# *A RADIOIMMUNOASSAY FOR THYROCALCITONIN*

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Thyrocalcitonin, the recently discovered polypeptide that produces hypocalcemia and hypophosphatemia, has been found in the thyroid of man and many mammalian species by bioassay of tissue extracts.[1, 2] The complete amino acid sequence of porcine thyrocalcitonin has been reported.[3] Extensive physiological and pharmacological studies have shown that the peptide is extremely potent; millimicrogram quantities lower blood calcium and phosphate, particularly in young animals, by inhibiting bone resorption.[1, 2, 4, 5] An assay method sufficiently sensitive to measure the concentration of the peptide in peripheral blood and thereby detect secretory responses to physiological stimuli is necessary to thoroughly establish the hormonal character of thyrocalcitonin. We have developed a sensitive radioimmunoassay for thyrocalcitonin with which we have measured the concentrations of the peptide in the peripheral blood of the normal rabbit and followed changes in hormone concentration in response to an infusion of calcium. Through the use of the radioimmunoassay procedure we can readily detect $10^{-12}$ $M$ concentrations of porcine thyrocalcitonin and human and bovine thyrocalcitonin in tissue extracts.

*Materials and Methods.*—Porcine thyrocalcitonin was purified as previously described.[6-8] Highly purified preparations of the peptide were used to immunize a series of guinea pigs, and homogeneous preparations were used to prepare radioactively labeled hormone and to serve as the assay reference standard.

Thyrocalcitonin was labeled by the method of Hunter and Greenwood.[9] Specific activities of 200–400 $\mu c/\mu g$ could be achieved routinely. Labeled thyrocalcitonin was separated from the unincorporated iodine and the hormone damaged during labeling by adsorption to a column of cellulose followed by elution with plasma obtained from thyroidectomized subjects.[10]

Five guinea pigs were injected subcutaneously at 2-week intervals with 2 mg of a highly purified preparation of thyrocalcitonin (50–75 MRC U/mg)[1] suspended in complete Freund's adjuvant. Each animal received a minimum of ten injections, providing at least 1000 Medical Research Council (MRC)[1] units per animal. Plasma was obtained from each animal by cardiac puncture before and at repeated intervals during the course of immunization. These samples were tested for their capacity to bind labeled thyrocalcitonin by separating free from bound $I^{131}$-labeled thyrocalcitonin ($I^{131}$-TC) by chromatoelectrophoresis[11] or charcoal.[12] No significant binding was detected in the plasma samples taken prior to immunization. One animal, guinea pig 2 (GP2), showed a progressive rise in binding capacity during the immunization. The other animals showed much lower binding capacity (Fig. 1). We employed several methods to show that the binding of $I^{131}$-TC by guinea pig plasma was due to the presence of specific antibody. After incubation with GP2 plasma, the bound fraction of the labeled thyrocalcitonin migrated with the gamma-globulin fraction of the plasma during electrophoresis.[11] The bound fraction of $I^{131}$-TC was precipitated with the plasma globulins by 50% saturated ammonium sulfate[13] and by addition of sheep anti-guinea-pig gamma globulin serum.[14] Finally, the binding of $I^{131}$-TC by gamma globulin was readily inhibited or reversed by the addition of an excess of unlabeled thyrocalcitonin.

The high degree of selectivity of the assay for thyrocalcitonin was demonstrated by showing that labeled hormone could not be displaced from antibody by as much as a

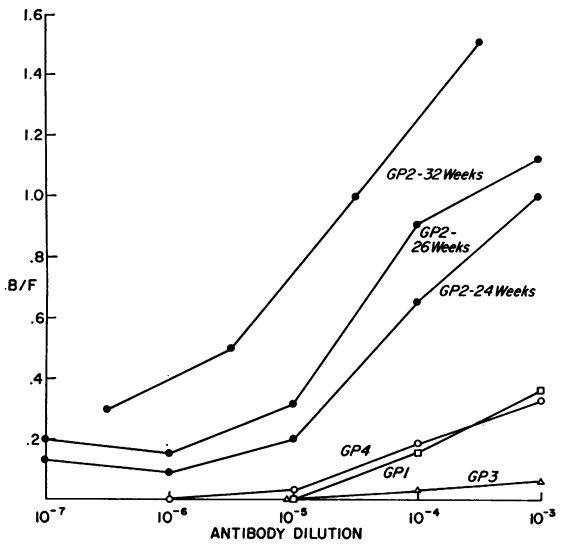


FIG. 1.—Binding capacity of $I^{131}$-TC by plasma from guinea pigs immunized with porcine thyrocalcitonin. The ratio of bound ($B$) to free ($F$) $I^{131}$-TC is shown on the vertical axis, and the final dilution of the antibody is shown on the horizontal axis.

