma). After centrifugation of the charcoal plasma mixture at 4000 r.p.m. for 1 h, radioactivity concentration in the supernatant was found to be less than 10% of initial, indicating that most of any residual GH had been adsorbed to the charcoal. Fine charcoal particles were removed by passing the depleted plasma through millipore filters (Milex-GS Millipore, France). Assay incubations were performed at 4°C as follows: 50 μl antiserum (1:50 000), 100 μl normal monkey serum (1:100), 75 μl assay buffer and 25 μl sample were mixed. Standard tubes were prepared in the same way as the samples, except that 50 μl of buffer rather than 75 μl was added. Twenty-five μl was added as 'GH-free plasma' in order to provide an identical protein environment as for samples.

After a 20 h incubation period [¹²⁵I]GH (~ 500 pg) was added together with 150 μl buffer. The reaction mixture was allowed to equilibrate for an additional 24 h. Then, 100 μl of antimonkey IgG antiserum at a dilution giving maximal precipitation was added. After an overnight incubation the tubes were centrifuged at 4000 r.p.m. for 20 min and supernatants were aspirated.

Tubes, for the assessment of total binding (absence of unlabelled hormone), total activity and non-specific binding (no anti-GH antiserum) were run in quadruplicate. All other tubes were run in duplicate. Radioactivity in the pellet was counted in a well type counter (Beckman). Pipetting was performed with a semiautomatic pipette, operating on the basis of a two syringe system which allows two different reagents to be added to the assay tubes simultaneously (Automatic Pipette, Micromedic Systems Inc., Philadelphia, PA 19105, USA).

6. Calculation and statistics

Standard curve fitting and calculation of hormone concentrations in samples were performed using a programmable desktop unit (TI95 and Pc-100c Printer, Texas Instruments). The logit-log transformation, as described by Rodbard & Lewald (1970) [ordinate (y): logit B/Bo = log (B/Bo); abscissa (x): log hormone concentration] was used for least squares fitting of the standard curve and calculation of hormone concentrations in samples. (B =
binding at a given hormone concentration, \( B_0 = \) binding in the absence of unlabelled hormone; \( B \) and \( B_0 \) corrected for unspecific binding.

Standards covering the full range of the dose response curve were run for each assay. Within- and between-assay variance was calculated as described by Abraham et al. (1971).

For other statistical analysis Student’s \( t \)-test was employed.

7. Source of plasma specimen and in vivo tests

Blood specimens were obtained from experimental dogs, from patients and normal pet dogs.

Jugular venipuncture and all in vivo tests were performed after an overnight fast. Blood was transferred into polystyrene tubes containing dipotassium ethylene diaminotetraacetic acid (EDTA), centrifuged immediately and plasma was stored at \(-20 \) to \(-30^\circ\text{C} \).

L-ornithine and L-arginine were dissolved at pH 7.4 at a concentration of 0.25 g/ml and were given iv as a bolus at a dose of 0.5 g/kg. Regular insulin was given iv at a dose of 0.4 U/kg. Clonidine was diluted with saline to a concentration of 20 \( \mu \)g/ml and given iv at a dose of 10 \( \mu \)g/kg.

Results

The first three immunizations did not lead to any detectable precipitation in Ouchterlony plates. Multiple site intradermal immunization, using complete Freund’s adjuvant, was then performed. Sera of 2 monkeys after this booster injection yielded a strong precipitation line. When the monkeys were given another booster with incomplete adjuvant, the precipitation pattern remained unchanged. The antiserum exhibiting the heaviest precipitation bands was used for assay procedures.

GH iodinations yielded specific activities ranging from 29 to 70 \( \mu \)Ci/\( \mu \)g GH. As shown in Fig. 1, chromatography of the radioiodination mixture resulted in 3 peaks. A large molecular one (Fra. 15–28, peak I), a peak eluting with the inner bed volume (Fra. 29–40, peak II) and a small molecular peak representing free \( ^{125}\text{I} \) and probably iodination damage products (Fra. 45–48, peak III).

For assay procedures pooled peak II was used. When \( ^{125}\text{I} \)GH was stored frozen and used in assay procedures without previous re-purification on a Sephadex column, assay sensitivity was found to be appreciably lower than in the case where re-purifi-
cated was performed. The loss of sensitivity was found to be associated with de novo generation of peak I (Fig. 1) occurring during the freezing process and/or during storage.

