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We have produced a library of monoclonal antibodies of various affinities by immunizing mice with synthetic calcitonin (CT) 1-32. These monoclonal antibodies defined two antigenic determinants on the molecule of CT. The first was located in the 11-17 region of the hormone; the second was present on the 26-32 portion of CT. The C-terminal epitope was restricted to the mature form of the hormone and immunologically silent on synthetic peptides with sequences analogous to the biosynthetic precursors for CT. Using two high-affinity monoclonal antibodies, designated as CT07 and CT08, we developed a two-site immunoradiometric assay (m-IRMA) for serum CT. This m-IRMA provided a sensitivity of 10 pg/mL using a one-step overnight incubation at room temperature. Gel filtration analyses of serum samples from patients with medullary thyroid carcinoma (MTC) demonstrated that the CT07-CT08 m-IRMA was specific for the circulating mature form of CT. (Henry Ford Hosp Med J 1987;35: 129-32)

Since the discovery that medullary thyroid carcinoma (MTC) is a calcitonin (CT)-secreting tumor (1,2), numerous radioimmunoassays (RIAs) have been described for the measurement of CT in the serum (3,4). Heretofore, these RIAs were based on the use of polyclonal antisera. Obtaining an anti-CT antiserum is dependent on the structure of CT: a disulfide bridge links amino acids one and seven. Five out of these first seven amino acids are hydrophobic (Cys, Gly, Asn, Leu, and Cys), and this sequence is engaged in a ring of constrained conformation (5). This conjunction defines a region of poor immunogenic potential (6,7) and the production of antibodies directed against the 1-10 sequence has not been reported. In contrast, polyclonal antisera raised against synthetic CT recognize epitopes present on the 11-32 region of CT. Various groups have disagreed about normal immunoreactive CT levels in serum because the different immunoreactive species of CT present in the circulation are variably recognized by different CT assays (4). The selective measurement of monomeric CT was only possible using extraction-concentration methods (8) that remain difficult for a routine purpose.

The hybridoma technology (9) allows the production of monoclonal antibodies of particular interest in the construction of sensitive (10) and specific (11) immunoassays for tumor-associated antigens. To improve the detection methods for serum CT, we investigated the potential of monoclonal anti-CT antibodies for the construction of immunoassays for this molecule.

Methods

Synthetic peptides and immunogen

Synthetic human 1-32 CT was a gift from Drs. Scheibli and Andreatta (Ciba-Geigy, Basel, Switzerland). Synthetic peptides corresponding to various fragments of this hormone or to fragments of pro-CT were assembled by the solid phase method (12) using an Applied Biosystems (Foster City, CA) model 430A peptide synthesizer. The purity of these peptides was checked by reverse-phase, high-pressure liquid chromatography; amino acid composition was determined using amino acid analysis and/or fast atom bombardment spectrometry. Amino acid sequences of these peptides are depicted in Table 1. CT was conjugated to tetanus toxoid (TT) using glutaraldehyde as a coupling agent.

Production, selection, and characterization of monoclonal anti-CT antibodies

High-responder mice (13) were immunized with the CT-TT conjugate according to a previously described protocol (14). The mice received four injections consisting of 15 μg peptide each during a schedule of up to 90 weeks. Three days after the last intravenous injection of the conjugate, the splenocytes of the mice were fused with the myeloma cell line NS1 according to previously described experimental procedures (15). The supernatants of growing hybridoma cells were screened for anti-CT antibody production using a previously described (16) ELISA system with CT-coated immunotitration plates as a solid phase. Positive cells were cloned twice by limiting dilution and expanded in nude mice. Monoclonal antibodies were purified from ascites fluid using protein-A sepharose (Pharmacia, Uppsala,

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Table 1
Amino Acid Sequences of the CT-Related Synthetic Peptides Used in This Study: One Letter Code Was Used

CT 1-32	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP
CT 1-10	CGNLSTCMLG
CT 11-23	TYTQDFNKFHTFP
CT 17-32	NKFHTFPQTAIGVGAP
CT 24-32	QTAIGVGAP
CT 26-32	AIGVGAP
PQN-34	QTAIGVGAPGKKRDMSSDLERDHRPHVSMPQNAN
PTN-47	TYTQDFNKFHTFPQTAIGVGAPGKKRDMSSDLERDHRPHVSMPQNAN

Sweden) affinity chromatography (17). The isotype of monoclonal antibodies was determined as previously described (17). Epitope mapping of monoclonal anti-CT antibodies was performed using both 1) direct binding ELISAs with CT-, PQN-34, and PTN-47 coated plates; and 2) hapten inhibition experiments in which the residual anti-CT activity of a monoclonal antibody preincubated with various synthetic peptides was measured by ELISA (18).

