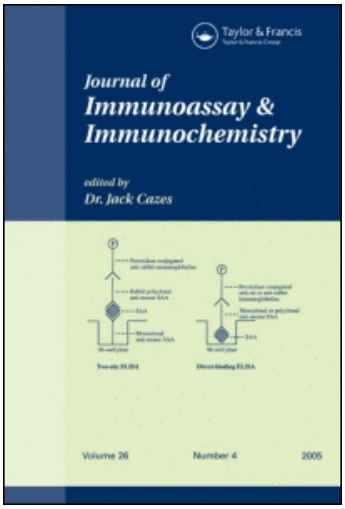

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A "CARBOXYL TERMINAL" CLINICAL RADIOIMMUNOASSAY FOR PARATHYROID HORMONE WITH APPARENT RECOGNITION PREFERENCE FOR THE INTACT HORMONE

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ABSTRACT

A radioimmunoassay for Parathyroid Hormone which is used in a clinical setting was characterized by (1) immunoreactivity with various synthetic fragments of the hormone, (2) serum parathyroid hormone response to oral calcium intake in normocalcemic calcium stone-formers, and (3) ability to detect fragments of parathyroid hormone secreted by abnormal human parathyroid tissue in vitro. Although almost all of the recognition sites for the antiserum were within the 53-84 carboxyl terminal amino acid sequence of the hormone, the radioimmunoassay mainly detected the "intact" hormone rather than the carboxyl terminal fragment(s) which most "carboxyl-terminal" assays of parathyroid hormone are claimed to preferentially detect. Differences in tertiary structure between the intact hormone and its fragments probably account for the relative inability of this antiserum to detect the carboxyl terminal fragment(s). (KEY WORDS: Parathyroid Hormone radioimmunoassay, PTH immunoheterogeneity).

INTRODUCTION

The radioimmunoassay (RIA) of Parathyroid Hormone (PTH) in a clinical setting has been made complicated by the immuno-heterogeneity of the circulating hormone, as first described by Berson and Yalow (1). The two main forms of PTH detected in plasma or serum by RIA are the

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intact, biologically active, 84 amino acid molecule (PTH 1-84) and biologically inactive carboxyl terminal fragment or fragments (C-PTH) of PTH 1-84 which appear to be present in much greater concentration than PTH 1-84 (2). This circulating C-PTH is secreted directly by the parathyroid gland (3,4) and is also formed by peripheral tissue metabolism of PTH 1-84 with subsequent release of C-PTH back into the circulation (5). Since PTH is a relatively small, linear peptide, with less than 20% of its structure in alpha-helix at physiological pH and at the slightly basic pH used in most RIA systems (6), one would expect most RIA's to have recognition sites based on amino acid sequences of different regions of the molecule. Antisera which recognize sequences in the carboxyl terminal portion of PTH will detect both C-PTH and PTH 1-84. However, the predominance of, and prolonged half-life of C-PTH in plasma, make C-PTH assays more useful as indicators of the chronic parathyroid secretory state, with clinical application in the detection of primary hyperparathyroidism (7,8). Antisera which recognize the amino terminus should have the ability to preferentially detect PTH 1-84, and be better able to detect acute changes in PTH secretion as has been shown by Arnaud et al (8). This is particularly true with respect to patients with renal failure, in whom the C-PTH levels reach extremely high levels, and effectively mask the detection of changes in PTH 1-84 by a carboxyl terminal specific RIA (C-RIA).

As so elegantly pointed out by Segre et al (9), most antisera used in animals injected with extracts of parathyroid tissue (containing PTH 1-84 and other PTH fragments), have a mixed population of antibodies, each

with its own specific binding of a region of the PTH molecule. A "carboxyl terminal" RIA antiserum would therefore have its largest population of antibodies capable of binding amino acid sequences in the carboxyl terminal portion of the PTH molecule.

Parthemore et al (10) described a C-RIA which was able to readily detect acute changes in PTH secretion. They were only able to use a PTH 53-84 fragment to characterize the carboxyl terminal recognition sites, but since they achieved nearly 100% displacement of 1-84 PTH tracer with this fragment, it is reasonable to assume the major antigenic recognition sites for their antiserum resided in the 53-84 region (carboxyl terminal) of the PTH molecule. They were able to detect changes in serum PTH in response to calcium or EDTA infusion which were similar to those reported by Arnaud (8) for his amino terminal specific antiserum.

