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Combining Provocative Agents of Calcitonin to Detect Medullary Carcinoma of the Thyroid†

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The presence of clinically undetectable medullary thyroid cancer can be revealed by characteristic patterns of calcitonin in serum after the injection of a provocative agent, pentagastrin or calcium ion. However, in some reports, medullary thyroid cancer was uncovered in a test with pentagastrin but not in one with calcium ion, and vice versa. To reduce the number of erroneous responses, each provocative agent has been employed in separate tests. Combining pentagastrin and calcium into one stimulus should give the advantage of each agent while requiring less time and fewer analyses in a single test.

We combined pentagastrin injection and calcium ion infusion into a single stimulus of calcitonin secretion. Normal responses to each pharmacologic agent and to the combination were established. In patients with medullary carcinoma, the combined stimuli usually, but not always, elicited a greater rise in serum calcitonin concentration than did the more potent of the two agents alone. However, we encountered no false negative results in any of the methods used: pentagastrin alone, calcium ion alone, or pentagastrin combined with calcium. Still, normal responses to both *agents theoretically give greater assurance that medullary cancer and hereditary disease are absent.*

The symptoms associated with the provocative tests were the same as, or no worse than, those reported for each agent alone. Serum calcium concentrations increased for a few minutes after calcium gluconate was administered, but, with practice at infusions, maximum concentrations were held below 14.2 mg/dl.

In the proper clinical setting, abnormal concentrations of serum calcitonin (CT) reliably indicate the presence of medullary carcinoma of the thyroid gland (MCT) or its precursor, C-cell hyperplasia. These cancers and premalignant cells usually secrete CT at rates greater than normal. Since abnormal basal CT levels may already be associated with incurable metastases, provocative stimuli — particularly pentagastrin and calcium ion — have been used to produce diagnostic secretory patterns of CT when basal concentrations are normal and the neoplasm is confined to the gland (1-6).

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Such provocative tests are of special value in detecting affected relatives of patients with multiple endocrine neoplasia type 2a (1-3) and type 2b (7). Screening programs of this kind have uncovered MCT in about 50% of family members, a figure predicted by the autosomal dominant mode of inheritance. Because the diagnoses were established at early stages, complete resection of the cancers has apparently been possible (1-3, 6). Nevertheless, there are reports in which a test using pentagastrin revealed the cancer, but the injection of calcium ion gave a false negative result, and vice versa (1). To reduce errors of this type, some physicians employ each pharmacologic agent in separate rate tests on individuals at risk for MCT.

To ascertain the effectiveness of a diagnostic-therapeutic program for hereditary MCT, patients must be retested periodically. Equally important are reevaluations of family members who responded normally to the stimuli in the initial screening procedures. At present, it seems prudent to reassess people who are still at risk every one to two years. However, recurrent testing imposes a burden on affected families, physicians who administer the studies, and laboratories that process the samples. Indeed, one group found it logistically impossible to employ both calcium and pentagastrin in separate provocative tests and has therefore

chosen to use only the latter agent (1). A single yet reliable method that effectively exploits the stimulating qualities of both pentagastrin and calcium would be welcome.

Wells, et al (3) reported that combining the stimuli, pentagastrin and calcium ion, into a single procedure detected MCT more frequently than when either provocative agent was employed alone. Here, using a comparable method in which injected pentagastrin is immediately followed by an infusion of calcium gluconate over one minute, we report a similar but not identical experience: 1) generally, but not invariably, the combined pharmaceuticals provoked responses equal to or greater than those of the more potent single agent; 2) no false positive patterns; 3) no false negative patterns when compared to a single stimulus; and 4) patient acceptance.

Materials and Methods

Controls and Patients

Control data were obtained from 38 paid healthy volunteers, aged 19-35 years. Some individuals served as controls in all three test methods, while others were employed for only one or two.

Patients were from nine families in which MCT had been found in more than one member, and all were considered to manifest multiple endocrine neoplasia 2a (1,2). Nine were evaluated before thyroidectomy. Two other patients were considered to have sporadic MCT because the tumor was unilateral and no relatives had developed the disease; one was tested before operation, after her diagnosis was established by needle biopsy.

Methods

Tests were carried out while the patients and controls were fasting except for some individuals who were given pentagastrin alone several hours after a meal. We used three methods: 1) pentagastrin (Peptavlon,® Ayerst Laboratories) 0.5 μg/kg was injected intravenously over five seconds; 2) calcium, 2 mg/kg, as calcium gluconate, was infused over one minute at as constant a rate as possible; 3) pentagastrin given over five seconds was immediately followed by the infusion of calcium over one minute. Blood specimens were obtained before and at 2, 5, and 15 minutes after the injections were started.

