Differential Diagnosis of Hypercalcemia by Measurement of Parathyroid Hormone, Calcitonin, and 25-hydroxy-vitamin D

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We have measured the blood levels of parathyroid hormone (PTH), calcitonin (CT), and 25-hydroxy-vitamin D (25-OH-D) in 24 cases with hypercalcemia from various causes, and have estimated the usefulness of the assays of these calcium-regulating hormones in differentiating the hypercalcemic conditions. In ten cases with primary hyperparathyroidism, PTH levels were markedly elevated. On the other hand, CT levels were within normal range except in one case with complicating renal failure. In seven cases with bone metastases from primary neoplasms, PTH levels were characteristically low or normal, presumably being suppressed by hypercalcemia, whereas CT levels were higher than normal. Four patients with remote malignant tumors produced a PTH-like substance with resulting hypercalcemia. In these cases, not only PTH but also CT levels were markedly raised, but 25-OH-D levels were within the normal range. From the tissues of the primary tumors, a PTH-like substance was identified, but not CT. In one case with hypercalcemic multiple myeloma, the PTH, CT, 25-OH-D, and ionized calcium levels all fell within the normal range. This elevated level of serum calcium was thought to be due to a large amount of myeloma protein, which could bind additional calcium. Two cases with vitamin-D intoxication were easily differentiated by extremely high 25-OH-D levels coupled with normal PTH and CT levels.

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Hypercalcemia occurs in primary hyperparathyroidism, in the neoplastic invasion of bone, in the production of parathyroid-hormone-like substance by an isolated neoplasm, and in sarcoidosis, multiple myeloma and vitamin-D intoxication.

Differential diagnosis of hypercalcemia is essential since the treatment consists of control of the primary disease. We have measured the blood levels of parathyroid hormone (PTH), calcitonin (CT), and 25-hydroxy-vitamin D (25-OH-D) in 24 cases with various causes of hypercalcemia, and have estimated the usefulness of the assays of these calcium regulating hormones in differentiating the hypercalcemic conditions.

MATERIALS AND METHODS

Basal plasma levels for PTH, CT, and 25-OH-D were measured in 24 patients with hypercalcemia, involving ten cases with histologically proven primary hyperparathyroidism (eight cases with parathyroid adenoma and two with hyperplasia), two cases with vitamin-D intoxication caused by ingestion of excessive amounts of vitamin D_2, and 12 cases with malignant diseases: six with bone metastases from primary neoplasms in the lung, kidney, ureter, and breast, four with ectopic PTH syndrome, one case with hepatoma without bone involvement, and one case with multiple myeloma. Serum calcium concentration in these cases ranged from 11.0 to 15.3 mg/

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TABLE 1. PROTOCOL FOR RADIOIMMUNOASSAY FOR PTH

Procedure		Volume (μl)
Bovine PTH standard or unknown serum		100
Antiserum		100
Hypoparathyroid serum		0 or 100
Assay buffer		100 or 200
	Total	400
Incubate at 4°C for 3 days; add tracer.		100
	Total	500
Incubate at 4°C for 2 days; add dextran-coated charcoal.		500
	Total	1,000
Shake and stand for 20 min; centrifuge at 3,000 rpm for 20 min; count radioactivity in supernatant and precipitate.		

100 ml. The urea nitrogen and the creatinine were normal in all patients except one with primary hyperparathyroidism, in which the urea nitrogen was 40 mg/100 ml and the creatinine 2.5 mg/100 ml.

Serum calcium, urea nitrogen, and alkaline phosphatase levels were estimated by an automated analyzer. Serum creatinine level was determined by Jaffe's reaction. Normal range for serum calcium was 8.5–10.5 mg/100 ml, urea nitrogen 8–20 mg/100 ml, alkaline phosphatase 30–85 mU/ml, and serum creatinine 0.8–1.7 mg/100 ml. Ionized calcium was measured by a calcium ion analyzer. Normal values were 1.85–2.25 mEq/l. Radioimmunoassay of CT was performed using synthetic human CT M and its goat antibody (*1*). The assay was sensitive to 0.1 ng/ml.

