THE USE OF REGION SPECIFIC RADIOIMMUNOASSAYS FOR CHARACTERIZATION OF CIRCULATING CALCITONIN IN PATIENTS WITH MEDULLARY CARCINOMA OF THE THYROID GLAND

By

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ABSTRACT

Two antisera with known region specificities have been used to characterize calcitonin immunoreactivity (iCT) in serum of patients with medullary thyroid carcinoma (MCT). Antisera I which was raised against the synthetic hormone (1–32 amino acid residues), contained heterogeneous populations of immunoglobulins directed predominantly against carboxy-terminal sequences of the hormone, but the antisera reacted also with the amino-terminal fragment (1–10 amino acid residues). Antiserum II, which was raised against the carboxy-terminal hormone fragment (11–32 amino acid residues) reached equally well with the intact hormone and the C-terminal fragment, but showed negligible binding of the amino terminal fragment. Antiserum I measured therefore both amino-terminal and carboxy-terminal sequences of calcitonin while antisera II measured only carboxy-terminal amino acid sequences.

In 40 patients with MCT, antiserum I measured usually the highest concentration of serum iCT suggesting the presence of non-uniform hormone immunoreactivity. The different molecular forms of circulating iCT in 7 MCT patients were explored by using antisera I after gel filtration on Sephadex G-100. The patients who were selected on basis of iCT measurement in serum using antisera I and II, could be divided into 3 groups which showed characteristic iCT profiles. Group 1, in which antiserum II measured a higher concentration of serum iCT, contained predominantly (60–70 %) small fragments of calcitonin immunoreactivity. On the other hand, in the sera of group 3 in which antisera I measured an equal or the highest concentrations, the dominant form of the hormone...
The hypocalcaemic and hypophosphataemic peptide hormone, calcitonin, is present in the circulation of patients with medullary carcinoma of the thyroid gland (MCT) as a non-uniform group of immunoreactive substances (Neher et al. 1968; Deftos et al. 1975; Sizemore & Heath III 1975; Snider et al. 1977; Silva et al. 1977). The demonstration at various forms of circulating immunoreactive calcitonin (iCT) is critically dependent on the specificity and the sensitivity of the antiserum used. Therefore, the partly controversial results that have been presented regarding the nature of iCT in serum may be due to differences in the antigenic specificities of various antisera which have been incompletely characterized.

In the present study we have used two antisera with known antigenic specificities and measured serum iCT in patients with chronic hypercalcitoninaemia due to MCT. In addition, 7 sera from MCT-patients have been subjected to gel filtration in order to characterize further the molecular forms of immunoreactive calcitonin.

We demonstrate that serum iCT is heterogeneous and consists mainly of peptides with carboxy-terminal sequences. Moreover, gel filtration of sera revealed characteristic iCT profiles when an antiserum which reacted with both amino-terminal and carboxy-terminal regions of CT, was used. These 7 patients had all, but one, normal concentrations of immunoreactive parathyroid hormone in serum, and they were normocalcaemic.

**MATERIALS AND METHODS**

*Serum sampling*

Sera from 40 patients with medullary carcinoma of the thyroid gland were obtained during fasting conditions and drawn from the antecubital vein. The patients were 20-60 years old and consisted of 26 females and 14 males.

*Production of antisera*

Antisera against the intact hormone (1-32 amino acid residues) and against the carboxy-terminal fragment (11-32 amino acid residues) were produced in rabbits after
repeated intracutaneous injections of synthetic peptides (40–200 μg/injection) (Ciba-Geigy, Limited). Antiserum I was raised against the intact hormone and used in a final dilution of 1 : 75 000. Antiserum II was raised against the carboxy-terminal fragment and used in a final dilution of 1 : 3000.

Characterization of the antisera

Antiserum I contained populations of antibodies with affinities directed against both amino-terminal and carboxy-terminal sequences of calcitonin. Antibodies which reacted with amino-terminal regions of CT were demonstrated in two ways using the intact hormone as tracer. Firstly, displacement of [125I]CT (1–32) from antibody binding by increasing amounts of CT (1–10) was studied. Secondly, the antiserum was pre-incubated with the carboxy-terminal fragment (25 ng/tube) and subsequently used in a standard curve with the intact hormone as tracer and standard. Immunoglobulins with specificities towards the N-terminal part of calcitonin would then, if present, give rise to a standard curve. Antibodies with affinities directed against carboxy-terminal parts of CT were demonstrated in a similar way. Both the binding of [125I]CT (11–32) to antiserum I and the ability of the carboxy-terminal CT fragment to displace [125I]CT (1–32) from antibody binding were tested. Pre-incubation experiments were then carried out where antiserum I and excess CT (1–10) were mixed and such “blocked” antiserum tested for the presence of antibody binding against the intact hormone. The amount of carboxy-terminal iCT present in serum was measured in a system using antiserum II which was raised against the carboxy-terminal fragment [125I]CT (11–32) and employing the same fragment as standard.

