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Isolation of Circulating Immunoreactive Human Parathyroid Hormone: Comparison with a 1-34 N-Terminal Synthetic Fragment (40029)

C. DESPLAN, A. JULLIENNE, M. S. MOUKHTAR, D. RAULAIS, P. RIVAILLE, AND G. MILHAUD

Unité 113, INSERM, hôpital Saint Antoine, 184 rue du Faubourg Saint Antoine, 75012 Paris, France

The sequence of the 1-34 amino terminal biologically active fragment of human parathyroid hormone (hPTH) is not unequivocally elucidated as three residues are controversial (1, 2). Immunochemical studies involving these two sequences have yielded somewhat conflicting results. Fisher *et al.* (3), using antibodies raised against extracted hPTH, showed a complete cross-reaction between extracted hPTH and the 1-34 N-terminal fragment synthesized according to the structure proposed by Brewer *et al.* (1-34 hPTH B) (1). Segre and Potts (4), using several different heterologous antibodies raised against bovine parathyroid hormone, showed that 1-34 fragment synthesized according to the sequence proposed by Niall *et al.* (2) (1-34 hPTH N) and highly purified hPTH displaced the bovine PTH tracer in an identical manner. In their system, low or no displacement was observed with 1-34 hPTH B.

We have shown (5) that extracted hPTH is immunochemically identical to 1-34 hPTH N, using a homologous radioimmunoassay specific for this peptide. This radioimmunoassay could eventually be used for the measurement of hPTH present in tissues or plasmas. It was however essential to compare 1-34 hPTH N with circulating hPTH, preferably normal. Therefore we have extracted hPTH from normal plasma by affinity chromatography and compared its immunochemical properties with the synthetic peptide. Simultaneous comparisons were also carried out with extracted hPTH, with Quso extracts of adenoma cultures and with plasma obtained from hyperparathyroid patients.

Materials and methods. Homologous RIA for 1-34 hPTH N. A sensitive radioimmunoassay (7 pg per tube) was developed using antibodies raised in the goat against the 1-34 hPTH N. The synthetic peptide (5) was labeled with ^{125}I using the chloramine T method (6). The labeled tracer was purified by Quso G.32 (7) and by chromatography on Sephadex G-25 (Pharmacia). Fifty picograms of the tracer was incubated in barbital buffer, pH 8.6, 0.05 *M*, containing 17% (by volume) of normal human plasma to minimize adsorption of hormone, sodium azide (0.2%), and antibody G10/017 at a final dilution of 1/250,000 and increasing amounts of unlabeled peptide (0.008-16 ng). All preparations tested were lyophilized and dissolved in the standard buffer, and multiple dilutions were added in the radioimmunoassay. Final incubation volume was 500 μl and incubation was carried out for 7 days at 4°. Separation of bound and free tracer was achieved by dextran-coated charcoal (8).

Affinity chromatography. Affinity chromatography was performed using either antibodies against the 1-34 hPTH N (column 1) or antibodies against bovine PTH (column 2). The two types of antibodies were selected for their high cross reaction with hPTH. Coupling to Sepharose 4B (Pharmacia) was performed by the cyanogen bromide technique (9) according to the procedure we have used for anticalcitonin antibodies (10). At the end of coupling, unreacted material was removed by several alternate washes of standard buffer and acetic acid 1 *M*. The coupled antibodies were finally resuspended in the standard buffer and packed in two small chromatographic columns (Ø: 0.9 cm, L: 15 cm). Specificity of the affinity columns was established by the specific binding of labeled 1-34 hPTH N (column 1) or labeled bovine PTH (column 2), and by the absence of binding of unrelated labeled peptides such as porcine, salmon or human calcitonin. Nonspecific binding never exceeded 5%. The antibovine antibodies used in column 2 were predomi-

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nantly C-terminal as they did not retain the labeled 1-34 hPTH N.

Plasma extracts. Aliquots (300 ml) of normal plasma were passed on each column; after a distilled-water wash, fixed immunoreactive material was eluted using 1 *M* acetic acid. In order to minimize the adsorption of immunoreactive material, effluents of the columns were directly collected on vessels cooled by dry-ice. The acid extracts obtained from both columns were separately freeze-dried and dissolved in 10 ml of distilled water; the purification factor was 300. Affinity chromatography (column 1) was also performed on plasma samples obtained from a totally parathyroidectomized patient (PTX) and a hyperparathyroid subject.

