Calcitonin Resistance: Clinical and Immunologic Studies in Subjects with Paget's Disease of Bone Treated with Porcine and Salmon Calcitonins

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ABSTRACT 15 patients with Paget's bone disease were treated with varying schedules of porcine (3.8–157.5 MRCU/kg per wk) and/or salmon (1.5–210 MRCU/kg per wk) calcitonins over periods ranging from 4 to 24 months. All of the subjects experienced a striking decrease in serum alkaline phosphatase during the first 4 months of treatment. In six patients, however, resistance to these peptides was suggested by a subsequent elevation of alkaline phosphatase activity in spite of continued and augmented hormone administration. These rebounds in alkaline phosphatase levels correlated with the appearance of calcitonin-binding substances and neutralizing material in serum. Incubations of calcitonins-^{125}I and sera from these six subjects resulted in the association of radioactivity with material whose behavior on chromatoelectrophoresis (6/6), sucrose density ultracentrifugation and immunoelectrophoresis (one subject) was identical with that of 7S immunoglobulin. Specific, reversible in vitro binding of salmon calcitonins-^{125}I was observed in sera obtained from these patients 5 to 12 months after initiation of salmon calcitonin therapy. All six of these subjects' sera acquired the capacity to neutralize salmon calcitonin's hypocalcemic effect in rat bioassay. Neutralization titers correlated with maximal binding capacities, which ranged from 0.042 to 6.6 mg/liter of serum. Competitive displacement of calcitonins-^{125}I from the sera of one patient treated with both porcine and salmon calcitonin indicated separate populations of antibodies to these hormones. In spite of return of disease activity comparable to baseline levels, 3/5 resistant subjects treated with salmon calcitonin failed to develop hypocalcemia after injection of 300–1000 MRCU of salmon calcitonin, but two of these patients developed hypocalcemia in response to the porcine hormone. The disappearance of total radioactivity from the circulation after intravenous administration of salmon calcitonin-^{125}I was retarded and the amount of serum radioactivity precipitable in 50% $(NH_4)_2SO_4$ greater in 3/3 resistant patients compared to control subjects. These observations on the incidence of significant titers of neutralizing antibodies to salmon (40%) and porcine (66%) calcitonins during their chronic (> 4 months) administration to man clearly indicate that an appraisal of this possibility be included in studies involving protracted use of these hormones.

INTRODUCTION

Calcitonins (CT)[1] from several species have been administered for prolonged periods by several groups of investigators in attempts to suppress the rapid skeletal turnover exhibited by subjects with Paget's disease of bone (1–5). Porcine, human, and salmon calcitonins have been used with subsequent observation of reduced urinary hydroxyproline excretion and decreased serum alkaline phosphatase. The precise amino acid sequences of these calcitonins are known to differ (6–8), and salmon calcitonin is recognized to be a more potent hypocalcemic agent in mammalian bioassay systems (9). We have observed rebound increases of serum alkaline phosphatase in 6/15 subjects during chronic administration of calcitonin. This paper describes the appearance of circulating, neutralizing antibodies and apparent

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[1] *Abbreviations used in this paper:* CT, calcitonin; HCT, human calcitonin; KAU, King-Armstrong units; MRCU, Medical Research Council units; PCT, porcine calcitonin; SCT, salmon calcitonin.

resistance to salmon and porcine calcitonin in subjects with Paget's disease of bone.

METHODS

Patients. 15 patients with Paget's disease of bone received varying amounts of partially purified (30% pure) porcine calcitonin[2] and/or extracted salmon calcitonin,[3] and/or synthetic salmon calcitonin[4] in varying dosage schedules over periods ranging from 4 to 24 months. Baseline evaluation, skin testing, and initial administration of the hormones were carried out in a clinical research center. Serum calcium and alkaline phosphatase activity (King-Armstrong units, normal adult range 7–17 KAU) were determined on a Technicon AutoAnalyzer. Original metabolic observations with porcine calcitonin in three of the subjects have been previously reported (2). Since 1970, self-administration of calcitonin was monitored with out-patient visits and periodic admissions to the clinical research center. In general, subjects receiving the hormone had severe, extensive disease with pain not well controlled by usual analgesics. Nine men and seven women, ranging from 47 to 86 yr, received the hormone. 14/15 subjects had bone pain as a part of their initial presentation and 14/15 had serum alkaline phosphatase elevations 2-31 times higher than normal. All subjects had >4 months continued exposure to salmon calcitonin and 3/15 had >4 months of porcine calcitonin injections.

Materials. AL0831, AL1025, and AL0977 were supplied in powdered form and solubilized in sterile isotonic saline or a diluent.[5] Room temperature storage of the dry form was allowed, but storage at 4°C was advised following solubilization in either vehicle. Injections were given subcutaneously, usually at a single lateral thigh site. Purified porcine calcitonin (PCT) (200 MRCU/mg) and synthetic salmon calcitonin (SCT) were supplied by Armour Pharmaceuticals. Synthetic human calcitonin (calcitonin "M") (HCT) was supplied by Ciba Ltd. (Basel).