Radioimmunoassay of PTH. We used a commercially available guinea-pig antibovine PTH serum. Highly purified bovine PTH (1,200 units/mg) was used to prepare iodinated tracer and for the standards in the assay system. Iodination with I-125 was carried out by the chloramine-T method of Hunter and Greenwood (*2*), and the iodinated PTH was purified with silica* according to the method of Yalow and Berson (*3*), and stored at −20°C. The labeled tracer was repurified by gel filtration on Biogel P-10 before use. The assay protocol is given in Table 1. Serial dilutions of bovine PTH containing 0–5,000 pg were made in 100 μl of assay buffer (10% hypoparathyroid serum, 0.05 *M* barbital buffer, pH 8.6). To these or to 100 μl of test serum were added 100 μl of diluted antiserum (at final dilution of 1:100,000) and 100 or 200 μl of the assay buffer. To equalize the protein concentrations in all standards and samples, 100 μl of hypoparathyroid serum were added to the standard tubes. Tubes containing the labeled hormone without antiserum served as controls with each standard curve.

The assays were preincubated at 4°C for 3 days without the labeled hormone. After addition of the tracer, the reaction mixture was incubated for an additional 2 days before separation. Phase separation was performed by the method of adsorption with dextran-coated charcoal (*4*). The reaction tubes were equilibrated at 4°C for 1 hr after addition of 500 μl of dextran-coated charcoal suspension. Tubes were then centrifuged for 20 min at 3,000 rpm, separated by decantation, and counted in an automated counter for a period sufficient to yield a counting error of less than 1%.

Assay of 25-OH-D. Competitive binding assay of 25-OH-D was performed by the method of Belsey et al. (*5*) with a minor modification. The 25-OH-D_3† diluted with absolute ethanol was used for the standards. The standard was stable for several months when kept 4°C. For the tracer, [23,24-^{3}H]25-OH-D_3‡ in absolute ethanol was used. The purity of radioactive material was checked before use by thin-layer chromatography on silica gel in ethyl acetate/n-hexane (1:6 or 1:1).

As a protein for the competitive binding assay, we used whole sera from Wistar rats fed a vitamin D-

TABLE 2. PROTOCOL FOR RADIOASSAY FOR 25-OH-D

Procedure		Volume (μl)
Unknown serum		250
Absolute ethanol		1,000
	Total	1,250
Shake and stand for 30 min; centrifuge at 3,000 rpm for 15 min.		
Supernatant or crystaline 25-OH-D_3 in 75% ethanol.		100
[23,24-^{3}H]25-OH-D_3 in absolute ethanol (100 pg).		50
0.01% β-lipoprotein in assay buffer.		500
	Total	650
Shake and add vitamin-D-deficient rat serum (1:1,000).		500
	Total	1,150
Incubate at 4°C for 30 min; add dextran-coated charcoal.		500
	Total	1,650
Shake and stand for 40 min; centrifuge at 3,000 rpm 4°C 15 min; count radioactivity in 1,000 μl of supernatant.		

deficient diet for 4 weeks. The binding protein was diluted 1:1,000 with barbital buffer, pH 8.6. The assay procedure is shown in Table 2. Test serum, 250 μl, was extracted with 1 ml of absolute ethanol. To the tubes containing 50 μl of labeled 25-OH-D_3 (about 100 pg), were added 500 μl of 0.01% β-lipoprotein in barbital buffer, 500 μl of binding protein, and 100 μl of aliquots of the extract or of crystalline 25-OH-D_3 standards (0 to 1,000 ng) in 75% ethanol. They were then mixed in a Vortex mixer and incubated at 4°C for 30 min. At the end of the incubation, 500 μl of dextran-coated charcoal suspension were added to each tube, mixed thoroughly, and left for 20 min. One-milliliter aliquots of the supernatant were transferred to vials with 10 ml of Bray's solution§ and counted in a liquid-scintillation counter with automatic external standardization. The efficacy of extraction was assessed in each assay using labeled 25-OH-D_3. The extraction of test serum with ethanol had a recovery rate of 85 ± 1% (mean ± s.e.) and was found to be independent of the test serum and its 25-OH-D_3 concentration.