1000-fold excess of highly purified preparations of bovine parathyroid hormone, glucagon, corticotropin, oxytocin, and vasopressin. Two mg/ml of bovine pituitary powder also caused no displacement. The thyrocalcitonin antiserum did not bind purified bovine parathyroid hormone, nor did parathyroid antiserum bind labeled thyrocalcitonin.[15]

A typical incubation mixture for the assay contained the following: $I^{131}$-TC sufficient to provide 5–10,000 cpm, GP2 antiserum at a final dilution of 1:50,000, and aliquots of the standard or unknown solution of thyrocalcitonin at multiple dilutions. All incubation tubes were adjusted to a final volume of 0.5 ml with plasma from a surgically thyroidectomized patient. This plasma was first diluted 1:10 in 0.2 $M$ phosphate buffer, pH 7.5; the same plasma-buffer mixture also served as a diluent for the tracer, antibody, and standard or unknown solution. The reaction mixtures were incubated for 48 hr at 4°C. Labeled thyrocalcitonin damaged during incubation (which spuriously fractionates as antibody-bound hormone) was estimated separately for each test sample by incubating the tracer and the test solutions without added antibody.[16] Separation of antibody-bound from free $I^{131}$-TC was accomplished by chromatoelectrophoresis[11] on 3MC filter paper (Whatman) at 700 v for 45 min in Veronal buffer 0.05 $M$, pH 8.60, at 4°C. Strips were scanned for radioactivity with an automatic recording radiation detector equipped with an integrator (Baird-Atomic). It was subsequently shown that satisfactory estimates of free, antibody-bound, and damaged $I^{131}$-TC could be made through the use of dextran-coated charcoal;[12] the latter method proved more convenient for routine assays. When charcoal was used, 200 $\mu$liters of the suspension were added to the incubation mixture, which was then centrifuged for 15 min at 3000 rpm. Both the supernate and the charcoal-dextran were counted in an auto-gamma counter (Packard) for a time sufficient to provide a counting accuracy of 1.5% at a confidence level of 95%.

For assay of tissue extracts, fresh surgical specimens of rabbit and human thyroid glands, metastatic medullary thyroid carcinoma, and other normal and malignant human tissue were quick-frozen, lyophilized, and extracted with urea as previously described for the extraction of thyrocalcitonin from porcine thyroid glands.[17] Extracts of porcine thyroid tissue obtained at various stages of purification of thyrocalcitonin[8] and one partially purified preparation of bovine thyrocalcitonin were tested. Bioassay of the porcine and bovine fractions[18] had established that the preparations varied in purity by 1000-fold. Biological activity is expressed in terms of the unit defined by the British Medical Research Council.[1]

Rabbits were chosen as an experimental animal in which to study the effect of calcium on endogenous thyrocalcitonin production because of the ease of obtaining repetitive arterial blood samples and maintaining an intravenous infusion in the conscious animal for prolonged periods.[19] Young New Zealand white rabbits were restrained in a box designed for this purpose; a 23-gauge needle was placed in a marginal ear vein and a polyethylene catheter (i.d. 0.023 in.) was then threaded into the central ear artery under local anesthesia. The animal was heparinized to prevent the cannulae from clotting. Blood samples were drawn from the arterial catheter, and infusions of calcium or dextrose were performed through the marginal ear vein at a rate of 14 ml per hour. Each experiment consisted of a 1-hr control period (infusion of 5% dextrose in water) followed by a 100-min infusion of 2% calcium chloride (140 mg/kg/hr), and terminated by a 2- to 3-hr infusion of 5% dextrose in water (while plasma calcium returned to normal). Five normal and two thyroidectomized animals were studied. Calcium concentrations were determined by atomic absorption spectrometry (Perkin-Elmer).[20] One of the intact rabbits was given a single injection of 90 MRC units of porcine thyrocalcitonin (9 U/mg) after which frequent samples of blood were taken for thyrocalcitonin assay over a 5-hr period.

