Heterogeneity of immunoreactive calcitonin in human milk

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Abstract. Immunoreactive calcitonin (ICT) has been detected in human milk by radioimmunoassay (RIA), using antibodies raised against synthetic human calcitonin (hCT). The level of ICT was 10–40 times higher than the reference level of human serum. Gel chromatography of human milk disclosed a molecular weight heterogeneity of ICT, with at least two forms larger than the monomer (Mr > 70 000 and 30 000 approximately). The 30 000 peak constituted the major fraction of ICT. No detectable amount of ICT was coeluted with [125I]CT. Reduction with disulphide cleaving agents, denaturation or acidification did not alter the gel chromatographic properties of ICT, while tryptic digestion caused partial degradation of ICT and formation of new ICT molecules with an approximate molecular weight of 6000.

The mechanism concentrating calcium to the calcium-rich human milk is not known but supposed to be endocrine. Calcitonin has been found to increase the renal excretion of calcium (Ziegler et al. 1967). In analogy with this finding we suspected calcitonin to be of possible importance for the calcium enrichment in human milk. Thus in search for a hormonal prerequisite for a calcium concentration mechanism we have found high concentrations of immunoreactive calcitonin in human milk (Bucht et al. 1980), later confirmed in our laboratory (Werner et al. 1982). In the present paper we report the presence of ICT of heterogenous molecular weight in human milk.

Materials and Methods

Milk was obtained from 37 healthy women during the first week after delivery. The samples were stored at −20°C and radioimmunoassay (RIA) was performed within 3 weeks after collection. Before RIA and gel filtration the milk was centrifuged at 2000 × g for 15 min at 4°C.

RIA-method

ICT was measured with a RIA kit from Immuno Nuclear Corporation, Stillwater, Minnesota. The antibodies were produced in rabbits against pure synthetic human calcitonin and were specific for the amino acid sequence 17 to 32. The antibodies had no significant cross-reactivity with parathyroid hormone, insulin, thyroid stimulating hormone, adrenocorticotropic hormone, growth hormone or prolactin.

Synthetic hCT was used as a standard and for labelling. The RIA was a sequential saturation method. After pre-incubation of a 0.1 ml sample or standard with 0.2 ml antiserum for 24 h at 4°C, 0.1 ml [125I]CT was added, followed by another 24 h incubation at 4°C. Bound and unbound radioactivity was separated by precipitation in 0.5 ml goat anti-rabbit precipitating reagent, consisting of pre-precipitated goat anti-rabbit immunoglobulin antiserum, normal rabbit serum and polyethylene glycol (6–8000) in a 0.4% BSA in 0.1 M borate buffer, pH 8.4 (RIA buffer). The milk was at most occasions diluted 1:6 with RIA buffer or CT-free serum before RIA. The sensitivity of the assay was 1.5 pg/tube (15 pg/ml). The intra-assay coefficient of variation was 10% and the inter-assay variation 15%.

Dilution curves were obtained by diluting milk in CT-free serum. Synthetic hCT was used as a reference.

Stability of [125I]CT in milk

To test whether milk contains proteolytic substances capable of degrading [125I]CT, samples of milk were incubated with the tracer for 24 h at 4°C. After incubation the [125I]CT was gel chromatographed on a Sephadex G-50 column (1.5 × 80 cm) at 4°C. The column was equilibrated and developed with 0.05 M phosphate buffer, pH 7.4 with 0.2% NaNO₃ and 0.2% bovine serum

Acidification. Reduction weight Dissociation before samples Attempts captoethanol ized, G-50 Three experiments were described the final albumin ing G-50 compared 80 ml of milk. Size heterogeneity of ICT Milk was gel chromatographed on a column of Sephadex G-50 (1.5 x 80 cm) and a Sephadex G-75 column (2.0 x 80 cm). The columns were equilibrated and developed with 0.1 M ammonium acetate, pH 6.8 and 3 ml fractions were collected, lyophilized and re-suspended in the RIA buffer.

The columns were calibrated with dextran blue (V₀), ovalbumin, chymotrypsinogen A, ribonuclease A, [123I]-insulin, [125I]ICT, unlabelled synthetic hCT and sodium chloride (Vₕ). Synthetic hCT was supplied by Ciba Geigy.

Dissociation studies of ICT
Attempts were made to dissociate the high molecular weight forms of ICT in milk by acidification, reduction and denaturation.

Acidification. 0.3 ml 5 M acetic acid was added to 1.2 ml milk (final pH 3.2). After 2 h incubation at 4°C the samples were centrifuged at 2000 x g for 15 min and chromatographed with 1 M acetic acid on a Sephadex G-50 column. The fractions were lyophilized, neutralized, re-lyophilized and re-suspended in the RIA buffer before measurement of ICT by RIA.

Reduction and denaturation. Milk was treated with either: 1) dithiothreitol (10 mM, final concentration); 2) mercaptoethanol (10%, final concentration); or 3) urea (6 M, final concentration). The samples were incubated overnight at 4°C. After centrifugation at 2000 x g for 15 min the samples were applied to a Sephadex G-75 column as described above.

After gel filtration the fractions from all experiments were analysed for ICT by radioimmunoassay. Control experiments consisted of gel chromatography of each agent used in the dissociation studies on the corresponding columns and analysis of ICT by RIA were made on the fractions obtained.