The immunoreactivity of antiserum I and antiserum II were tested against the following hormones which were used in amounts of 10 ng and 100 ng per tube: TRH, Gn-RH, TSH, FSH, LH, PRL, GH, insulin, secretin, glucagon, gastrin, bovine pancreatic polypeptide, human gastrointestinal polypeptide and neurotensin. Neither of these hormones showed any cross-reactivity in the radioimmunoassay using antiserum II. However, FSH gave a small displacement of [125I]CT (1–32) from antiserum I, but the curve was not parallel to the standard curve. Moreover, both antisera failed to detect iCT in sera which had been absorbed with charcoal or were derived from athyreot non-MCT-patients.

Radioimmunoassay of calcitonin (1–32 amino acid residues) and the carboxy-terminal fragment (11–32 amino acid residues)

The intact hormone and the carboxy-terminal fragment were labelled with 125I (Amersham, England) as described previously by Gautvik et al. (1976). The hormones were measured in a non-equilibrium radioimmunoassay where the lower limit of detection was 0.05 μg/l (0.1 × 10−10 mol/l) and the upper normal border set to be 0.5 μg/l (1.4 × 10−10 mol/l) when tested with antiserum I (Gautvik et al. 1976).

Radioimmunoassay of parathyroid hormone

A highly purified preparation of bovine parathyroid hormone (Wilson & Co., USA) was labelled with 125I (Amersham, England) as described by Gautvik et al. (1979). The hormone was measured in a non-equilibrium radioimmunoassay where the lower limit of detection was 0.1 μg/l (0.11 × 10−10 mol/l) and upper normal border in normocalcaemic persons, is set to be 0.5 μg/l (0.66 × 10−10 mol/l (Gautvik et al. 1979).
a) The binding of labelled intact hormone, $[^{125}\text{I}]\text{CT} \,(1-32)$ and of the carboxy-terminal hormone fragment, $[^{125}\text{I}]\text{CT} \,(11-32)$ using different dilutions of antiserum I m I which was raised against the intact hormone. Binding expressed as per cent bound und of total radioactivity added.

b) The binding of $^{125}\text{I}$-labelled intact and carboxy-terminal hormone fragment using sing different dilutions of antiserum II, which was raised against this fragment.
**Gel filtration**

Serum from patients with medullary carcinoma was concentrated by lyophilization 2 to 3 times before gel filtration on Sephadex G-100 columns (1.2 x 40 cm or 0.9 x 80 cm) after application of 1 ml aliquots. The elution buffer was 0.1 mol/l Tris-HCl (Sigma), pH 7.5 containing 0.1 % egg albumin. The columns were calibrated with different reference proteins: rat glandular kallikrein (molecular size about 34,000), bovine parathyroid hormone (molecular size about 9500) as well as with labelled calcitonin (1-32) (molecular size about 3500) and calcitonin fragment (11-32) (molecular size about 2000). Fractions of 1 ml were collected and the presence of iCT measured, using antiserum I since it turned out that also amino-terminal CT sequences were recognized by this antiserum.

**RESULTS**

**Characterization of the specificity of the two antisera used**

Fig. 1a and 1b show the binding of $^{125}$I-labelled intact hormone (1-32 amino acid residues) and of the $^{125}$I-labelled carboxy-terminal fragment (11-32 amino acid residues by antiserum I and II which were raised against the intact hormone and against the carboxy-terminal fragment, respectively. Both antisera were able to bind the labelled peptides. Fig. 2a shows standard curves obtained with antiserum I, and Fig. 2b shows similar curves using antiserum II. Antiserum I gives typical standard displacement curves when both the intact hormone and the carboxy-terminal fragment are used in competition with $^{125}$I]CT (1-32). In addition, a partial displacement of $^{125}$I]CT (1-32) from antiserum I was observed using the amino-terminal fragment (1-10 amino acid residues). At low concentrations of this fragment, the curve was parallel with the other standard curves, but its levelling out indicated the presence of several populations of antibodies with non-uniform antigen specificities. Antiserum II showed parallel standard curves using the intact hormone and the carboxy-terminal fragment, but no significant immunological reactivity was demonstrated against the amino-terminal hormone fragment (Fig. 2b).