We wish to report a clinical RIA for PTH which has almost all of its recognition sites in the carboxyl terminal one-quarter of the molecule, but which is able to detect acute changes in PTH in response to an oral calcium load in humans, and is relatively unable to detect C-PTH released by abnormal human parathyroid tissue in vitro.

METHODS

Radioimmunoassay. The guinea pig antiserum (GP-468) was raised by one of the authors (D.H.), in the laboratory of Dr. Louis M. Sherwood. Parathyroid TCA powder (Inolex, Glenwood, Ill.) was mixed with Freund's Adjuvant for immunization of the guinea pig using the method of

Vaitukaitis et al (11). Highly purified bovine PTH 1-84 (Inolex, lot #G009) was used for standards, and was also labelled with ^{125}I (chloramine T method) for use as tracer in all assays. The tracer was purified by passage over a 1.0 x 25 cm column of Ultrogel AcA 54 (LKB, Bromma, Sweden), using 0.1M sodium barbital pH 8.6 with 5% PTH-free human serum (charcoal absorbed) as elution buffer. The assay buffer was 0.01 M Veronal, 0.01 M EDTA pH 8.6, containing (1:5, v/v) PTH-free serum to minimize non-specific binding to the glass tubes. Serum samples were assayed in triplicate (0.25 ml/tube) with a final volume of 0.5 ml in each tube. GP-468 antiserum was used in a final dilution of 1:500,000. Non-equilibrium conditions at 4°C were used, with ^{125}I-PTH being added for 2 additional days after 4 days incubation with antibody. Separation of bound from free was by double antibody using goat anti-guinea pig gamma-globulin serum incubated at 4°C for one day. Tubes were centrifuged at 2,500 x g for 30 minutes and the precipitates counted in a Rackgamma II 1270 gamma counter (LKB-Wallac, Turku, Finland). The initial bound (Bo) counts divided by total counts (T) averaged 20% and non-specific binding (N) less than 5% of bound counts. Standards and unknowns were expressed as Bx-N/Bo-N; where Bo = maximal binding of ^{125}I-PTH, N = non-specific binding, and Bx = binding of ^{125}I-PTH in the presence of known or unknown amounts of immunoreactive PTH. A typical standard curve is shown by the displacement curve for PTH 1-84 in figure 1. Intra-assay and inter-assay coefficients of variation averaged 8 and 15% respectively. The range of values for serum from 60 normal subjects was up to 160 ng/L, with about 10 of such samples having

undetectable levels of PTH. Least detectable dose was 40 ng/L. For characterization of the antiserum the following peptides were used: bovine PTH 53-84 (kindly donated by Dr. H. Keutmann, Boston, MA); two sources of bovine PTH 1-84, which gave comparable standard curves (National Institutes of Health Standards, Bethesda, MD and Inolex, Glenwood, Ill.); human PTH 28-48 and human PTH 44-68 (Bachem, Torrance, CA), and human PTH 39-84 (Peninsula, Belmont, CA).

Patients. Patients with surgically proven primary hyperparathyroidism, patients on chronic hemodialysis, and patients with non-parathyroid hypercalcemia had serum PTH measurements performed.

During the assessment of patients with a history of calcium nephrolithiasis, 108 individuals were tested as outlined by Pak et al (12), with respect to their response to an oral calcium load. Serum was made available for PTH and total calcium, from blood samples drawn just before, and two hours after an oral intake of 25 mM (1 gram) elemental calcium in the form of a mixture of calcium lactate, gluconate, and carbonate (Calcium-Sandoz, Sandoz-Canada, Dorval, Quebec). Serum total calcium was measured as part of a SMAC-20 analysis (Technicon, Tarrytown, N.Y.). Statistical significance of changes in PTH and calcium was tested with Student's t test.

Analysis of Secreted PTH by Abnormal Human Parathyroid Tissue In Vitro. Fresh parathyroid adenomas were made available through the cooperation of the Department of Pathology, Foothills Hospital. This tissue was sliced into 1-2 mm^3 pieces and placed in a perifusion chamber as previously decribed for bovine tissue (4). During these experiments,

perifusion medium was collected continuously in 15 minute aliquots, for measurement of PTH release in response to various stimuli to PTH secretion (such as high or low calcium concentration) in the perifusion medium. 1.0 ml of perfusion medium was removed, made 6 M with respect to guanidine hydrochloride, and stored frozen at -70°C. Later, these samples of perifusion medium were applied to a 2.0 x 100 cm column of Ultrogel AcA 54. Elution buffer was 0.15M ammonium acetate, pH 4.5 with 10% human serum. Fractions of 2 ml were collected, lyophilized and re-constituted in the radioimmunoassay buffer described above. Radioimmunoassay of the PTH in the eluted fractions was carried out with GP-468 as described above, and also with the carboxyl terminal specific antiserum, GP-TCA5, used and characterized in earlier studies (4). This antiserum differed from GP-468, in that all of its recognition sites were in the 53-84 sequence of PTH, and it recognized that fragment on an equimolar basis with the PTH 1-84.