The three methods were carried out in all patients and controls within a six-week period except for three patients who required a few months (up to ten) before all tests could be completed.

We measured calcitonin in sera using a radioimmunoassay developed by one of us (BGE). The method is a non-equilibrium assay that uses antibody developed in a rabbit immunized against synthetic human calcitonin (syn-hCT). We prepared radio-labeled tracer by labeling syn-hCT using a modification of the chloramine-T method of Hunter and Greenwood (8), a World Health Organization syn-hCT standard (70/234), and a double antibody separation using a 2.5% solution of polyethylene glycol and sheep anti-rabbit gamma globulin. Unlabeled ligand (standard or subject's sample) and antibody were pre-reacted for 24 hours before the radio-labeled tracer was added. The reaction, contained in an incubation volume of 800 μl, was allowed to proceed at 4°C for 48 hours before the separating reagent was added. The separation reaction is complete within 10 minutes, and separation of antibody-bound and free tracer was accomplished by centrifugation at 800 g for 30 minutes.

Assay sensitivity, based upon the variation observed in the zero standard tubes, was approximately 5 pg/tube with a lower limit of detection in serum of approximately 50 pg/ml of serum, based upon the use of a serum sample size of 100 ul. A second estimate of assay sensitivity, the lowest point on the standard curve, yielded 10 pg/tube, or 100 pg/ml of serum. The standard curve consisted of 10 points extending from 5 ng/tube to 10 pg/tube with a 50% inhibition point of 142 pg/tube and a slope of -2.2 Results were calculated from a weighted least square linear regression analysis of the binding data after a logit-log transformation. Interassay coefficient of variation for the 50% point on the inhibition curve for 12 assays was 9.0%. For 3 quality control sera that bound at 65%, 47%, and 25% on the standard curve, the coefficient of variations were 7.7%, 18.1%, and 16.6%, respectively.

Parallelism checks for one patient (No. 15), shown in Fig. 1, represent stimulation studies before and after thyroidectomy. The assay results from the samples of these times appear parallel to the standard curve. We cannot explain the source of calcitonin detected in the sera of patients who have undergone thyroidectomy. In the early phase of the study we determined parallelism for all samples, but since parallelism seemed the rule, only duplicate measurements were made on blood specimens from later subjects.

We measured calcium by an automated method using orthocresolphthalein to develop color (9); the 95% confidence limits of the normal range were 8.8-10.9 mg/dl.

Results

Calcitonin values acquired from control subjects in the three methods are summarized in Table I. Increments of CT above baseline concentrations conveniently depict responses at two and five minutes after injections, times when maximal changes have been observed. The basal concentration of CT in normal men was slightly higher than that of women, but the difference was not statistically significant. In the two methods with pentagastrin as a

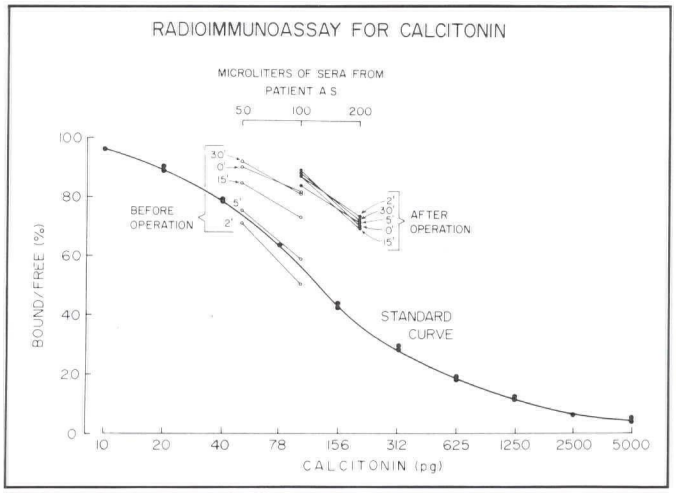

Fig. 1

A standard curve for the radioimmunoassay of CT. Groups of five samples of sera were obtained from patient No. 15 before and after thyroidectomy for MCT. Each group constituted a study in which pentagastrin was the provocative stimulus; the time of each sample relative to the injection of pentagastrin is indicated. When the concentrations of calcitonin in different volumes of each serum are plotted, parallelism with the standard curve is inscribed.

stimulus, the increments of CT in men exceeded those in women, but this difference too was not significant. No detectable response was observed in many normal subjects.

Normal ranges were therefore established to be the same for both sexes: 97.5% of basal values were below 450 pg/ml; but those above this concentration (550 pg/ml) were considered abnormal. Although increments in normal women were slightly less than those in normal men, upper limits of normal were designated from the studies of men: in pg/ml, for pentagastrin +110, for calcium +130, and for pentagastrin/calcium +220.