Nonspecific binding of labeled 25-OH-D_3 in the absence of binding protein was assessed for standards and for serum extracts in each assay.

Extraction of tumor PTH and CT. Twelve patients with malignant tumors were evaluated for the ectopic PTH and/or CT production.

The surgically removed tumors, along with control tissue, were frozen on dry ice within 5 min after removal. The specimens were homogenized in a Waring blender at 4°C for 30–60 sec with 10 ml of 0.1 *N* hydrochloric acid/g. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The opalescent supernatant was refrigerated until assayed for PTH and CT (*6*).

The recovery of the extraction, checked by labeled PTH and CT, was 40% for PTH and 30% for CT. The extracts, both tumor and control, were assayed directly by radioimmunoassay in multiple dilutions: 1–100 μl of extract; 10 μg to 1 mg of protein. Control assays of the hydrochloric acid extracting buffer alone showed no reactivity or interference with the assay. Protein concentrations in the tissue extracts were determined by the method of Lowry (*7*).

Bone scintigraphy. Bone scintigraphy was performed 3 hr after the i.v. injection of 7–15 mCi of Tc-99m HEDP, the dose being adjusted according to body weight. AP and PA whole-body skeletal images were obtained with a dual whole-body scanner with 3.5-in., low-energy collimators, and/or a gamma camera with a high-resolution, medium-sensitivity collimator.

Evaluation of methods. Figure 1 illustrates a typical standard curve for the radioimmunoassay for

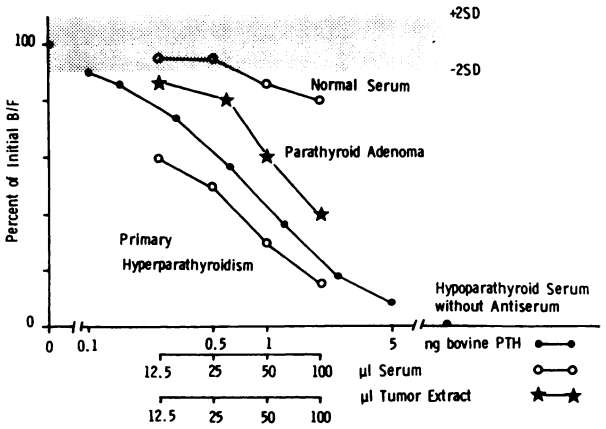

FIG. 1. Representative standard curve of bovine PTH (solid circles) and dilution curves of serum (open circles) from a patient with primary hyperparathyroidism and a normal subject, and extract from a parathyroid adenoma (asterisks).

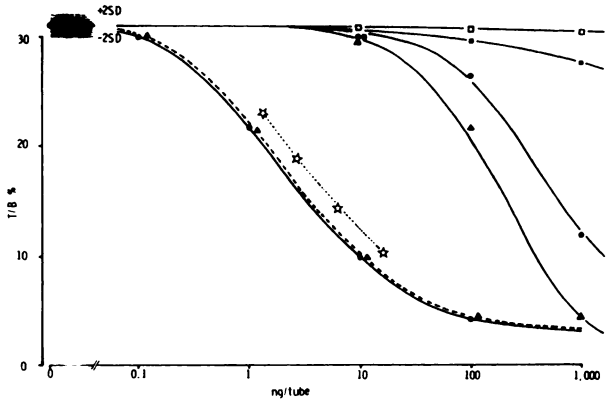

FIG. 2. Representative standard curve of 25-OH-D_3 (solid circles) and a dilution curve for serum from a patient taking large doses of D_2 (asterisks). Displacement curves for D_3 analogs: 24,25-$(OH)_2$-D_3 (solid triangles); 1,25-$(OH)_2$-D_3 (open circles); 1α-OH-D_3 (solid squares); D_3 (open triangles); and cholesterol (open squares).