Iodination of calcitonins with ^{125}I and purification of the reaction mixtures were carried out as previously described (10) to specific activities of 50–100 mCi/mg. Chromatoelectrophoresis was performed on Whatman filter paper. Screening of sera for presence of antibody was done by incubating sera and calcitonin-^{125}I in 0.05 M sodium phosphate buffer containing 2% egg albumin, pH 7.4, for 1 hr at 25°C and 2–4 days at 4°C. Separation of the bound and free peptide was effected with Dextran-70 coated charcoal (11), chromatoelectrophoresis (12), or use of rabbit anti-human gamma globulin sera.[6] Incubations were also layered onto linear 4.8 ml 5–20% sucrose gradients prepared in 0.05 M sodium phosphate, pH 7.4, and spun for 14 hr.[7] Bottom puncture eluates were collected into tubes and counted in a well-type gamma spectrometer.[8]

Serum maximal binding capacities for calcitonin. Sera (1–10 μl/tube) were incubated with calcitonin-^{125}I and increasing amounts of the unlabeled peptide. After 72-hr incubations at 4°C, coated charcoal was used to adsorb "free" hormone. The reciprocal of the total amount of hormone (1/T; abscissa) was plotted against the reciprocal of the amount of hormone bound (1/B). A linear slope was drawn by the method of least squares, and the ordinate intercept was taken as the reciprocal of the maximal binding capacity for the amount of serum tested (13).

Serum neutralization of calcitonin in rat bioassay.[9] Sera were heated at 56°C for 20 min to inactivate enzymes, and then diluted with saline in siliconized glass tubes. Calcitonin (20 mU/tube) was dissolved in 1% gelatin-saline and mixed with the diluted serum at a concentration of 20 mMRCU/ml. Tubes were incubated at room temperature for exactly 30 min, and the contents injected into 100–130 g female Sprague-Dawley rats that had been fasted for 16 hr. Six rats were used for each group, each rat receiving 10 mU/100 g (0.5 ml) subcutaneously. 30 min after injection each group of rats was anesthetized with ether and bled from the abdominal aorta. Serum calcium was determined on a Technicon AutoAnalyzer (14). As controls, calcitonin was added to normal sera treated as described above, and injected into separate groups of rats at 5, 10, and 15 mU/100 g subcutaneously.

Administration of intravenous SCT-^{125}I. SCT-^{125}I was eluted from micro-fine silica[10] with 33% acetone in 1% acetic acid. After evaporation of the acetone under a stream of nitrogen, the eluate was passed through a Millipore filter into a vial of sterile isotonic saline. Before and after injection, syringes, needles, and their contents were counted in an Armac spectrometer[11] in order to calculate precise dosages. In resistant subjects, no calcitonin had been administered for at least 5 days before the study. All subjects were given 25 μg of triiodothyronine four times daily for 4 days before and 3 days after the study. An indwelling needle was placed in the vein of one arm and kept patent with a slow infusion of 5% dextrose solution. Preinjection sera were obtained and the SCT-^{125}I was rapidly (5 sec) injected into a vein in the contralateral arm. Serial blood and urine samples were obtained thereafter, with no interruption of meal schedules. Blood was allowed to clot for 30 min at room temperature, and serum was obtained by centrifugation at 5°C. Portions of serum were assayed for total radioactivity and that radioactivity precipitated by exposure of serum to equal volumes of saturated $(NH_4)_2SO_4$. Urine samples were collected into plastic bottles, and their radioactivity was measured in the Armac counter. The data were collated by taking into account the relative efficiencies of ^{125}I measurement by the Armac (6%) and well-type counter (33%) and expressed as percentage of the dose administered.

RESULTS

Clinical observations. The limited availability and early interdiction of out-patient administration of calcitonin preparations, as well as patients' choices, resulted in an irregular sequence of administration and dosage of the porcine and salmon hormones. 15 patients received calcitonin injections daily or thrice weekly for 4 months or longer and are reported in this study.

[2] AL0831, 60 MRCU/mg; kindly supplied by Armour Pharmaceuticals, Kankakee, Ill.

[3] AL1025, 1500 MRCU/mg; Armour Pharmaceuticals.

[4] AL0977, 4000 MRCU/mg; Armour Pharmaceuticals.

[5] Gelatin U. S. P. 160 mg and Phenol U. S. P. 5 mg/ml; Armour Pharmaceuticals.

[6] Gateway Immunosera Co., Cahokia, Ill.

[7] Beckman Ultracentrifuge L2-65B, SW 50.1 rotor, 40,000 rpm at 5°C.