RESULTS

Characterization of GP-468, in a manner similar to that employed by Segre et al (9), is depicted in figure 1. The assay was highly sensitive for detection of the intact PTH 1-84, and although the PTH 53-84 fragment caused approximately 90% displacement of ^{125}I-PTH, it only did this at a concentration increased over PTH 1-84 by about 100-fold (50% displacement response for PTH 1-84 was seen at 7.4 x 10^{-15} M, compared with 1.6 x 10^{-12} M for PTH 53-84). The assay was more sensitive to PTH 39-84, a peptide which is probably quite similar to the

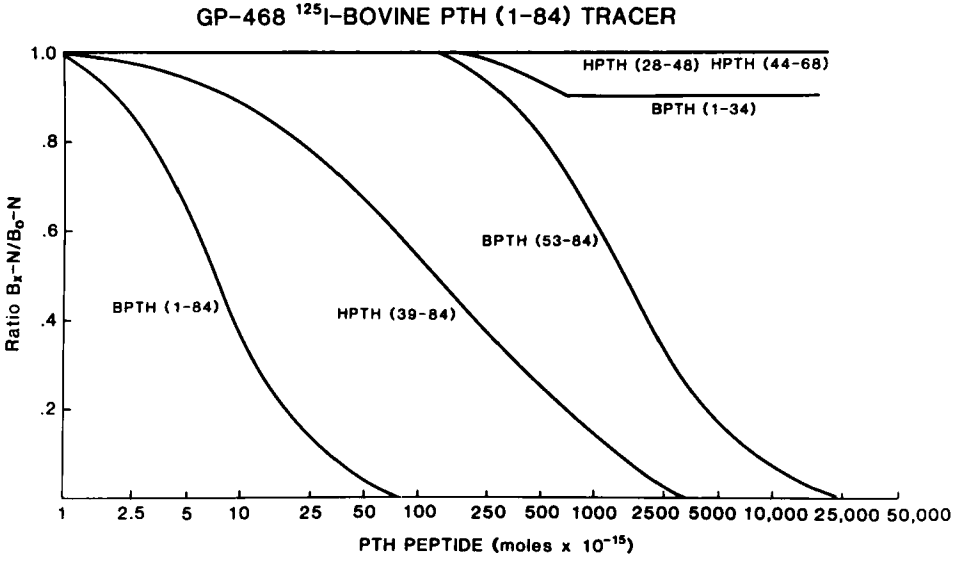

FIGURE 1. Characterization of GP-468. Using ^{125}I-bovine PTH 1-84 as radioligand, fragments of PTH were compared to PTH 1-84. Note the increased sensitivity of the assay system to the PTH 1-84 peptide compared to PTH 53-84 and PTH 39-84. The numbers on the abscissa, indicating moles of PTH peptide, are the actual dose levels of peptide used per assay tube. The letters "B" and "H" before PTH indicate bovine and human peptides, respectively.

circulating fragments of PTH, but again the 50% displacement response to PTH 39-84 was 1.2×10^{-13}, approximately 15-fold less sensitive than that for PTH 1-84. The mid-region PTH fragments, 28-48, and 44-68 were not capable of causing significant displacement of tracer. A small displacement of PTH 1-84 tracer was caused by the amino terminal

fragment of PTH, but only at a much higher concentration than the intact hormone.

Table 1 depicts the assay's performance in several clinical disorders of calcium metabolism. The patients with secondary hyperparathyroidism had been on chronic hemodialysis for an average of about 3 years, and blood samples for PTH measurement were drawn before dialysis. Of the 41 patients with surgically proven primary hyperparathyroidism, 11 had values in the upper end of the normal range (lowest value 124 ng/L).

Table 2 indicates that this RIA is capable of detecting an appropriate, statistically significant, decrease in PTH levels in response to a modest rise in serum calcium induced by an acute oral intake of calcium. Of the 130 patients tested, 101 showed a decrease in serum PTH in response to the oral calcium load.