Tissue extracts. Human parathyroid adenomas obtained by surgery and immediately frozen in dry ice were pooled, defatted, and extracted by the phenol-TCA method (11). Quso extracts of adenoma culture medium were obtained from the British Medical Research Council. In addition, extracts of human kidney and muscle were prepared. Protein contents of the extracts were determined by the Folin-Wu technique.

Statistical treatment of data. The ratio of bound tracer to bound tracer at O dose of cold hormone (B/Bo) was computed for each dose level. Radioimmunoassay curves were linearised using the arc Sine transform of $\sqrt{B/Bo}$ and the log of the dose. Regression curves were then performed using the method of least squares.

Results. Figure 1 shows that whether immunoreactive material present in normal plasma is extracted by antibodies directed towards the 1-34 hPTH N column 1 (N terminal) or bPTH (column 2) multiple dilutions of the two extracts are superimposable on the standard curve. The only difference is that total amount of immunoreactive material extracted by column 1 (anti 1-34 hPTH N) was four times higher for the same sample of normal plasma than the material extracted by column 2 (anti bPTH).

Concentration by affinity column of nonspecific factors interfering with the assay could be excluded as the extract of 30 ml of

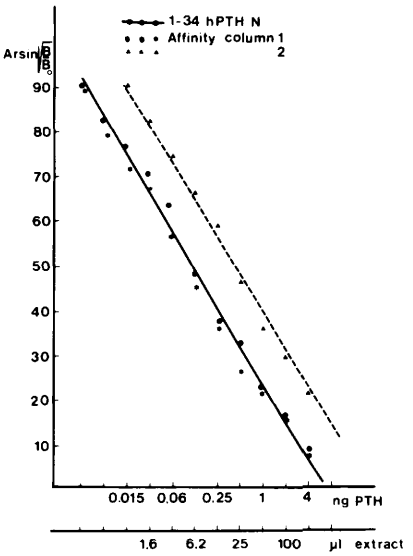

FIG. 1. Cross reaction of hPTH extracted from normal plasma by affinity column anti 1-34 hPTH N (★) or anti bPTH (▲), and 1-34 hPTH N (●) in a homologous radioimmunoassay: antiserum goat 10/017 1/250,000 anti 1-34 hPTH N, ^{125}I labelled I-34 hPTH as tracer.

PTX plasma was unable to displace the tracer; in contrast the extract of a hyperparathyroid plasma (30 ml) shows total cross-reaction (Fig. 2). In addition, multiple dilutions of a plasma obtained from a hyperparathyroid patient (Fig. 2) as well as extracts of adenomas or Quso extract of adenoma culture medium (Fig. 3) were superimposable on the standard curve. On the other hand, no displacement of the tracer was observed with extracts of muscle or kidney, even at concentration 10^4-fold higher than the adenoma extract.

Discussion. The total cross-reaction observed with hPTH extracted from adenoma or tissue culture medium of human adenoma and the 1-34 hPTH N using a homologous assay for this fragment suggests that the natural hormone or its N-terminal fragments share common antigenic sites with the peptide synthesized according to the sequence proposed by Niall *et al.* (2). Such common antigenic sites are also present in the hormone or its fragments circulating in hyperparathyroid plasma. In the case of normal plasma, concentration of immunoreactive material was a necessary prerequisite before carrying out cross reaction stud-

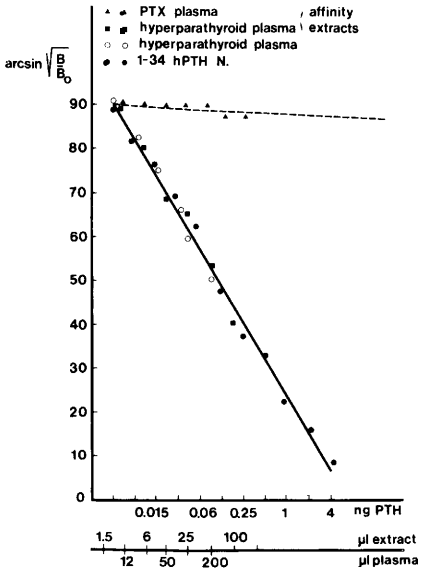

FIG. 2. Displacement of labelled 1-34 hPTH N by hyperparathyroid plasma (○), extracts from plasma of hyperparathyroid patient (■) or from plasma of a parathyroidectomized subject (▲) and 1-34 hPTH N (●) in a homologous R.I.A. anti 1-34 hPTH N.