[8] Nuclear Chicago, Des Plaines, Ill.

[9] We are grateful to Dr. J. P. Aldred of Armour Pharmaceuticals for kindly performing the rat bioassay neutralization studies.

[10] Quso G-32, Philadelphia Quartz Co., Philadelphia, Pa.

[11] Packard Model 446 Armac Spectrometer.

Occasional "flush" sensations and xerostomia were reported by several of the patients after intravenous injections of calcitonin, but no abnormalities of the urine sediment, hemogram, or chemical profile were observed in any of the subjects. Changes in the radiographic appearance of the skeleton were not observed during the period of hormone administration.

Fig. 1 depicts the serum alkaline phosphatase responses of 14/15 patients during calcitonin administration. One subject not included in the figure had a normal alkaline phosphatase level before SCT administration, and her serum did not acquire the ability to bind SCT-^{125}I after 6 months of hormone injections. All 14 subjects shown experienced a reduction in serum alkaline phosphatase activity (mean decrease of 49%) within 4 months after initiating therapy. Within the next 4 months, however, a rise in enzyme activity was observed in four patients. Two additional patients exhibited increased phosphatase activity as high as or above pretreatment levels by the end of 1 yr. Of the eight other patients shown, six have been followed for 8 months or longer. Five have shown either continued mild suppression or a plateau in phosphatase activity, and one subject has experienced a sustained decrease to $< 20\%$ of pretreatment levels (158–25 KAU). Nine of the 14 subjects reported some relief of bone pain during therapy, and four of these later exhibited a rebound in phosphatase activity in spite of continued hormone administration. Two patients experiencing rebound elevations of phosphatase and two patients exhibiting continued suppression of phosphatase reported a return of bone pain during therapy. One resistant subject (M. I. S.) reported new pain relief during placebo injections after cessation of calcitonin administration. Of the six patients showing a rebound in phosphatase activity, three (M. I. S., T. H. C., H. E. L.) had interrupted regimens wherein second courses of treatment were initiated after an interval of no treatment or administration of the other calcitonin species. Of the nine patients experiencing continued suppression of serum phosphatase activity, three had similar interruptions. All subjects (except B. I. P.) had some hormone injections in the gelatin vehicle. B. I. P. also was the only subject to experience erythema (lasting 12–24 hr) around injection sites. Table I lists data on the six patients who exhibited rebound elevations of phosphatase during therapy.

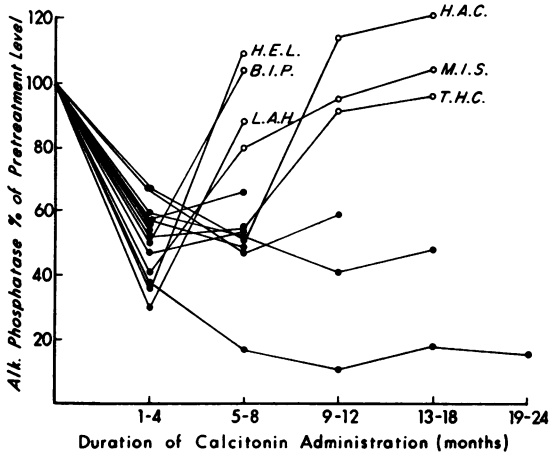

FIGURE 1 Serum alkaline phosphatase responses to calcitonin administration in 14 of the 15 subjects studied are depicted by open and closed circles. Each point reflects the mean of 3–10 of the lowest values observed during each period. When serum in vitro binding of calcitonin-^{125}I was observed, the alkaline phosphatase level at that period is depicted by an open circle. Closed circles indicate no detectable in vitro binding of calcitonin-^{125}I by sera from that subject at that time.

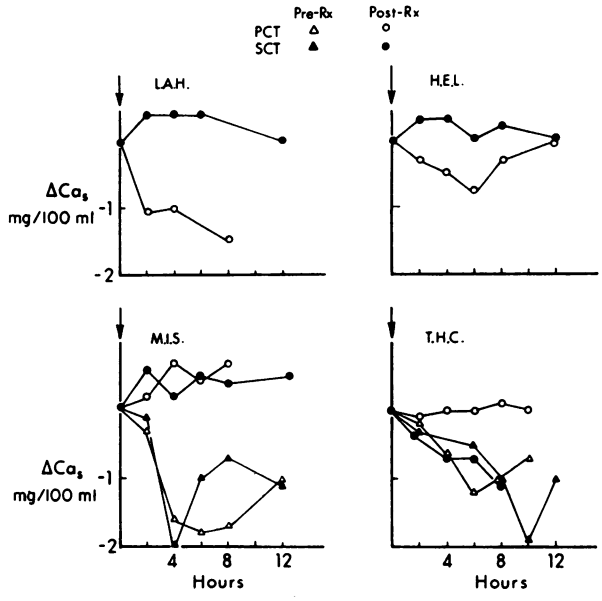

FIGURE 2 Changes in total serum calcium after intravenous injection of calcitonin in four resistant subjects. Triangles depict the responses before therapy with that calcitonin, and circles indicate responses after its chronic administration. L. A. H., H. E. L., and T. H. C. received 400 MRCU of SCT and 300 MRCU of PCT. M. I. S. received similar pretherapy doses, but later did not respond to 500 MRCU of PCT and 1000 MRCU of SCT.