The relative inability of this RIA to detect the synthetic carboxyl terminal fragments of PTH is also seen with respect to secreted C-PTH as shown in figure 2. RIA of eluted chromatography fractions, using the carboxyl-terminal specific antiserum GP-TCA5 (4), detected a large amount of C-PTH being released by the parathyroid adenoma in vitro. Although GP-468 is also a "carboxyl-terminal" RIA, its assay of the same chromatography fractions suggested that very little fragment, compared to intact PTH, was being released by the adenoma tissue. Although the C-PTH detected by GP-TCA5 in the perifusion medium has not yet been proven to be identical to the circulating fragments of PTH, it elutes in the same position as the C-PTH detected in serum from patients with chronic renal failure (data not shown).

TABLE 1

Serum Calcium and PTH in Patients with Primary and Secondary Hyperparathyroidism, and Non-Parathyroid Hypercalcemia

	N	Ca (mM/L)	PTH (ng/L)
Primary Hyperparathyroidism	41	2.92 ± 0.04	227 ± 14.4
Chronic Hemodialysis (Secondary Hyperparathyroidism)	52	2.52 ± 0.03	634 ± 43.7
Non-Parathyroid Hypercalcemia*	10	2.91 ± 0.07	49 ± 15.5

Values for Ca and PTH measurements are Mean ± Standard Error of the mean.

* 8 patients with hypercalcemia of malignancy, 1 patient with milk-alkali syndrome and 1 patient with sarcoidosis.

TABLE 2

Serum Calcium and PTH Response to the Acute Ingestion of 25 mM Calcium in 130 Normocalcemic Patients with Calcium Nephrolithiasis

	Pre-Dose	2 Hours Post-Dose	Normal Value
Serum Ca (mM/L)	2.42±.01	2.55±.01*	2.12 - 2.62
Serum PTH (ng/L)	87±4	67±3*	< 40 - 160

Values for Ca and PTH measurements are Mean ± Standard Error of the Mean.

* Significant change, $p < 0.01$

DISCUSSION

The characterization of this antiserum (GP-468) using synthetic fragments of PTH places it in the "carboxyl terminal" category. However, in common with the radioimmunoassay reported by Parthemore et al (10), we have demonstrated that our radioimmunoassay detects acute changes in PTH secretion induced by small increases in serum calcium