Plasma samples were also collected from several normal and thyroidectomized human subjects.

*Results.*—The sensitivity and reproducibility of the radioimmunoassay are illustrated by the two standard curves shown in Figure 2. As little as 15–20 $\mu\mu$g of pure porcine thyrocalcitonin could be measured consistently in assays when antibody was incubated for 48 hours at a final dilution of GP2 antibody of 1:50,000.

Excellent agreement was evident when the results obtained by bioassay and immunoassay were compared with preparations of porcine and bovine thyrocalcitonin which differed in purity by 1000-fold (Fig. 3). This shows that the many protein components present in the crude preparations of porcine and bovine thyrocalcitonin did not react in the immune system. Taken together with the lack of interference in the assay by the several peptide hormones tested, these findings strongly attest to the specificity of the immunoassay for thyrocalcitonin.

*Measurement of endogenous thyrocalcitonin in the rabbit:* Repetitive plasma samples from five normal and two thyroidectomized rabbits were obtained during the infusions. Hormone was not detectable in the blood of the thyroidectomized rabbits before, during, or after the infusion of calcium. When urea extracts[17] of the excised thyroid gland were made, thyrocalcitonin could be detected with aliquots of a 1:1000 dilution of the extract. The estimated concentration of thyrocalcitonin (in porcine equivalent units) in the rabbit glands was 0.1–0.3 $\mu$g per gram. Salivary gland (taken as a control from the same animal) did not contain any detectable thyrocalcitonin.

The hormone could be readily detected in the blood of each of the five normal rabbits. The mean concentration in these animals was 0.14 m$\mu$g/ml (SD 0.07). The responses obtained in the assay with aliquots of plasma from normal and thyroidectomized rabbits prior to calcium infusion are shown in Figure 4. Aliquots of plasma from thyroidectomized animals did not displace $I^{131}$-TC from antibody, in marked contrast to the progressive displacement caused by similar aliquots of plasmas from normal animals. The insert in Figure 2 illustrates the displacement of $I^{131}$-TC from antibody by aliquots of plasma from a rabbit during a period of hypercalcemia. Infusion of calcium to intact rabbits caused 3- to