Before and during therapy, the serum calcium response to an intravenous injection of calcitonin (200–400 MRCU) was measured over 8–12 hr in 12/15 patients. A decrease in serum calcium of at least 0.8 mg/100 ml was observed in each instance. Fig. 2 shows the acute serum calcium response to calcitonin before and during therapy by 4/6 subjects who exhibited a rise in alkaline phosphatase in spite of continued hormone administration. L. A. H. and H. E. L. showed

TABLE I

Clinical and Serum Binding Data of Resistant Patients

Patient	Age/sex	Body wt	Alkaline phosphatase	Hormone given	Total weekly dose*	Duration of administration	Serum calcium response‡	In vitro binding of CT-^{125}§	Per cent neutralization of SCT-induced hypocalcemia in rats/serum dilution	Maximal binding capacity
		kg	*KAU*		*MRCU/kg*	*months*				*mg/liter*
M. I. S.	62/F	68	303	Pretreatment		0	+(PCT)	0		
			118	PCT	52.5–157.5	0–4.4	+(PCT)	--		
			185	SCT‖	14	4.4–5.9	+(SCT)	--		
			267	Placebo	—	5.9–6.4	—	0		
			250–306	SCT	10.5–42.2	6.4–16.4	0(SCT)	+	80%/1:200	2.0 (SCT)
			304	PCT	42.2	16.4–17.4	0(PCT)	+		6.6 (PCT)
			318	SCT	210	17.4–18.4	0(SCT)	+		
			324	No treatment		18.4–22.4	—	+	80%/1:10	0.41 (SCT)
B. I. P.	53/F	68	50	Pretreatment		0	+(SCT)	0		
			33	SCT	35	0–0.3	--	0		
			29	SCT	2.5–15	0.3–3.0	--	+		
			51	SCT	2.5–5	3.0–8.0	--	+	72%/1:50	2.5 (SCT)
T. H. C.	47/M	80	207	Pretreatment		0	+(PCT)	0		
			130	PCT	60.9	0–2.0	+(PCT)	--		
			97	PCT	17.5	2.0–5.0	+(PCT)	0		
			120	SCT‖	3.8	5.0–9.0	+(SCT)	0		
			176	SCT	3.8	9.0–13.0	+(SCT)	+	45%/1:10	0.32 (SCT)
			188	PCT	3.8–11.4	13.0–16.0	0(PCT)	+		0.21 (PCT)
L. A. H.	86/F	48	103	Pretreatment			+(SCT)	0		
			49	SCT	21–42	0–2.5	--	--		
			29	SCT	18	2.5–5.5	--	+		
			86	SCT	15	5.5–8.5	0(SCT) +(PCT)	+	73%/1:50	1.1 (SCT)
			111	No treatment		8.5–12.5	--	+	50%/1:50	
H. E. L.	51/F	60	60	Pretreatment		0	+(SCT)	0		
			22	SCT	14–102	0–2.0	--	0		
			31	PCT	12	2.0–4.0	+(PCT)	0		
			47	SCT	9	4.0–6.5	--	+	55%/1:50	
			60	SCT	21–35	6.5–13.5	0(SCT) +(PCT)	+	39%/1:50	0.45 (SCT)
H. A. C.	61/M	78	149	Pretreatment		0	+(SCT)	0		
			83	SCT‖	6–17.5	0–3.5	--	—		
			70	SCT	3	3.5–7.5	--	0		
			165	SCT	1.5	7.5–13.5	+(SCT)	+	40%/1:10	0.042 (SCT)
			156	SCT	6	13.5–17.5	--	+		

* Doses were given daily or thrice weekly.

‡ Serum calcium decrease in response to intravenous CT (+ = hypocalcemia, 0 = no response, -- = not tested).

§ + = binding, 0 = no binding, — = not tested.