The affinity constant of monoclonal anti-CT antibodies was calculated from RIA binding results using ^{125}I-CT (19) according to the method described by Van Heyningen et al (20).

Two-site immunoradiometric assay for CT

A two-site immunoradiometric assay for CT was developed as a "one step" simultaneous sandwich. A "capture" solid phase was prepared by incubating polystyrene beads (Precision Plastic Balls, Chicago, IL) with a 1:500 dilution of CT07-containing ascites fluid in phosphate buffer solution (PBS) pH 7.4. Radiolabeled indicator antibody was prepared by radioiodination of purified monoclonal antibody CT08 using the Iodogen reagent (21). Antibody-coated solid phase, CT-containing serum sample (200 μL), and radiolabeled indicator antibody (100,000 cpm as 50 μL in PBS) were simultaneously incubated overnight at room temperature. The beads were then washed with distilled water and bound radioactivity was measured in a gamma counter. Results were expressed as a signal-to-noise (S/N) ratio where noise was the mean cpm of negative controls run in the same experiment.

Immunoreactive profile in serum

Serum from patients with MTC was passed through a Sephadex G75 column equilibrated and eluted with a 0.1 M PBS, pH 7.4, containing 10 mM sodium acetate, 10 mM barbital and 0.1% sodium azide. Collected fractions were assayed using the CT07-CT08 assay. In some experiments, guanidine and/or dithiotreitol denaturation of eluates was performed according to the method extensively described by Goltzman and Tischler (22). The resulting products were again analyzed by gel filtration chromatography.

Results

A library of monoclonal anti-CT antibodies was produced. Among 178 culture supernatants positive for anti-CT antibody, six were selected that displayed a significant binding at a high dilution of supernatant in the ELISA system. The immunochemical characteristics of these antibodies are shown in Table 2. Hapten-inhibition experiments demonstrated that these antibodies bound to two antigenic regions on the molecule of CT (14). CT01, CT06, and CT08 monoclonal antibodies recognized an epitope located in the 11-17 portion of CT; CT02, CT03, and CT07 monoclonal antibodies recognized an antigenic determinant present in the 26-32 region of the hormone. The accessibility of these two epitopes on the biosynthetic precursors of the hormone was studied by both direct binding assay (Table 3) and hapten-inhibition experiments using synthetic peptides with sequence mimicking the C-terminus of pro-CT. The results of such experiments have been extensively reported elsewhere (18). Whereas the 11-17 antigenic determinant was found to be accessible on both the mature form of the hormone and biosynthetic precursors, the 26-32 epitope was found to be only expressed on the mature form of CT, ie, the 32 amino acid polypeptide bearing a proline amide as the C-terminal residue. Indeed, the binding of the CT02, CT03, and CT07 monoclonal

Table 2
Immunochemical Characteristics of Six Monoclonal Anti-CT Antibodies

Monoclonal Antibody	Isotope	Affinity Constant (M^{-1})	Antibody Binding Site
CT01	IgG_1	ND*	CT 11-17
CT02	IgG_1	5.8×10^8	CT 26-32
CT03	IgG_1	2.0×10^9	CT 26-32
CT06	IgG_1	1.1×10^{10}	CT 11-17
CT07	IgG_2	0.9×10^{10}	CT 26-32
CT08	IgG_1	3.0×10^{10}	CT 11-17

*Not determined: CT01 did not bind to ^{125}I-CT.