During repurification, the height of peak I was, occasionally, found to be as high as peak II. However, repurification and the addition of identical trace amounts of [125I]GH allowed reproducible assay sensitivity for at least 3 months after iodination.

Binding studies, performed with the antiserum exhibiting the strongest precipitation line, resulted in binding values of 90, 73, 62 and 42% at antiserum dilutions of 1:500, 1:50000, 1:100 000 and 1:500 000, respectively. For assay procedures the dilution 1:500 000 was chosen. Total binding values in 20 assays, performed over several months, yielded a variation of 40 to 50%. This variation however, did not appreciably influence the lower detection limit of the assay. As a rule, the assay reaction mixture was allowed to equilibrate for 24 h after the tracer had been added. When this incubation was prolonged for another 24 h, no increase in total binding was observed. Addition of the second antibody was shown to result in maximal precipitation after 5 to 6 h, indicating that under the described conditions assays were performed at equilibrium. As can be seen from Fig. 2, linearization of the standard curve was obtained for concentrations between 0.39 and 50 ng/ml. When assay incubations were performed using 50 µ of plasma and standard solution instead of using 25 µ, standard curves ranging from 0.19 to 25 ng/ml were obtained. When serial dilutions of plasma from normal dogs were used in order to displace [125I]GH, a straight line, parallel to the standard curve, resulted (Fig. 2).

An identical dose response curve was obtained with plasma dilutions from acromegalic dogs. When different known amounts of GH, ranging from 1.54 to 50 ng/ml were added to the assay, recovery was found to be 97 ± 1.92% (mean ± SEM, n = 8). The values found were 98, 104, 97, 95, 90, 89 and 102%. Canine prolactin exhibited a cross-reactivity of 2%. The within-assay coefficient of variation (CV) for 35 samples with values between 0.5 and 48 ng/ml was 3.8%. The between-CV for 23 samples ranging from 3 to 50 ng/ml, was 7.2%.

GH values were obtained in 63 normal dogs. Their mean concentration was 1.92 ± 0.14 (mean ± SEM). GH concentrations in acromegalic dogs were found to be appreciably higher (Fig. 3).

Several stimulation tests were performed in normal dogs. Intravenous administration of the amino
Discussion

Using immunologic and physicochemical methods, 2 major subclasses among mammalian growth hormones have been identified. GH of one subclass, the primates exhibits a low isoelectric point (IEP) and virtually no immunocross-reactivity with GH from the other subclass (e.g. dog, ox, pig, rat). GH from the latter subclass exhibits a higher IEP and virtually no cross-reactivity with primate GH (Wilhelmi 1974). These findings indicate appreciable structural difference between the two types of GH and also explain why the monkey is considered to be an ideal host for immunization procedures with canine GH.

It is difficult to know why the monkeys did not respond until the third GH injection. The following factors should be taken into consideration: a) emulsions with incomplete Freund’s adjuvant and given sc are likely to evoke only a weak immune response; b) anti-GH antibodies may have been present, though undetected by immunodiffusion.

The radioiodination yield (specific activity) we obtained had proven satisfactory and no attempts to increase specific activity were undertaken. A standard curve, ranging from 0.39 to 50 ng/ml, had proven practical for determinations of normal or elevated levels of GH. Using a standard curve ranging from 0.39 to 50 ng/ml, accuracy for low levels is less. When predominantly low levels are expected, the dose-response curve should range from 0.19 to 25 or from 0.09 to 12.5 ng/ml in order to increase accuracy in the lower range. This is easily achieved by performing assays with 50 or even 100 μl volumes of plasma and standard. For the determination of normal levels such a limited-range standard curve should be employed. In general, we used a standard curve extending up to 50 ng/ml since almost all plasma GH levels, having clinical or pathophysiologic significance, lie above the low range. Moreover, when GH deficiency is to be demonstrated, the finding of low GH concentrations alone is insufficient and stimulation tests must be performed. Our observation that repurification of $[^{125}\text{I}]$GH is important for reproducible assay sensitivity is in agreement with the report of Hampshire et al. (1975). Repurification of $[^{125}\text{I}]$GH before use is also recommended for the assay of human GH (Peake 1974). Our finding and the findings of other seems to be in contrast with those of Coccola et al. (1976) and Tsushima et al. (1971)

Fig. 5.