‖ Natural SCT comprised part of the course.

a rise in alkaline phosphatase during SCT therapy, and, as shown, failed to respond to SCT at a time when their alkaline phosphatase was back to or above levels obtained before treatment. They were observed to respond to PCT, however (Table I and Fig. 2). Both M. I. S., and T. H. C. received PCT initially, and later received SCT injections. They both displayed a sharp hypocalcemia in response to each hormone before its continued use. After their phosphatase levels climbed back to their pretherapy range, M. I. S. failed to respond to large doses (1000 MRCU of SCT, 500 MRCU of PCT) of either hormone. T. H. C. did not respond to PCT (300 MRCU), but did respond to SCT (400 MRCU). Of the other two subjects showing rebound hyperphosphatasia during therapy, H. A. C. continued to show hypocalcemia in response to SCT, and B. I. P. was not tested in this way. All seven of the subjects not showing rebound phosphatase elevations who were tested showed a hypocalcemia (0.8 mg/100 ml or greater) in response to SCT before therapy and >4 months later when their serum phosphatase activity had fallen to 18–60% of their pretherapy levels.

Serum binding of calcitonin. 11 control sera (pretreatment sera, patients with Paget's disease not treated with calcitonin, normal volunteers) have been employed in incubations with calcitonins-^{125}I, and no significant binding ($<10\%$ of tracer) has been observed in any of these samples. Similar findings were also noted

with the sera from the treated subjects who experienced sustained lowering of alkaline phosphatase activity (8/8) and sustained hypocalcemic responsiveness (7/7). In contrast, the sera from the six patients who experienced rebound elevations of serum phosphatase acquired the ability to bind labeled calcitonin (Table I and Fig. 1).

Chromatoelectrophoresis of incubations of patients' sera with SCT-^{125}I is depicted in Fig. 3. Migration of radioactivity to strip segments of the gamma globulin region was observed in H. E. L., L. A. H., and M. I. S., whereas SCT-^{125}I and control serum incubations resulted in little anodal migration of radioactivity. The reversible association of SCT-^{125}I is indicated by the lack of anodal migration of radioactivity when L. A. H.'s serum incubation contained 100 mμg of unlabeled SCT. Similar results (not shown) were obtained with sera from T. H. C., H. A. C., and B. I. P.

Incubations of M. I. S. serum with SCT-^{125}I resulted in the sedimentation of radioactivity in a region of the sucrose density ultracentrifugation pattern like that of human 7S gamma globulin (Fig. 4). Addition of 100 mμg of unlabeled SCT resulted in the failure of sedimentation of the SCT-^{125}I, similar to the results observed with analyses of control serum.

Competitive displacement of SCT-^{125}I and PCT-^{125}I from the serum of M. I. S. was studied (Fig. 5). The binding of SCT-^{125}I was easily reversed by the addition of unlabeled SCT, but not by PCT or HCT. Similarly, the binding of PCT-^{125}I was sensitively antagonized by

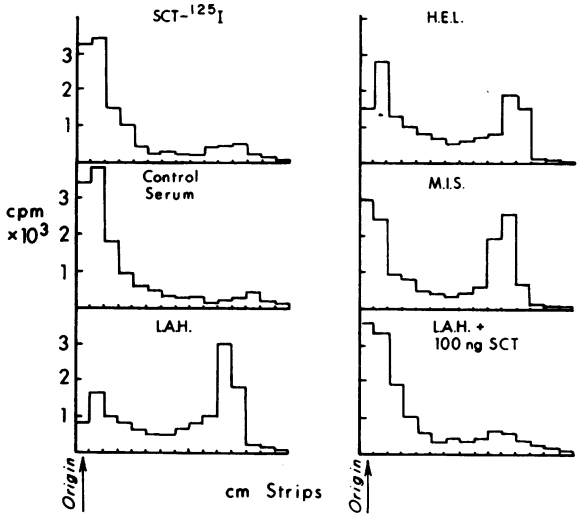

FIGURE 3 Paper radioelectrophoretograms of incubations of SCT-^{125}I with various sera. Incubations were done at 5°C for 48 hr, and portions applied to paper strips. After chromatography with the tank cover open for 1 hr, electrophoresis was initiated. Strips were counted in a gamma spectrometer.

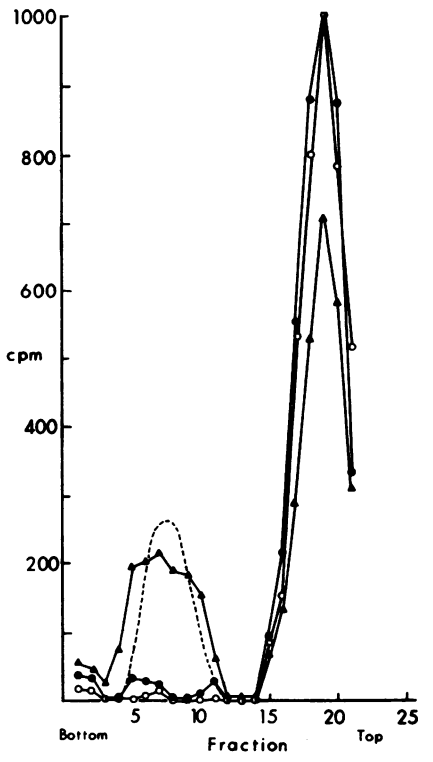

FIGURE 4 Linear sucrose gradient (20–5%) ultracentrifugation of SCT-^{125}I (○——○), SCT-^{125}I and 1 μl of MIS serum after incubation for 1 hr at 37° (▲——▲), SCT-^{125}I and 1 μl of M. I. S. serum plus 100 ng of unlabeled SCT after incubation for 1 hr at 37° (●——●), and human 7S immunoglobulin (absorbance at 280 nm, dotted line). After centrifugation at 40,000 rpm on a SW 50.1 rotor for 14 hr, 0.24-ml fractions were collected through bottom punctures.