PTH. The assay system was sensitive to 100 pg/ml of bovine PTH, at which percentage the binding of the tracer by antibody was significantly lower ($p < 0.05$, Student's t-test) than that at the zero point of the standard curve. About 70% of normal fasting subjects had detectable PTH concentrations ranging from 100 to 800 pg/ml. The interassay coefficient of variation in ten assays was 13.0% for pooled hyperparathyroid serum, assayed to 4.2 ng/ml. The intra-assay coefficient of variation of the pooled hyperparathyroid serum in 20 consecutive assays was 9.4%. As shown in Fig. 1, sera from a normal subject, a patient with hyperparathyroidism, and an aliquot of extract from a parathyroid adenoma gave curves parallel to the standard curve (-●-●-) upon serial dilution. Figure 2 shows a representative standard curve for 25-OH-D radioassay. The de-

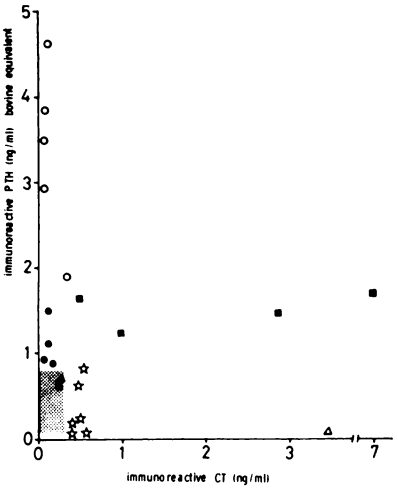

FIG. 3. Relation between plasma immunoreactive PTH and CT levels in hypercalcemia. In primary hyperparathyroidism: bone type (open circles) and stone type (solid circles). In malignant tumor: with bone metastases (asterisks), ectopic PTH syndrome (squares), hepatoma with neither bone metastases nor ectopic PTH syndrome (open triangle), and multiple myeloma (closed triangle). Stippled area represents normal range.

tection limit, which was defined as the point 2 s.d. below the zero point, was 0.1 ng/tube. Plasma levels of 25-OH-D were estimated in 20 normal healthy individuals. The normal range of 25-OH-D was 20–60 ng/ml. Figure 2 also shows displacement curves for the natural metabolites of vitamin D. Cross reactivity of 24,25-$(OH)_2$-D_3 with the binding protein was 100% at 50% displacement of the labeled 25-OH-D_3; that of D_3 was 1.9%; and that of 1,25-$(OH)_2$-D_3 was 0.7%. Cholesterol and 1α-OH-D_3 were much less potent in displacing labeled 25-OH-D_3. If 24,25-$(OH)_2$-D_3 or 1,25-$(OH)_2$-D_3 are considered to be a problem, they must be separated from 25-OH-D_3 by chromatography. Serum from a patient with vitamin-D intoxication gave a curve parallel to the standard curve upon serial dilution (Fig. 2). The coefficient variation within assay was 5% for pooled serum in ten assays containing 30 ng/ml. The interassay coefficient of variation for the pooled serum in ten consecutive assays was 11%.

RESULTS

Figure 3 shows the relation between plasma immunoreactive PTH and CT levels in ten cases with primary hyperparathyroidism and 12 cases with malignant tumors.