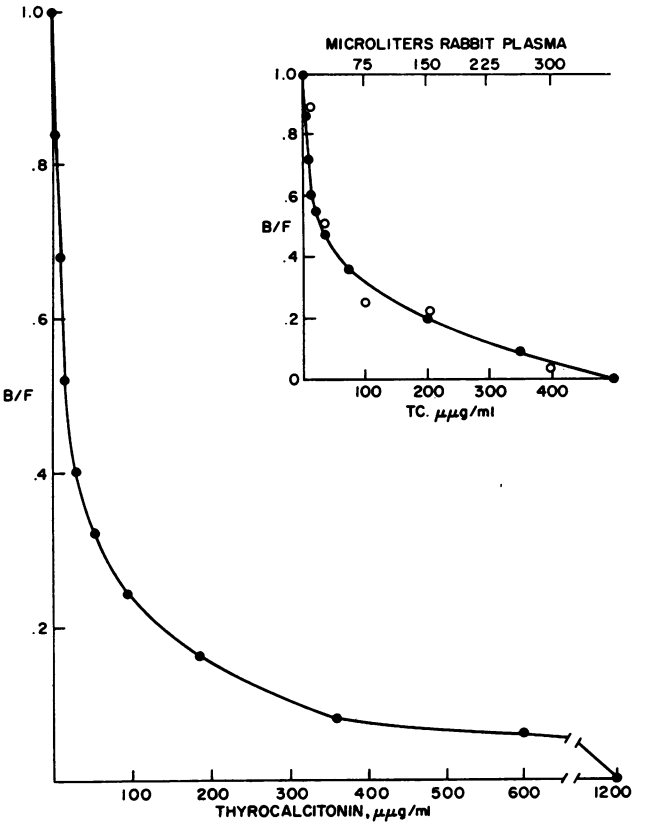


FIG. 2.—Sensitivity of the radioimmunoassay for thyrocalcitonin. The effect on the ratio of antibody-bound (*B*) to free (*F*) $I^{131}$-TC of unlabeled thyrocalcitonin (●——●) is illustrated. A second standard curve (●——●) is shown in the insert. A detectable decline in the *B/F* ratio is seen in both standard curves with as little as 15–20 μμg of thyrocalcitonin. The insert figure also shows a similar effect produced by increasing aliquots of plasma taken from a rabbit during a period of induced hypercalcemia (*open circles*).

15-fold increases in the concentration of thyrocalcitonin. The increased concentration of hormone was detected within 15 min of initiation of the infusion of calcium, peak concentrations were achieved during the period of maximal hypercalcemia, and hormone concentration rapidly returned to normal when the infusions of calcium were stopped (Fig. 5). In the rabbit injected with a large single dose of porcine thyrocalcitonin, a concentration of 2500 mμg/ml was achieved ten minutes after the injection. The concentration had fallen below 3 mμg/ml within 60 minutes. This observation, taken together with the rate of fall in endogenous thyrocalcitonin following the cessation of calcium infusion in the normal rabbit (Fig. 5), suggests a rapid turnover of the hormone in blood (estimated half life 5–15 min).

These observations in the rabbit show the usefulness of this animal for further studies on the regulation[21, 22] of thyrocalcitonin secretion.

*Detection of human thyrocalcitonin:* We have not yet established the relative

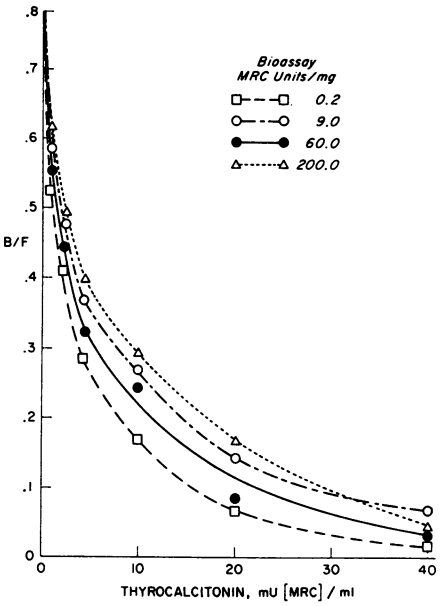


FIG. 3.—Ratio of antibody-bound (*B*) to free (*F*) $I^{131}$-TC as a function of the concentration of unlabeled thyrocalcitonin in extracts of porcine thyroid glands that vary in biological activity by 1000-fold. GP2 antisera 1:10,000, 18 hr incubation.

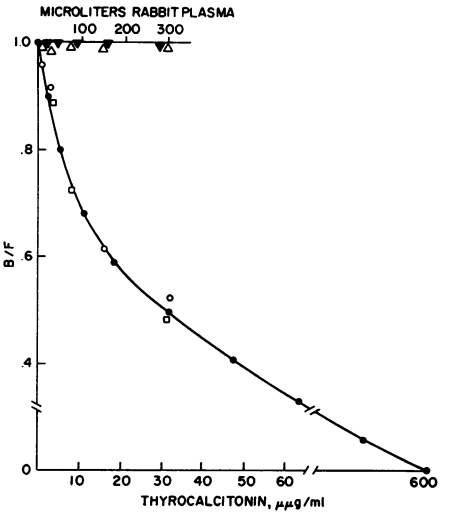


FIG. 4.—Effect of increasing aliquots of plasma from thyroidectomized rabbits (▼, △) and normal rabbits (○, □) on the ratio of bound (*B*) to free (*F*) $I^{131}$-TC compared with the response produced by increasing amounts of pure thyrocalcitonin (●——●).