PCT. However, both SCT and HCT were weakly competitive to this binding. Sera from T. H. C. also bound SCT-^{125}I and PCT-^{125}I well, but SCT was equally capable of displacing both tracers. In contrast, sera from H. E. L., B. I. P., and L. A. H. only weakly bound PCT-^{125}I, and this binding was easily reversed by addition of PCT or SCT in amounts < 10 mμg, but not by 1 μg of HCT. Rabbit anti-human gamma globulin serum precipitated radioactivity from incubations of M. I. S. and T. H. C. sera with PCT-^{125}I and SCT-^{125}I, as well as incubations of H. E. L., B. I. P., H. A. C., and L. A H sera with SCT-^{125}I.

Fig. 6 shows the results of a study in which in vitro incubations of M. I. S. sera with calcitonins-^{125}I were subjected to agar electrophoresis and allowed to diffuse against rabbit anti-human serum (Fig. 6, upper). After extensive washing of the gel slides, large amounts of radioactivity in the gamma globulin precipitation lines were detectable in the autoradiograph (Fig. 6, lower).

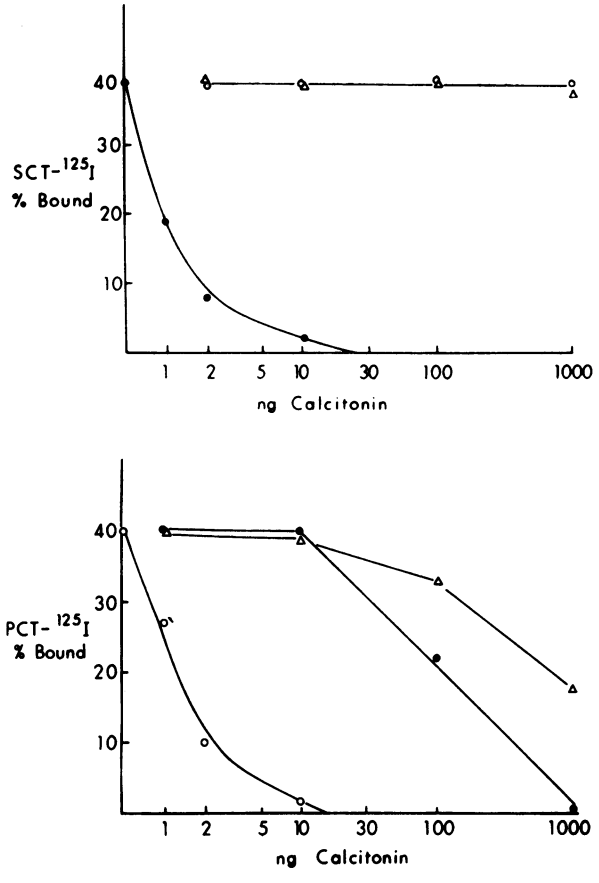

FIGURE 5 Competitive displacement of ^{125}I-labeled SCT and PCT from binders in the serum of M. I. S. In each tube, 1 μl of serum was incubated with either of the labeled peptides and varying amounts of salmon (●), porcine (○), or human (△) calcitonin for 72 hr at 5°. Bound and free peptide were separated with dextran-coated charcoal. Each point is the mean of duplicate analyses.

The maximal binding and neutralizing capacities of the sera from the patients with apparent resistance to calcitonin are shown in Table I. Although sera for in vitro studies were always obtained >20 hr after prior injections of calcitonin, the possibility of occupied binding sites in such sera suggests our estimates of maximal binding to actually be low. After cessation of therapy, the binding capacity and neutralizing ability of M. I. S. and L. A. H. sera have been observed to decrease. As shown in Table I, the rat bioassay neutralization data correlate reasonably well with the estimates of serum binding capacity and phosphatase levels.