Among those with primary hyperparathyroidism, there were five cases with prominent bone resorption in which immunoreactive PTH levels were markedly elevated. In the other five cases with renal stones and no overt bone resorption, plasma immunoreactive PTH levels were distributed from normal into a slightly higher range. Plasma immunoreactive CT levels were within the normal range, except in one patient who developed renal failure. No significant differences were seen between parathyroid adenoma and hyperplasia in terms of the plasma values of immunoreactive PTH and CT. Bone scintigrams showed high uptake of Tc-99m HEDP within the entire skeleton in all five cases with prominent bone resorption. In the other five cases, however, with renal stones, bone scintigrams were read as normal.

In six hypercalcemic cases with bone metastases from primary neoplasms in the lung, kidney, ureter, and breast, plasma immunoreactive PTH levels were characteristically low except in two cases with relatively high normal PTH, whereas plasma immunoreactive CT levels were distributed into a higher range. In addition, neither immunoreactive PTH nor CT were extracted from tumor tissues. Bone scintigraphy revealed multiple metastatic lesions, including the ribs, vertebrae, and pelvis.

There were four cases with malignant tumors that produced a PTH-like substance with resulting pseudohyperparathyroidism (Table 3). Surgical speci-

TABLE 3. SERUM BIOCHEMISTRY AND IMMUNOREACTIVE PTH AND CT IN TUMOR EXTRACTS IN ECTOPIC PTH SYNDROME

	Serum biochemistry			Tumor tissue	
	Calcium (mg/100 ml)	Phosphate (mg/100 ml)	Alkaline phosphatase (mU/ml)	Immuno-reactive PTH (ng/wet g)	Immuno-reactive CT (ng/wet g)
1. Renal-cell carcinoma	12.7	2.7	182	20	N.D.*
2. Squamous-cell carcinoma of lung	13.2	3.3	118	15	N.D.
3. Oat-cell carcinoma of lung	15.1	2.4	86	100	N.D.
4. Bronchogenic squamous-cell carcinoma of neck	12.3	2.0	132	20	N.D.

* N.D. = nondetectable.

mens of the tumors, extracted with acid, were found to contain abnormally elevated concentrations of PTH. The extracts gave dose-response curves superimposable on those of bovine PTH. No immunoreactive CT was detected in these extracts. Adjacent control samples—e.g., from the kidney, lung, and muscle not involved by the tumor—did not have demonstrable PTH or CT. As shown in Fig. 3, these four cases with the ectopic PTH syndrome showed high plasma levels of both immunoreactive PTH and CT. Bone scintigrams were read as normal in these cases. In spite of the high PTH levels, no increased uptake of Tc-99m HEDP by the skeleton was seen.

In one case of hepatoma, with serum calcium concentration of 15.3 mg/100 ml, phosphate of 2.5 mg/100 ml, and alkaline phosphatase of 177 mU/ml, the plasma immunoreactive PTH level was too low to be detected by the assay, whereas the CT level was markedly raised. This pattern was similar to that seen in the hypercalcemic cases with bone metastases. However, no metastatic bone lesions were identified, either by repeated scintigraphic bone survey or at autopsy. In addition, neither immunoreactive PTH nor CT was identified in the hepatoma tissue.

In one case with hypercalcemic multiple myeloma, serum calcium concentration was 14.8 mg/100 ml, phosphate was 4.8 mg/100 ml, and alkaline phosphatase was 82 mU/ml. No clinical symptoms of hypercalcemia were recognized. Ionized calcium concentration in the serum was 2.20 mEq/l, which is within the normal range. Plasma immunoreactive PTH level was 0.7 ng/ml, CT was 0.2 ng/ml, and 25-OH-D was 20.0 ng/ml, all within the normal range. Total serum protein was 10.2 g/100 ml and immunoglobulin G was markedly increased to 5.7 g/100 ml.

Figure 4 shows the distribution of plasma 25-OH-D levels in the hypercalcemic cases. In most of the cases with primary hyperparathyroidism, 25-OH-D levels were distributed within the normal range. In seven of the 12 cases with malignant tumors, with or without bone metastases, 25-OH-D levels were lower than normal, probably due to malnutrition. Two cases with vitamin-D intoxication showed the extremely high 25-OH-D levels. Bone scintigrams showed a markedly increased uptake of the nuclide within the entire skeleton.