Three categories of patients are tabulated in Table II. Medullary carcinoma was verified histologically, either before or after testing, for patients in the first two categories (except for patient No. 5, who has not yet undergone operation but whose test abnormalities were so marked as to be strong evidence of MCT). Patients with borderline high or high basal concentrations of CT were allocated to the first category; normal basal CT values characterized those in Category 2.

In the third group are subjects whose responses were normal by all methods. They have had MCT completely removed by operation or are at 50% risk of developing MCT because of family affiliation.

The highest increment of CT was seen after the combined stimuli in six of the 11 patients in Categories 1 and 2. In several patients the increment of calcitonin concentration after injection of pentagastrin or calcium alone was less than 50% of that obtained with the agents together (e.g., patient No. 10). Also, the five individuals (patients No. 1, 3, 6, 7, and 9) whose maximum increment was highest with a single agent exhibited rises in calcitonin after the combined stimuli (62-97%) that approached the highest value achieved by any procedure. Even though the combined stimuli did not always provoke the largest increments of CT, this method never gave normal (false negative) responses when either of the other methods elicited abnormality. Indeed, no method gave a false negative result.

Pentagastrin produced the well known symptoms of upper abdominal and lower chest cramping associated with nausea. Calcium caused sensations of generalized warmth and occasionally nausea. Both control subjects and patients

TABLE I
Calcitonin Responses to Provocative Stimuli in Control Subjects

Basal Concentrations of Calcitonin (pg/ml) Mean ± SD						
Men (22) 274 ± 135				Women (16) 248 ± 79		
Provocative Tests: Increments in Calcitonin (pg/ml) Mean (Range)						
	Men			Women		
Test	No.	2 min	5 min	No.	2 min	5 min
Pentagastrin	(11)	+35 (−70 to +110)	+14 (−90 to +80)	(8)	+9 (−40 to +60)	+5 (−30 to +60)
Calcium	(11)	+24 (−90 to +130)	+2 (−90 to +80)	(7)	+4 (−50 to +70)	+26 (0 to +60)
Pentagastrin plus calcium	(10)	+30 (−90 to +180)	+50 (−70 to +220)	(11)	+17 (−50 to +150)	+8 (−50 to +100)

TABLE II
Calcitonin Responses to Provocative Stimuli in Patients

Patient	Age	Sex		Calcitonin (pg/ml) as Maximum Increment*/Basal		
				Pentagastrin†	Calcium†	Pentagastrin plus Calcium
Category 1: Patients with basal concentrations of >550 pg/ml						
1.‡	30	M		12,400/760	26,500/930	24,200/1,180
2.‡	27	M		64,600/1,020	36,400/1,040	96,100/950
3.‡	55	F		3,700/580	86,200/960	63,100/680
Category 2: Patients with basal concentrations of <550 pg/ml and abnormal responses						
4.‡	22	F	(Preop)	12,200/370	11,500/520	20,600/360
			(Postop)	740/340	1,090/310	1,610/330
5.‖	15	M		650/300	400/350	990/350
6.‡	24	M		3,000/480	3,700/490**	2,900/300
7.‡	25	F		3,300/310	780/380	3,200/270
8.‡	34	F		1,040/130	310/170	1,170/110
9.‡#	40	F		7,100/150	9,500/200	5,900/200
10.‡	26	F		1,900/300	2,400/250**	5,000/160
11.‡	50	F		15,300/390	5,400/430	19,700/270
Category 3: Patients with normal basal concentrations and normal responses						
12.§	19	F		20/290	10/280	−10/310
13.§	13	F		−10/350	110/390	30/360
14.§	12	F		0/400	0/350	60/370
15.§	62	M		−10/500	−40/450	−40/490
16.‖	20	F		−20/310	−10/320	60/320
17.‖	23	F		−40/500	10/430	40/480
18.‖	17	M		−90/200	40/180	−140/260
19.§#	30	M		−30/460	−60/510	−30/440

* Maximum occurred at 2 or 5 minutes after injections were started.
† See text for dose schedules.
‡ MCT confirmed histologically.
§ MCT postoperative state, presumably cured
‖ At risk (50% risk)
Apparently sporadic MCT
** Calcium given 3 mg/kg over 10 minutes
ND Not done

reported that the method of combining provocative pharmaceuticals induced no more symptoms than did the more unpleasant of the single agents. Symptoms were severe in none and disappeared by four minutes in all.

Serum calcium concentrations were determined in blood specimens obtained from all control subjects following the infusion of calcium gluconate (Table III). These infusions produced serum calcium concentrations after two minutes that varied from no change over baseline to a value of 22.5 mg/dl in one woman of the control group. The serum calcium in this subject fell to 10.4 mg/dl by five minutes, and it is possible that some of the pharmaceutical was mixed with the first blood specimen in the catheter. Nevertheless, calcium concentrations reached 15-16 mg/dl in other individuals, and symptoms were generally related to the level attained. Values were invariably lower at five minutes and normal by 15 minutes. The calcium values after the last 13 infusions did not exceed 14.1 mg/dl. The infusions were carried out by hand, and with practice they were administered more smoothly.