Table 3
Binding of Anti-CT Monoclonal Antibodies to Synthetic Peptide-coated Plates in the ELISA System

Monoclonal Antibody	Coated Peptide		
	CT 1-32	PQN-34	PTN-47
CT02	1.495	< 0.010	< 0.010
CT03	1.510	< 0.010	< 0.010
CT07	1.805	< 0.010	< 0.010
CT08	1.950	< 0.010	1.850

Results are expressed as absorbance at 492 nm after subtraction of the nonspecific background. Monoclonal antibodies were tested at a $1:10^4$ dilution of culture supernatant.

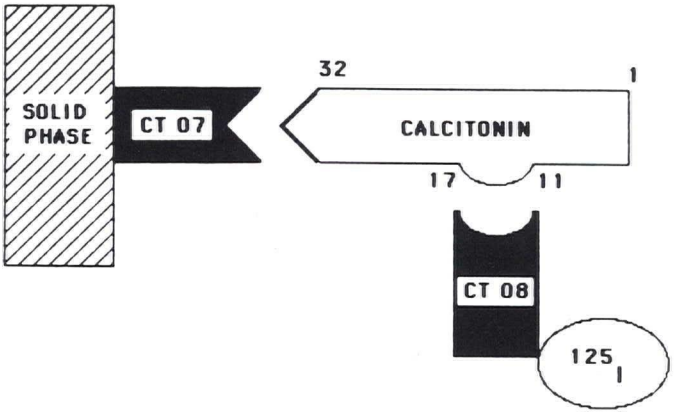

Fig 1—Schematic representation of the construction of the two-site monoclonal immunoradiometric assay for serum CT used in this study.

antibodies to the 26-32 epitope was found to be dramatically dependent on the presence of the carboxamide group on the C-terminal proline residue. In the sequence of pro-CT, this residue is bound via a peptide linkage to a glycine residue (23), and the C-terminal epitope is not accessible for antibody binding.

Using this library of monoclonal anti-CT antibodies directed against two distinct epitopes to develop a multisite radioimmunoassay, we studied the ability of two monoclonal antibodies to bind simultaneously to the molecule of CT. From these studies we concluded that the simultaneous binding of two monoclonal antibodies to CT was possible and mainly dependent on the affinity constant of both the antibody used as a capture solid-phase, and the antibody used as a radiolabeled indicator. These data led us to use CT07 as a capture antibody and CT08 as a radiolabeled indicator antibody in the development of a two-site immunoradiometric assay for CT (Fig 1). The binding of ^{125}I-CT08 to CT linked to the solid phase through CT07 was studied at different temperatures. At 20°C, equilibrium was reached after an 18-hour incubation (Fig 2). The limit of detection of the assay was 10 pg/mL.

The profile of serum immunoreactive CT detected using the CT07-CT08 m-IRMA was assessed by gel filtration analysis of serum from MTC patients. A typical result is shown in Fig 3. Several peaks of immunoreactivity were detected when analyzing the native serum; one corresponded to monomeric CT and the other to higher molecular weight (HMW) species. The composition of these HMW species was studied using chemical denaturation. The fractions corresponding to the first peak (eluting with the void volume of the column) were pooled (pool 1) and denatured using the guanidine-dithiotreitol protocol; the fractions of intermediate molecular weight were pooled (pool 2) and only submitted to the dithiotreitol denaturation. After these treatments, immunoreactive CT eluted at the elution volume of ^{125}I-CT. These data establish that the different CT molecular forms detected by the CT07-CT08 m-IRMA correspond to mature CT, to polymers of mature CT linked by disulfide bonds, and to mature CT bound to irrelevant proteins. In conclusion, 1) the immunochemical characteristics of CT07 monoclonal

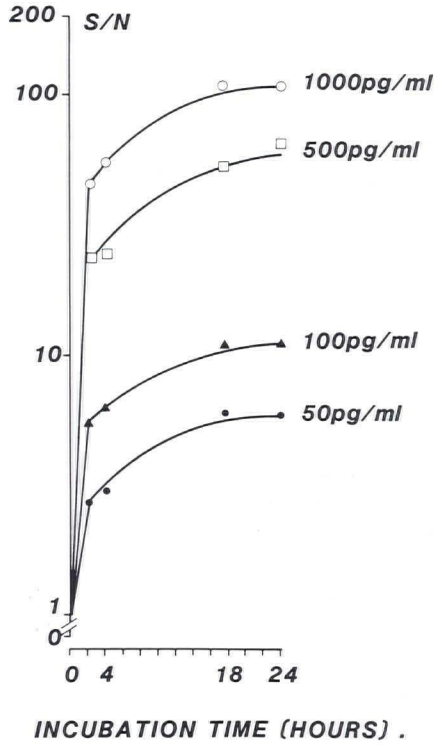

Fig 2—Kinetics of binding of the monoclonal m-IRMA at room temperature using various doses of CT.