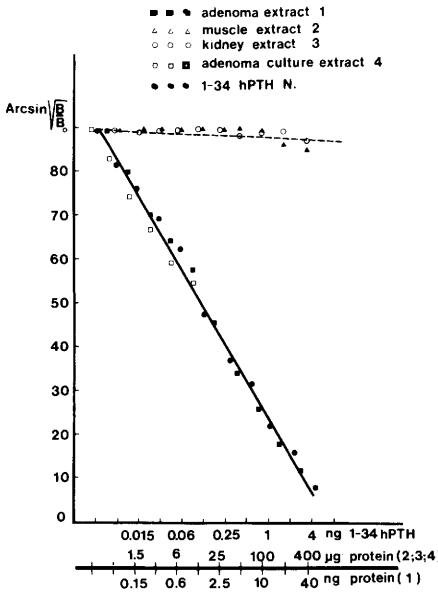

FIG. 3. Cross reaction of extracts from adenoma (■), adenoma culture medium (□), muscle (△) or kidney (○) and 1-34 hPTH N (●) in a homologous R.I.A: anti 1-34 hPTH N.

ies due to the low amount of immunoreactive hormone present. Extraction by column 1 (anti 1-34 hPTH N) shows that in the case of normal plasma, hPTH or some of its fragments are immunochemically identical to fragment 1-34 hPTH N. Immunoreactive material present in this extract could comprise 1-34 sequence of either smaller or larger peptides involving the N terminal antigenic site. Column 2 does not retain molecules $\leqslant$ 1-34 sequence and thus the antibodies coupled are presumably directed towards mid or C terminal regions of the molecule or its circulating fragments, and the extract tested was therefore devoid of molecules $\leqslant$ 1-34. The result of the radioimmunoassay of this extract and the total cross reaction observed between this extract and the 1-34 hPTH N demonstrates the presence in normal plasma of peptides > than 1-34 and intact molecules. This implies that radioimmunoassay using antibody G10/17 will measure all the biologically active fragments present in peripheral plasma and probably certain biologically inactive molecules in which N terminal amino acids may be missing. As we have already pointed out, extraction by affinity chromatography is not total as fragments of hPTH or even the intact hormone in which the antigenic sites are either masked or lacking will escape fixation. Notwithstanding these limitations, the affinity chromatography is usefull for the concentration and purification of immunoreactive species present in blood as well as for obtaining plasma free from immunoreactive material. We now use such plasma for minimising adsorption of the hormone instead of normal plasma in the standard assay.

Our results further substantiate the studies of Segre and Potts (4) which showed with heterologous radioimmunoassays, identical immunochemical reaction of 1-34 hPTH N and 1-84 hPTH. In addition our results extend this identity to hPTH or its fragments present in normal or hyperparathyroid plasma. Our findings do not at present disagree with those obtained by Fisher *et al.* (3) i.e., the immunochemical similarity of 1-34 hPTH B and 1-84 hPTH, as the antiserum used shows a high cross reaction with 1-34 hPTH B synthesized in our laboratory (12). Furthermore Fisher *et al.* (3) did not study the cross reaction of 1-34 hPTH N in their radioimmunoassay sys-

tems, and thus their antibodies like ours could have been directed toward common sequences of the two proposed structures.

The results obtained in this study imply that hPTH or its N terminal fragments present in parathyroid gland or in blood share in their N terminal region a high degree of similarity in their tertiary structure with the sequence proposed by Niall *et al.* (2). Though the exact structure of the 1-34 hPTH is still debated, the results reported here indicate that certain antibodies raised against the 1-34 hPTH N can be used in establishing radioimmunoassays specific for circulating hPTH or its fragments. Preliminary results using this radioimmunoassay show that immunoreactive hPTH can be measured in normal plasma in 65% of cases, and that a good discrimination between normal and hyperparathyroid plasmas is observed (13).

Summary. Immunoreactive human parathyroid hormone (hPTH) was isolated from normal plasma by Sepharose bound antibodies raised against either the synthetic 1-34 fragment of hPTH (Niall sequence) or bovine PTH. The isolated material was compared with extracted hPTH, Quso extracts of adenoma cultures, hyperparathyroid plasma and the synthetic fragment using a radioimmunoassay homologous for this fragment. Complete cross reaction of all preparations studied was observed in this system, indicating that immunoreactive material separated from normal plasma by this method was identical with the synthetic peptide and implying that hPTH or its fragments present in normal and hyperparathyroid plasma, in tissue extracts or released in culture medium share common tertiary structures with the 1-34 peptide, synthetized according to the structure proposed by Niall *et al.*

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