When antiserum I was pre-incubated with an excess carboxy-terminal CT fragment and then tested using the intact hormone both as label and standard, a large reduction in binding of $^{125}$I]CT (1-32) was observed and no standard curve could be produced (Fig. 3). A smaller reduction in binding of $^{125}$I]CT (1-32) was observed when antiserum I was blocked with excess of amino-terminal CT fragment, and a standard curve could still be produced. These findings combined suggested that antiserum I contained antibodies with non-uniform antigen specificities directed against both amino-terminal and carboxy-terminal sequences. In contrast, antiserum II would only measure carboxy-terminal CT sequences.
Fig. 2.

a) Standard curve obtained with antiserum I used in final dilution 1:75,000. The percent bound $[^{125}I]CT$ (1–32) in the zero sample is set as 100%.

b) Same as 2a, but using antiserum II in final dilution 1:3000.
Standard curves obtained with antiserum I after pre-incubation with excess amino-terminal CT fragment (1-10 amino acid residue) or carboxy-terminal fragment (11-32 amino acid residue) 25 ng/tube. [¹²⁵I]CT (1-32) and CT (1-32) were used as tracer and standard and the results expressed as per cent bound compared to total radioactivity added.

Calcitonin immunoreactivity in serum of patients with medullary carcinoma of the thyroid gland

Fig. 4 shows the measurement of iCT in serum from 40 patients using antiserum I with [¹²⁵I]CT (1-32) as tracer and intact CT (1-32) as standard, and using antiserum II with the carboxy-terminal fragment as tracer and standard. The antiserum raised against the intact hormone (antiserum I) measured in 35 out of 40 sera the highest hormone concentration. These results thus indicated that in most patients serum calcitonin immunoreactivity is heterogeneous and that peptides with both carboxy-terminal and amino-terminal sequences may be present.

Parathyroid hormone immunoreactivity (iPTH) in serum from patients with MCT

Sera from 36 MCT-patients were analyzed for iPTH and 33 had values
Serum calcitonin in patients with medullary carcinoma of the thyroid gland, measured with the two different antisera (Ab I and Ab II). The initials correspond to the sera which were gel filtered as shown in Figs. 5, 6 and 7.

- - - - - - = lower level of detection.

below 0.6 μg/l which is set to represent the upper normal limit (Gautvik et al. 1979). Only 3 out of the 36 patients had elevated iPTH. Other endocrine disorders were not found in these patients.

**Further characterization of the different molecular forms of iCT in serum from MCT patients**

Gel filtration on Sephadex G-100 of 7 different sera from patients with MCT gave three different elution profiles that are represented in Figs. 5–7. Fig. 5 shows that 3 of these sera contain, as the major immunoreactive form of CT, molecular species similar in size to the carboxy-terminal fragment. The
Gel filtration of MCT-sera (S.J., A.G., and I.B.) on Sephadex G-100 column (1.2 cm × 40 cm) and measurements of iCT in fractions using antiserum I. One ml fractions were collected and the elution of the radioactively labelled marker substances is indicated.

Fig. 5.

presence of carboxy-terminal fragments in these sera dominate to such an extent that antiserum II measured a higher concentration of iCT in serum than antiserum I (Fig. 4). In comparison, Fig. 6 shows two elution profiles where the major part of iCT reactivity is found more evenly distributed corresponding to peptides of molecular sizes between the intact hormone and the carboxy-terminal fragment. In these patients, antiserum I and II measured an equal concentration of serum iCT (Fig. 4). Fig. 7 shows a third elution profile where the major part of iCT elutes as molecules larger or equal to the intact hormone. In these patients antiserum I measured higher or similar concentration of serum iCT when compared to antiserum II (Fig. 4). Table 1 shows the distribution profile of serum iCT for the 7 MCT-patients given in per cent of total immunoreactivity in each serum so that the different patterns are easier to recognize.
Fig. 6.
Gel filtration of MCT-serum (G. W. and K. T. P.) on Sephadex G-100 column (1.2 cm × 40 cm) and measurements of iCT in fractions using antiserum I. See legend to Fig. 5 for further explanation.

Fig. 7.
Gel filtration of MCT-serum (S.G. and E.P.) on Sephadex G-100 column (0.9 cm × 80 cm) and measurements of iCT in fractions using antiserum I. For explanation see legend to Fig. 5.
Table 1.
Elution pattern of iCT after gel filtration on Sephadex G-100

<table>
<thead>
<tr>
<th>Patients</th>
<th>Percentage distribution of calcitonin immunoreactivity according to molecular size</th>
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<tr>
<td>Group 1</td>
<td></td>
</tr>
<tr>
<td>(small</td>
<td></td>
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<tr>
<td>fragments</td>
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<tr>
<td>I.B.</td>
<td>0</td>
</tr>
<tr>
<td>S.J.</td>
<td>3</td>
</tr>
<tr>
<td>A.G.</td>
<td>13</td>
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<tr>
<td>Group 2</td>
<td></td>
</tr>
<tr>
<td>(intermediate sizes)</td>
<td></td>
</tr>
<tr>
<td>K.T.P.</td>
<td>1</td>
</tr>
<tr>
<td>G.W.</td>
<td>4</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
</tr>
<tr>
<td>(mono- and polymeric forms)</td>
<td></td>
</tr>
<tr>
<td>S.G.</td>
<td>54</td>
</tr>
<tr>
<td>E.P.</td>
<td>71</td>
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</tbody>
</table>