GH response to iv clonidine administration (10 μg/kg body weight). ●●● = normal dogs, n = 7. Values represent the mean ± SEM. ■■■ = GH response in a pituitary dwarf dog. ——— = GH response in an adult hypopituitary dog (unknown aetiology). □□□ = GH response in an adult hypopituitary dog (brain tumour). ——— and □——□: diagnosis corroborated by histopathology. * = statistically different ($P < 0.05$) from control value (**).

Acids L-arginine and L-ornithine led to weak responses with great individual variations. The responses were found to be significantly different ($P < 0.05$) at 30 min for arginine and at 90 min for ornithine when compared with saline injection, in the same dogs (Fig. 4). Intravenous administration of insulin to 6 dogs was followed by a drastic fall in blood glucose in all dogs and induced elevated GH levels at 30 min in some dogs. The mean values obtained did not differ significantly from basal levels.

A much higher and more consistent response was obtained when clonidine was administered iv (Fig. 5). Also here individual variation occurred, but there was in every case a response. The illustration (Fig. 5) also shows the response obtained in 3 hypopituitary dogs (1 pituitary dwarf and 2 dogs with histologically proven hypopituitarism). Clonidine failed to evoke a GH response in 2 dogs and evoked a very poor response in the third.
who used $[^{125}]$I GH tracer for up to 4 weeks after iodination. We also used tracer without repurification and straight line standard curves were obtained. But this often resulted in a loss of sensitivity in the lower range and therefore samples had to be re-assayed. GH tends to aggregate in neutral solution and at moderate concentrations dimers tend to form (Wallis & Davies 1976). Thus a loss of sensitivity should be expected. Canine GH dissolves easily at acidic pH below 6.5 or at basic pH between 8 and 10 (Wilhelmi 1968). Therefore, we chose to perform chromatography at a basic pH and to store $[^{125}]$I GH in a basic buffer. It is unknown whether changes in ionic strength and pH would prevent aggregate formation of $[^{125}]$I GH. The slight cross-reactivity obtained with purified prolactin may have been caused by a contamination of our GH preparation with prolactin or may have been caused by contamination of the prolactin preparation with GH. The parallel dose response obtained with pituitary GH, and plasma GH from normal as well as acromegalic patients, demonstrates the identical immunoreactivity of all samples. The GH concentrations we found in normal dogs are in good agreement with those found by Hampshire et al. (1975). The values among acromegalic dogs were variable. In acromegalic man GH levels vary appreciably from one individual to another and values ranging from 4 to 400 ng/ml have been reported (Besser et al. 1978).

Suitable stimulation tests for GH in the dog have been rarely reported. Insulin-induced hypoglycaemia, which has been shown to be one of the better stimuli in man (Reichlin 1974), has proven to be of rather limited use in our laboratory. Other investigators reported dogs which failed to respond to insulin injections (Tsushima et al. 1971; Hampshire et al. 1975). Likewise, arginine and ornithine injections have given inconsistent results. Hampshire et al. (1975) could not demonstrate a significant increase after arginine while Tsushima et al. (1971) found a prompt and significant increase after infusion of arginine. It seems difficult to explain these different findings, but it is likely that the dog responds in a much more inconsistent way to amino acid administration than does man (Reichlin 1974). We do not know whether factors, such as sex and age differences, could account for the divergent results obtained in dogs.

Clonidine, an effective antihypertensive drug, has been shown to be a potent agent for provocation of GH release in man (Gil-Ad et al. 1979) and in the dog (Scott et al. 1978). Its effect on GH secretion is believed to be mediated by central $\alpha$-adrenergic action (Gil-Ad et al. 1979; Gold et al. 1978). In preliminary experiments 30 $\mu$g/kg body weight resulted in adverse reactions such as dizziness or aggressive behaviour. Ten $\mu$g doses per kg body weight caused an almost identical increase in GH response without severe adverse effects. Additional studies to evaluate the usefulness of clonidine in clinical cases in dogs are desirable. The drug seems to be a powerful stimulus for GH release even when used at a low dose. The following conclusions may be drawn from this study: 1) GH iodination preparations of a specific activity as low as $\sim 30 \mu$Ci/$\mu$g are suitable for RIA; 2) $[^{125}]$I GH, stored frozen, becomes less suitable for RIA procedures, unless freshly purified for each assay; 3) iv amino acid amistration or insulin-induced hypoglycaemia cause only very inconsistent GH release in the dog. Prompt GH release is evoked by the administration of a low dose of clonidine.

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