Disappearance of SCT-^{125}I from serum. The disappearance of total radioactivity from the serum after intravenous administration of SCT-^{125}I was delayed in those subjects displaying resistance to the hormone (Fig. 7). In addition, the percentage of serum radioactivity precipitable with 50% $(NH_4)_2SO_4$ was higher in these patients. The salt-precipitable radioactivity was considerable in the nonresistant subjects as well during the early postinjection period. In vitro incubation of preinjection serum with SCT-^{125}I for 37°C for 2 hr also showed more radioactivity to be salt-precipitable in the resistant groups. It is possible that some of this precipitated radioactivity reflects trapped peptide in addition to association with antibody molecules. The continued high percentage of precipitable radioactivity we observed in the resistant sera is similar to that reported by Berson et al. using sodium sulfate treatment of sera taken from insulin-treated diabetics after intravenous administration of insulin-^{131}I (12). In addition, significantly less radioactivity was recovered in the urine of the resistant subjects compared with those subjects who were sensitive or not previously exposed to calcitonin (data not shown).

DISCUSSION

The retardation of disappearance of SCT-^{125}I from the blood stream (3/3), its altered migration on paper electrophoresis and its precipitation by anti-human gamma globulin serum after incubation with these patients' sera (6/6) compared with control collectively provide strong evidence for the presence of antibodies to calcitonin in the subjects studied. In addition, these sera were capable of neutralizing the hypocalcemic potency of calcitonin in the rat bioassay. The presence of antibodies does not necessarily indicate hormone resistance unless their affinity, capacity, and nature of binding prevent the expected biologic response. Our estimates of binding capacity are probably underestimates, since calcitonin previously administered may have still occupied antibody sites. It is of interest, however, that the resistant subject with the least binding capacity (H. A. C.) exhibited increased phosphatase activity but continued to develop hypocalcemia in response to SCT.

The data presented in this report indicate that a calcitonin binding globulin is acquired by 40% (6/15) of human subjects in response to repeated administration of salmon calcitonin for longer than 4 months and 66% (2/3) of patients receiving PCT. The failure to observe hypocalcemia after calcitonin administration in subjects with Paget's disease of bone is a reasonable measure of resistance to the peptide, since the hormone's hypocalcemic potency through inhibition of bone resorption is in large part dependent on the rate of skeletal turnover (15). Although the alkaline phosphatase level and disease activity are known to be variable in Paget's disease of bone, our serial observations strongly suggest that skeletal turnover resumed pretreatment levels in spite of continued calcitonin administration. Bone pain and its relief did not correlate

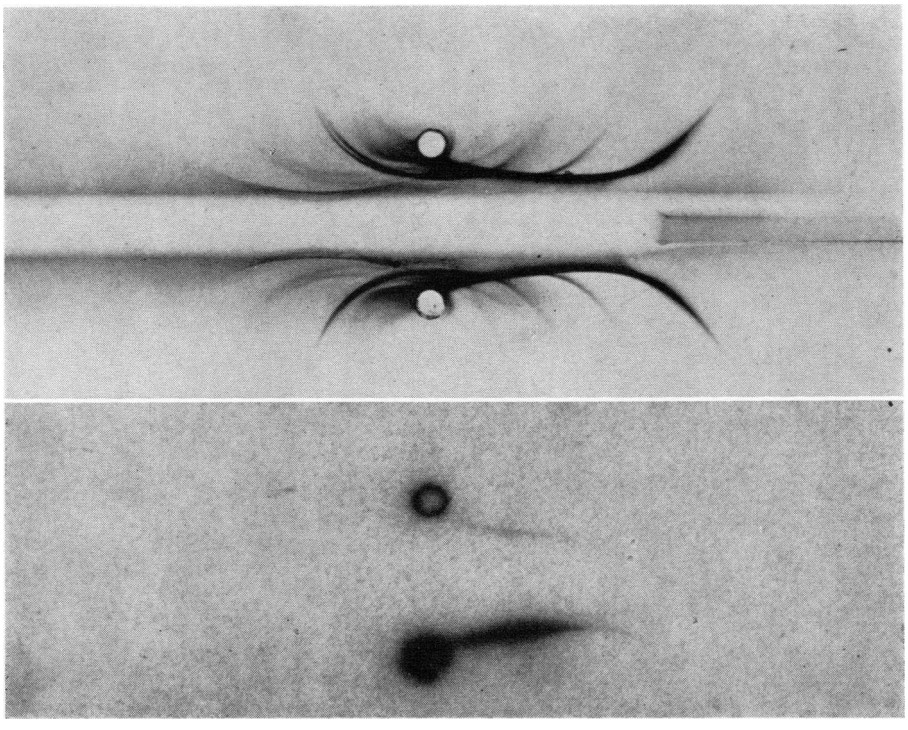

FIGURE 6 *Upper figure:* Serum from one resistant subject (M. I. S.) was separately incubated with SCT-^{125}I and PCT-^{125}I for 4 days at 5°. Portions were placed in center wells of 1.5% Noble agar-coated slides (veronal buffer, pH 8.2). Electrophoresis was carried out at 240 v for 2 hr. Rabbit anti-human serum was then added to the center trough and allowed to diffuse overnight. A photograph of the precipitin arcs is shown in the upper figure. *Lower figure:* The slide was then placed in a tank containing 500 ml of phosphate buffered 0.15 M saline and allowed to rinse with continued gentle stirring of wash solution for 72 hr. The buffer was changed each day with final rinse consisting of distilled water. After air-drying, the slide was placed under film and an autoradiograph was developed. The lower figure represents the autoradiograph (upper panel, PCT; lower panel, SCT) indicating association of radioactivity with the cathodal chevron of 7S gamma globulin.