immunological reactivity between porcine thyrocalcitonin and thyrocalcitonin from man and other mammalian species. However, preliminary experiments suggest that the immunoassay for porcine thyrocalcitonin permits detection of human thyrocalcitonin. The lymph node metastasis obtained from a patient with medullary carcinoma of the thyroid was found to contain 30–60 μg per gram of tissue; activity was readily detected in aliquots of a 1–10,000 dilution of the tissue extract.[23] Extracts of normal human thyroid glands had 0.2–3.6 μg of thyrocalcitonin per gram; this agrees closely with estimates based on bioassay.[1, 2] No activity was detected in extracts of other normal or malignant human tissues. A plasma sample was not available from the patient with metastatic medullary carcinoma. However, the finding that this metastasis contained thyrocalcitonin at a concentration 100-fold higher than that of normal human thyroid provides direct evidence by a specific immunochemical test that medullary thyroid carcinoma is a tumor of thyrocalcitonin-producing cells. Dr. P. M. Grimley has succeeded in growing this tumor in monolayer tissue culture; preliminary results with the immunoassay suggest that the tumor cells continue to produce thyrocalcitonin. Thyrocalcitonin activity could not be detected in preliminary tests with plasmas from normal adult human subjects. These plasmas caused severe damage[16] to thyrocalcitonin during incubation, thereby interfering with assay of the hormone in human blood. The failure to detect thyrocalcitonin in these samples from human subjects might also reflect true age or species differences or merely a lesser degree of

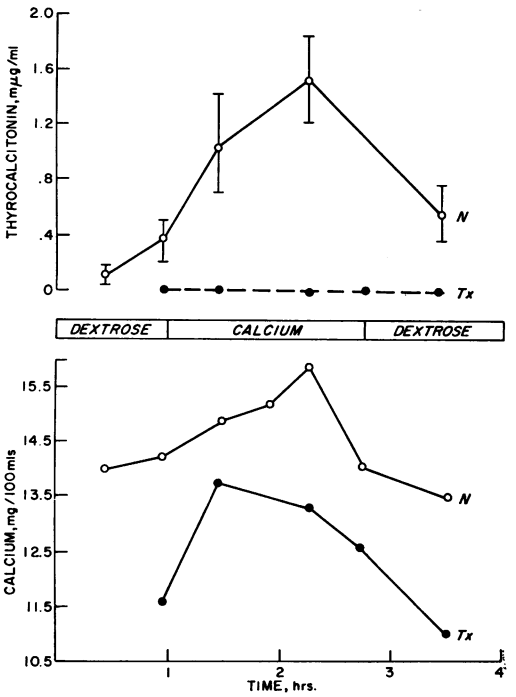


FIG. 5.—Change in plasma concentration of calcium and thyrocalcitonin during calcium infusion contrasting the response of a surgically thyroidectomized rabbit (*Tx*) with that of a normal rabbit (*N*).

immunologic response by human thyrocalcitonin in the porcine immunoassay system.

We have not yet established the ultimate sensitivity of the present immunoassay for porcine thyrocalcitonin. In most of the assays to date we used 48-hour incubations with 1:50,000 dilutions of antiserum taken after the eighth immunization of GP2 (26 weeks, Fig. 1). With continued immunization there has been a progressive increase in high affinity antibody (32 weeks, Fig. 1). More recent experiments suggest that greater sensitivity is obtained by using higher dilutions of immune plasma in incubations carried out for six days or more. Improved sensitivity will provide greater usefulness of the assay in future application in physiological and clinical studies.

*Summary.*—A highly sensitive and specific radioimmunoassay for porcine thyrocalcitonin has been developed whereby as little as 15–20 μμg of the porcine hormone can be reproducibly detected.

Studies with rabbits show that thyrocalcitonin circulates in the blood of the normal animal, that the secretion of the hormone increases rapidly in response to induced hypercalcemia, and that the turnover of hormone in the blood is rapid. Thyrocalcitonin was found in extracts of normal and malignant human thyroid tissue, indicating that human thyrocalcitonin can be detected by the assay.

These results lend considerable support to the hypothesis that thyrocalcitonin is an important mammalian hormone and suggest that extension of these studies

will help to clarify the physiological role of the hormone and possible derangements of thyrocalcitonin secretion in clinical disorders.

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