**DISCUSSION**

It is generally accepted that iCT is measurable in sera from normal subjects, although the absolute concentrations are not agreed on (Clark et al. 1969; Gudmundsson et al. 1969). Probably it is less than 0.45 μg/l (Gautvik et al. 1976; Pathemore & Deftos 1975), possibly as little as 0.1 μg/l (Deftos 1971; Deftos et al. 1971). The use of antisera with varying specificities and affinities will result in measurements of different concentrations of iCT in the same sample and this may explain some of the controversies regarding the range of serum iCT in normal individuals. Antisera with defined region specificities will not only make estimates of total hormone concentrations more meaningful, but is a necessary tool in order to identify the hormone related peptides which are present in circulation. This is highly relevant for serum iCT since carboxy-terminal fragments of the hormone are usually responsible for the main part of the measured immunoreactivity (Figs. 5 and 6). Antiserum I, which was raised against the intact hormone, contains populations of immunoglobulins which predominantly react with carboxy-terminal amino acid sequences. With this antiserum the 11–32 amino acid residue fragment of CT gives complete cross-reactivity in the radioimmunoassay for the intact hormone. Furthermore, when antiserum I is pre-incubated with an excess carboxy-terminal fragment prior to use in the radioimmunoassay, most of the immunoreactivity (about 85%) is lost (Fig. 3). However, the amino-terminal fragment (1–10 amino acid residues) does also show some degree of cross-reactivity using antiserum I, and
a fraction of the anti-CT immunoglobulins can be blocked by pre-incubation with this fragment (Fig. 3). Thus, antibodies with both carboxy-terminal and amino-terminal specificities are present in this antiserum and may account for the higher concentration of serum iCT usually measured in patients when compared to the carboxy-terminal directed antiserum II (Fig. 4). Also, if larger circulating calcitonin fragments with amino-terminal sequences exist, they will have a better immunoreactivity than the isolated 1–10 amino acid residue and will preferentially be recognized by antiserum I.

As expected, antiserum II shows a negligible binding to the amino-terminal calcitonin fragment as exemplified in displacement experiments, but gives equally well standard curves with the intact hormone and the carboxy-terminal fragment (Fig. 2b). Since most sera from patients with MCT shows a higher measurable concentration of serum iCT with antiserum I than with antiserum II (Fig. 4), this implies that hormone sequences other than those present in the 11–32 carboxy-terminal fragment frequently circulate. By using the combination of these characterized antisera and gel filtration, it is possible to examine the molecular forms of circulating iCT in MCT patients. The patients were chosen as to represent sera with equal concentration of iCT measured with antiserum I and antiserum II, sera in which antiserum I measured the highest concentration and sera in which antiserum II measured the highest concentration, respectively. This selection resulted in three different serum profiles as characterized by the dominant molecular form of calcitonin immunoreactivity. When the two antisera measure an equal amount of hormone, gel filtration shows that the predominant form of serum iCT is peptides of intermediate sizes between the intact hormone and the carboxy-terminal fragment (> 45% of total iCT).

However, in sera in which smaller peptides of sizes in the range of the carboxy-terminal fragment or less dominate (> 60% of total iCT), antiserum II detected the highest concentration of iCT. Finally, those sera in which antiserum I measures an equal or the highest concentration, poly- and monomeric forms of iCT dominate (more than 90%). However, the distribution of CT immunoreactivity obtained by gel filtration will depend upon the characteristics of the antiserum used. The seven gel filtered MCT-sera showed that only a fraction of the total circulating iCT had molecular size comparable to the intact hormone, and one patient did not have any (Table 1). These results are in agreement with Singer & Habener (1974) who found that only 9.7–44% of total iCT coincided with the elution of 125I-labelled synthetic calcitonin.

The chemical structure of the different circulating CT forms is unknown as is the biological mechanisms underlying the variation in circulating iCT profiles in patients with MCT. The appearance of serum iCT as a non-uniform group of polymeric and monomeric forms as well as peptide fragments may reside in different forms of CT being secreted and/or to peripheral degradation of the hormone.
However, this striking heterogeneity in the serum CT immunoreactivity is not related to the extent of disease, since the variation of total serum iCT within groups 1, 2 and 3 was greater than the between group variation. Also, it was not related to the presence of hyperparathyroidism since iPTH was elevated in only one of the patients in group 1 and the serum iCT profile was similar to the others in this group. Even if the molecular profile of circulating iCT showed this extensive variation, all serum iCT had low or no biological activity since the patients were normocalcaemic without hyperparathyroidism and since serum was unable to cause hypocalcaemia when injected to rats (Myhre & Gautvik, in preparation).

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