well with the level of serum phosphatase or serum binding and neutralization of calcitonin, but the protocol was not double blind, therefore limiting the validity of the reports of pain relief. The prompt response of hypocalcemia to the other calcitonin species (Fig. 2) indicates high skeletal turnover and shows that the failure to respond to the original calcitonin cannot be explained by secondary changes in mineral homeostasis.

Several investigators have reported their observations employing calcitonin in Paget's disease of bone, as well as other skeletal disorders (1–5). The failure to observe suppression of skeletal turnover to normal levels in Paget's disease during calcitonin therapy has been thought to indicate secondary hyperparathyroidism (16, 17), even though antibody formation to PCT and SCT has been reported (17, 18). Others have not detected antibodies but have observed elevations of serum alkaline phosphatase during continued PCT therapy (5). Although secondary hyperparathyroidism should play a role in homeostatic adjustment to repetitive hypocalcemic stimuli, it could not explain the resistance in our subjects, most of whom responded very well to a different calcitonin. It is possible, however, that resistance to calcitonin in some patients may reflect secondary hyperparathyroidism as well.

Of interest in the finding that human and salmon calcitonins share 14 identical amino acid positions and that the human sequence more closely resembles the salmon than that of the bovine and porcine hormones (8). Several subjects exposed to both hormones demonstrated binding in vitro of both PCT-^{125}I and SCT-^{125}I. Of those sera thus far tested, only M. I. S. demonstrates specific binding of both hormones, most likely demonstrating the presence of two distinct antibody populations in her serum. It is also not clear whether the slight cross-reactivity of her PCT antibody with SCT might indicate that her course of SCT therapy provided a stimulation of the antibodies to PCT.

Several heterologous species' hormones have been shown to elicit immunologic responses in human sub-

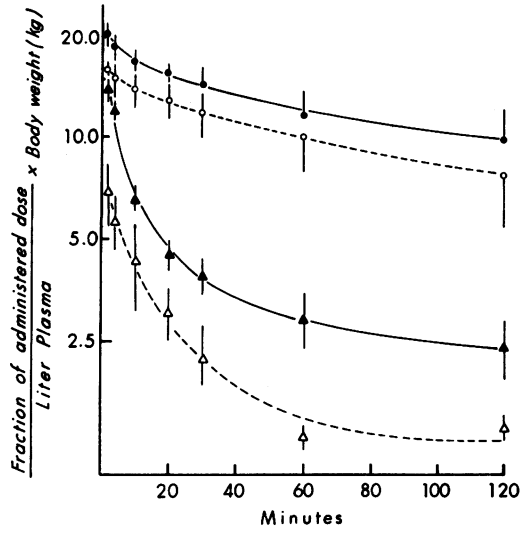

FIGURE 7 Disappearance of total radioactivity from sera of three resistant patients (●) (M. I. S., H. E. L., and L. A. H.) and three nonresistant subjects (▲) (one treated, responsive patient, an untreated patient with Paget's disease of bone, and a normal volunteer) after rapid intravenous injection of salmon calcitonin-^{125}I. Radioactivity precipitable in 50% $(NH_4)_2SO_4$ is depicted by the open circles (resistant subjects) and open triangles (nonresistant subjects). Vertical lines indicate the SEM.

jects (12, 19, 20). In the case of insulin, however, the immunologic response infrequently results in significant clinical resistance (21). As a 32 amino acid peptide, calcitonin ranks with adrenocorticotropin (39 amino acids) (20) and beta 1–24 ACTH (22) as one of the smaller peptides shown to elicit antibody formation in human subjects. The relatively high biologic potency of SCT (4000 MRCU/mg), compared with PCT (200 MRCU/mg) has prompted its synthesis on a large scale, and resulted in the use of smaller amounts of the hormone. It is possible, therefore, that larger doses of SCT would overwhelm the neutralizing titers of some resistant subjects. Recently, human calcitonin has also been shown to be effective in suppressing Paget's disease of bone (4) and increased utilization of this peptide in man is likely. It should be recalled, however, that chronic administration of human growth hormone to man has led to immunologic resistance as well (13, 23). The present study clearly indicates that an appraisal of the possible development of neutralizing antibodies be included during the protracted administration of porcine and/or salmon calcitonins to man.

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