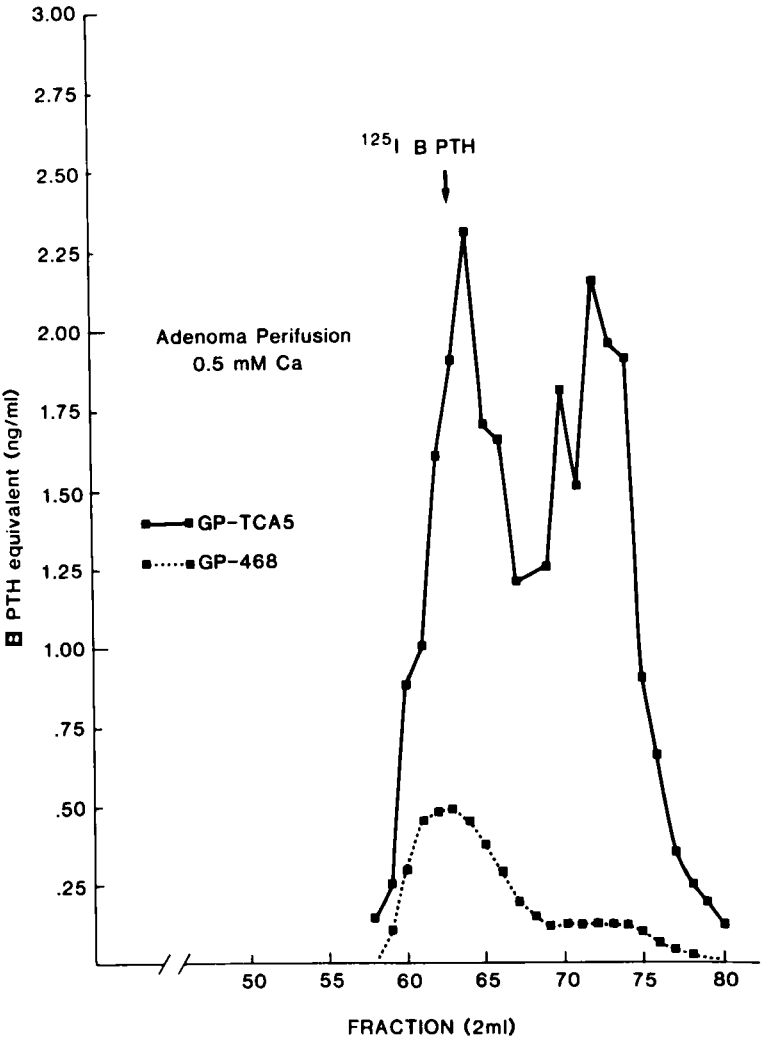

FIGURE 2. AcA 54 Ultrogel chromatography of perifusion medium from human parathyroid adenoma tissue exposed to 0.5 mM calcium (low calcium). Note the large amount of immunoreactive material in a second peak eluting later than intact PTH as detected by GP-TCA5 (solid line). GP-468 (dotted line) primarily detects a peak of immunoreactivity eluting in a position similar to ^{125}I-bovine PTH 1-84.

concentration (Table 2). When GP-468 is compared with another C-RIA in analysis of PTH released from human parathyroid tissue (figure 2), it becomes clear that the recognition preference for PTH 1-84 over the synthetic carboxyl terminal fragments, also holds for the "intact" PTH vs. C-PTH released by abnormal human parathyroid tissue in vitro. The overall levels of PTH measured by GP-468 (as bovine PTH equivalents), are much lower than those for GP-TCA5, both for in vitro studies as in figure 2, and when GP-TCA5 is used as a clinical RIA (13). Most of the range of normal values for serum PTH as measured by GP-468 is below the detection limit (150 ng/L) of GP-TCA5 (13). However, the normal range of PTH, as measured by GP-468 is still well above that presumed to represent circulating, biologically active, intact PTH as measured by the cytochemical bioassay (14). It is likely that the GP-468 RIA primarily detects a PTH immunoreactivity in serum which still has a significant C-PTH component, but has proportionally much more PTH 1-84 than is measured in other C-RIA's.

The isolated measurements of PTH in various clinical disorders of calcium metabolism (Table 1) are similar to most reported clinical PTH RIA's.

The finding of suppressibility of PTH in response to an acute oral calcium intake which increased the serum calcium (still within the range of normal values) is indicative of the assay's sensitivity to changes in PTH secretion during studies of calcium physiology. In contrast, more traditional C-RIA's are less consistent in showing a response to dietary calcium or calcium infusions (8,13). For example, GP-TCA5 as a clinical

RIA was able to show significantly lower levels of PTH for stone formers, but when these same patients were given a low calcium diet, the slight increase in PTH seen was not significant (13). In surveying the literature describing the use of the oral calcium load test, usually only urinary cyclic AMP excretion is reported as the indicator of parathyroid hormone secretion during the 4 hour test period. When serum PTH has been measured during an oral calcium loading test, typically no significant changes in serum PTH are found (15).

The observations in this paper are also consistent with the recent studies of Marx et al (16), who demonstrated the relative inability of a C-RIA to detect secreted fragments of PTH in parathyroid venous effluent. In contrast, their "mid-region" assay, which showed preferential recognition of determinants within the 44-68 region of the PTH molecule, detected large amounts of C-PTH being secreted by the parathyroid glands. They suggest these differences in recognition capabilities of the antisera being tested were due to differences in tertiary structure between 84-PTH and the PTH fragments. For a review of PTH tertiary structure, the reader is referred to the excellent paper by Cohn and MacGregor (6). It is likely that GP-468 recognizes a region in the carboxyl terminal portion of PTH which is partially "hidden" when PTH 1-84 is cleaved to C-PTH, resulting in a relative inability of this RIA to recognize the C-PTH fragment(s). As Marx et al suggest (16), RIA's which predominantly detect C-PTH, probably have their major recognition sites within the region comprising amino acids 44 through 68. This region was not "seen" by GP-468 (figure 1).

The results presented here, and those of Marx et al, confirm the suggestion of Parthemore et al (10) that characterization of PTH antisera by response to fragments of the hormone does not take into account the immunoreactivity of the tertiary structure of PTH in the circulation. The GP-468 RIA is clearly carboxyl terminal in its specificity, yet has an ability to detect acute changes in PTH in serum and a relative inability to detect PTH fragments; two features which would not be expected based on the early characterization studies of Arnaud et al (8). However, as Arnaud's paper clearly points out, characterization must also be based on clinical performance of the assay.

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