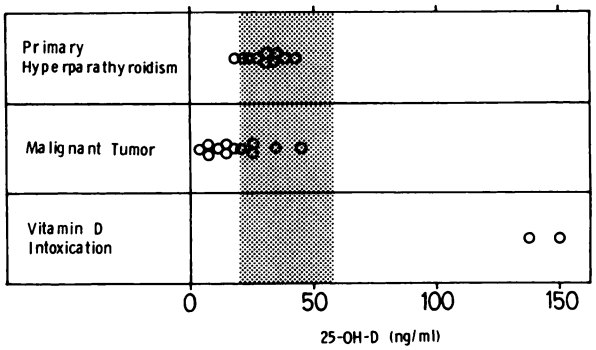

FIG. 4. Plasma 25-OH-D levels in various hypercalcemic conditions. Stippled area represents normal range.

DISCUSSION

Our data show that in patients with primary hyperparathyroidism, plasma immunoreactive CT levels are normal, regardless of the degree of hypercalcemia, unless coexisting renal failure is present. The exact mechanism for the coexistence of hypercalcemia and normal plasma immunoreactive CT level is not clear. In the majority of hypercalcemic patients with malignant tumor, there is spread from a primary neoplasm to bone, and the hypercalcemia is due to local destruction by the metastases. Elevation in blood calcium may act directly on the parathyroid glands to reduce PTH release and concurrently stimulate CT secretion from the C cells in the thyroid. This results in the characteristic findings of low to normal plasma immunoreactive PTH associated with high CT levels in patients with hypercalcemia due to bone metastasis. Immunoreactive PTH was not identified in the tumor extracts when 1 mg of tissue protein was used for the assay. The question still remains, however, whether the two cases with relatively high normal PTH in this group would not have shown ectopic PTH if a large amount of tumor extract had been measured.

Measurements of PTH and CT are also useful in recognizing the syndrome of pseudohyperparathyroidism. In all cases with tumors producing ectopic PTH, increased quantity of immunoreactive PTH was found in the circulation. Elevated plasma immunoreactive CT levels were thought to be the result of the direct action of hypercalcemia on the C cells in the thyroid, since no immunoreactive CT was identified in the tumor extracts. The combination of high levels of both plasma immunoreactive PTH and CT is a characteristic finding in the ectopic PTH syndrome, which can be distinguished from primary hyperparathyroidism by the elevated plasma immunoreactive CT. We speculate that the patient with hepatoma may have been hypercalcemic through secretion of prostaglandin E_2 (*8*), or vitamin-D-like sterol (*9*), or osteoclast-stimulating factor (*10*), and that the hypercalcemia in the myeloma patient was due to abnormal binding of calcium by immunoglobulin G (*11*).

Patients receiving an excessive amount of vitamin D_2 may develop the syndrome of vitamin-D

intoxication. Measurements of vitamin-D metabolites, such as 25-OH-D or 1,25-$(OH)_2$-D, are of particular value in differentiating vitamin-D intoxication from other causes of hypercalcemia.

In conclusion, the measurement of circulating immunoreactive PTH, CT, and 25-OH-D appears to be useful in the differential diagnosis of the hypercalcemic conditions.

FOOTNOTES

* QUSO G32, Philadelphia Quarz Co., Philadelphia, Pa.
† Philips-Duphar B.V., Amsterdam, Holland.
‡ Specific radioactivity 80 Ci/millimol, Radiochemical Center, Amersham, U.K.
§ Naphthalene 120 g, PPO 8 g, and dimethyl POPOP 0.4 g, dissolved in 200 ml of methanol, 40 ml of ethylene glycol and 1,760 ml of p-dioxane; counting efficiency 35%.

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