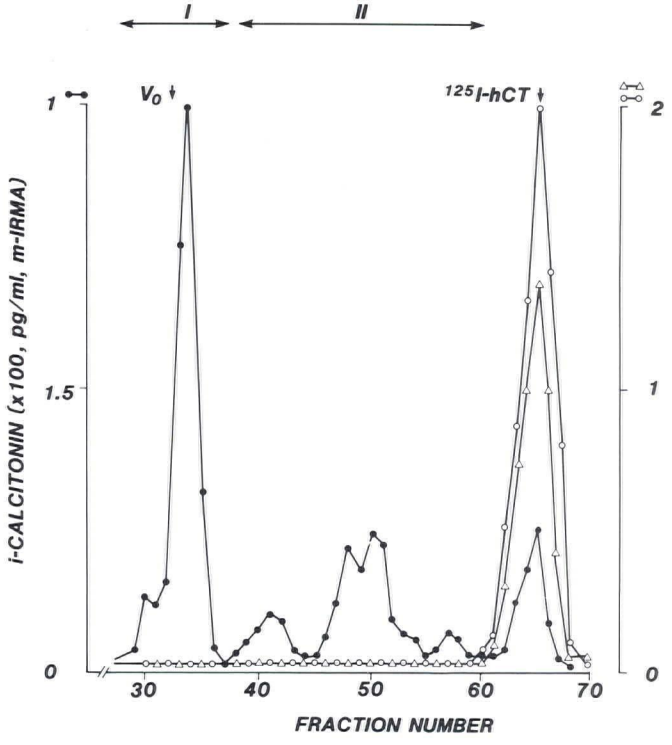

Fig 3—Gel filtration analysis of a serum sample from a patient with MTC using the CT07-CT08 m-IRMA for CT. (●), immunoreactive profile of the native serum; (△), immunoreactive profile obtained after denaturation of pool I using the guanidine/dithiotreitol protocol; (○), immunoreactive profile obtained after denaturation of pool II using the dithiotreitol protocol.

antibody, and 2) the data from gel filtration analysis of serum samples led us to the conclusion that the CT07-CT08 based m-IRMA is specific for the mature form of CT.

Discussion

The immunochemical heterogeneity of circulating CT has hampered the standardization of serum CT levels among different laboratories. Indeed, various sequential-saturation radioimmunoassays differ in the immunochemical characteristics of the polyclonal antiserum used in the construction of the assay, resulting in various normal ranges for CT and multiple immunoreactive profiles in the serum. In an attempt to develop new methods for both the measurement of CT and the diagnosis of MTC, we have produced a library of monoclonal anti-CT antibodies. These antibodies of various affinity constants defined two antigenic regions on human CT, one located in the internal sequence (11-17), and the other localized in the C-terminal region (26-32). By studying the molecular requirements for epitope-paratope binding, we found that the C-terminal epitope was restricted to the mature form of the hormone. Then, monoclonal antibodies directed against this epitope, particularly the high affinity CT07 antibody, provided tools for the selective recognition of the mature CT in the circulation. A solid-phase immunoradiometric assay was developed using CT07 (anti-CT 26-32) and CT08 (anti-CT 11-17) as capture antibody and indicator antibody, respectively. Such an assay was found to be sensitive (10 pg/mL), rapid (overnight incubation), practicable (one-step technique), and specific for the mature form of CT in the serum. The clinical relevance of this assay has been studied and the results showed that the m-IRMA allowed an earlier detection of either MTC or recurrence of the disease than did the conventional antiserum-based radioimmunoassay (24).

Since successful treatment of MTC requires an early diagnosis, reliable methods for the measurement of CT are of particular importance. The monoclonal antibody-based immunoradiometric assay described in this report may offer new insights in the diagnosis of this tumor.

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