Basal measurements of calcium were obtained in all patients to exclude the possibility of hypercalcemia. None had a value greater than 13.1 mg/dl at two minutes after the calcium infusion began.

Discussion

In our studies we did not observe the statistically significant differences between men and women in the basal concentrations or in stimulated increments of CT that others

TABLE III
Serum Calcium Concentrations After Calcium Gluconate Infusions* in Normal Subjects**

Time After Infusion (min)	Serum Calcium (mg/dl) −1	2	5
Men			
Concentration (Mean ± S.D.)	(16) 9.4±0.4	(18) 11.8±1.8	(18)10.7±0.7
Increment (Range)		−1.3 to +7.0	−0.4 to +2.7
Women			
Concentration (Mean ± S.D.)	(11) 9.6±0.7	(16)13.8±3.0	(16) 10.8±0.8
Increment (Range)		0.0 to +12.8	+0.5 to +3.6

* 2 mg calcium/kg over 1 minute
** Some subjects served in two methods, calcium alone or pentagastrin plus calcium.

have reported (10-12). Although the sensitivity of our CT assay may not be as great as that in other laboratories (10, 11), the increments following stimulation readily detect MCT that is localized and completely resectable. In fact, of 11 individuals who were first identified as abnormal by provocative testing over three years, two had C-cell hyperplasia, and all but two had localized disease. In follow-up evaluations subsequent to operation, only the two individuals with regional node involvement have exhibited abnormalities in annual provocative studies.

Combining the provocative agents, pentagastrin and calcium, into a single method usually induced increments in serum calcitonin as great as, or greater than, either stimulus used alone. In contrast to the results reported by Wells, et al (3), this was not an invariable observation. Variability in responsiveness probably does not relate to progressive disease over the time of testing (1), which was a few months in some cases. In fact, MCT generally grows slowly over years, and of the different methods employed in this investigation, the last often did not elicit the greatest response. This suggests that, in these individuals, we were not observing the changes of progressive disease.

When one agent alone appears more potent than the combination, the result very likely relates to random variations in individuals. No evidence exists that either calcium ion or pentagastrin inhibits the effects of the other.

Experience in many laboratories and clinics over the years records the significance of CT changes after the injection of pentagastrin or the infusion of calcium. Only one false positive result has thus far been reported (1). The combination of agents is a potent stimulus of CT secretion and, as such, could produce more false positive responses than has been the case when the two pharmaceuticals were employed separately. However, if used primarily as a screening method, subsequent evaluations of suspicious results with one or both pharmaceuticals alone should provide additional perspective to the status of an individual.

Although calcium concentrations greater than 15 mg/dl are disquieting, these levels lasted only a few minutes and were well tolerated. Others have reported the increment of serum calcium concentrations after infusions of 2 mg/kg over one minute only as a mean value, 2.1 mg/dl (3); the maximum increments were undoubtedly higher. Nevertheless, if calcium is to be used as a stimulating agent, then, by whatever technique of administration — including 3 mg/kg over 10 minutes (4) and 15 mg/kg over 4 hours (13) — hypercalcemia must be an accepted consequence. With practice in giving 2 mg/kg over one minute, elevations of serum calcium above 14 mg/dl can be avoided. Perhaps a safer alternative to manual administration from a syringe would be infusing the calcium by a rate-controlling pump. Precautions should include a preliminary measurement of calcium concentrations to avoid adding calcium to an already abnormal level of blood calcium, a state not infrequently found in these patients who are at risk for hyperparathyroidism. In addition, calcium should be avoided in patients with bradyarrhythmias and in those taking digitalis preparations.

Silva, et al (14) have proposed that a measurement of the calcitonin/creatinine ratio in a single urine specimen will serve as the initial evaluation of harboring MCT. While simplified for the patient, the laboratory technique is complex. If assays on urine are to provide an acceptable rate of false negative results, many more patients will need to be studied by this procedure.

In this study we encountered no false negative results in any of the three provocative methods used. Nevertheless, since pentagastrin and calcium ion have given false negative responses when used alone (1), the use of both agents together is attractive, especially when the physician wishes to assure his patient about the absence of cancer and hereditary disease. Combining the two pharmaceuticals into a single method minimizes the patient's and physician's time, the amount of blood removed, and the cost of

calcitonin assays. In view of the need for reevaluation over many years, particularly for members of families who remain at risk, the method of combined agents has great appeal.

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