Digestion with trypsin
Three ml of milk was lyophilized and re-dissolved in 1.0 ml of 0.1 M Tris/HCl, pH 8.2, containing 2 mM-CaCl₂. One mg Trypsin (Merck, 2000 U/g) dissolved in the Tris/HCl buffer was then added and digestion was allowed to proceed for 4 h at 37°C. The reaction was stopped by lowering the pH to pH 2-3 with 1 M HCl. After centrifugation the sample was applied to a Sephadex G-50 column as described above and the fractions were analysed for ICT by RIA. In a control experiment trypsin was filtered on the column and RIA was performed on the fractions.

Results
Methodological aspects
Dilution curves of milk were superimposable on those of synthetic hCT (Fig. 1). Incubation of [125I]ICT in milk did not alter the chromatographic properties of the [125I]ICT (Fig. 2) or the binding of [125I]ICT to the antibodies. Synthetic hCT added to milk was completely recovered, in all three different concentrations. The regression line y = ax + b gave y = 1.08 x - 0.88, where y = measured CT (pg/ml) in milk, a = slope; x = CT amounts added to the milk; b = intercept.

Fig. 1.
Standard curve for the RIA-determination of synthetic hCT and five different dilutions of milk.
Elution volume(ml)

**Fig. 2.**
Gel chromatography on Sephadex G-50 of 1) $^{125}$I-CT and 2) $^{125}$I-CT after 24 h incubation in milk. Aliquots of the peak fractions from $^{125}$I-CT and $^{125}$I-CT incubated in milk were subjected to RIA. In both cases 28% of the $^{125}$I-CT was bound to the antibodies thus indicating unaltered immunoreactivity of $^{125}$I-CT after incubation in milk.

**Levels of ICT**
The level of ICT in milk was 2.3 ± 1.1 ng/ml (mean ± SD) (range 0.5–5.1 ng/ml, N = 37), i.e. 10–40 times the reference serum value (<0.13 ng/ml). The levels were highest in the first produced milk and declined during the first week after delivery.

**Heterogeneity of ICT**
Gel chromatography of milk on Sephadex G-75 yielded at least two peaks of ICT larger than the monomer. Fig. 3 shows a representative pattern of ICT from milk. The largest form had a molecular weight of at least 70,000 daltons. The major peak eluted in the region between ovalbumin and chymotrypsinogen A with an approximate molecular weight of 30,000 daltons. No apparent peak of ICT was eluted with the volume corresponding to $^{125}$I-CT. The total recovery of ICT was 80–90%.

**Dissociation studies**
Neither acidification nor treatment with reducing or denaturing agents altered the elution profile of ICT as revealed by gel chromatography on Sephadex G-75.

**Digestion with trypsin**
After treatment of milk with trypsin about 35% of the high molecular weight forms was cleaved to smaller molecules that eluted in a region close to $^{125}$I-insulin (Fig. 4). The digestion resulted in a 5–10% loss of immunoreactivity. Trypsin showed no interference in the RIA system when RIA was performed on the fractions eluted from Sephadex G-50.
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This was used: pre-incubated after treatment with trypsin. The following markers were used: dextran blue (V₀), [¹²⁵I]insulin (a), [¹²⁵I]ICT (b) and sodium chloride (Vₛ). The detection limits of the assay are indicated by dotted lines.

Discussion
We have found that human milk contains large amounts of ICT. There seems to be an immunoreactive similarity between synthetic hICT and ICT in milk since dilution curves of milk were superimposable on CT standard curves in RIA when the milk was diluted 1:4 or more.

The prerequisite for this study was that CT is stable in milk. This seems to be the case, as [¹²⁵I]ICT was not degraded during 24 h of incubation in milk, as judged by gel filtration, and as the binding of pre-incubated [¹²⁵I]ICT to the antibody was unchanged. Added synthetic hICT was quantitatively recovered from milk. The stability of CT in milk could be explained by the presence of protease inhibitors (Lindberg 1979).

Gel chromatography disclosed molecular heterogeneity of the immunoreactive forms of ICT in milk. At least two forms of ICT larger than the monomer seem to exist. Size heterogeneity of ICT is known to exist in blood plasma (Singer & Habener 1974; Sizemore & Heath 1975) and urine (Snider et al. 1978) from patients with medullary thyroid carcinoma (MTC). Extracts from MTC tissue (Heath & Sizemore 1979) and culture medium exposed to MTC cells (Goltzman & Tischler 1978) also contain ICT forms with different molecular weight. Serum or plasma from patients with other forms of cancer, for example lung cancer, also shows heterogeneity of ICT (Becker et al. 1978).

The large forms of ICT may represent aggregates of the monomer, non-covalent binding of the monomer to another protein, high molecular weight complexes of CT formed by disulphide bridge formation or a complex of the monomer linked to a protein by disulphide bridges. They may also constitute precursor forms of CT. Goltzman & Tischler (1978) found that the largest forms of ICT probably constitute complexes of CT resulting from intermolecular disulphide bridge formation or CT bound to a protein by disulphide bridges. The structures of the ICT with intermediate molecular weight are still unknown.

In our study there was no dissociation with reducing or denaturing agents or by acidification of the large forms of ICT. Thus the ICT in milk does not seem to be bound to a carrier or to be an aggregate with S-S bridges or otherwise polymerized CT. The possibility remains that these forms may constitute precursor forms of CT. High molecular weight precursors of CT have been described by Jacobs et al. (1979) in cod and rat and by Goodman et al. (1979) in man.

Proteolytic cleavage of milk ICT produced smaller fragments with intact immunoreactivity. This indicates that the large ICT forms may be susceptible to biological transformation in a proteolytic environment i.e. the gastro-intestinal tract of the suckling baby. If so, ICT in milk may be of importance for gastro-intestinal function in the newborn infant.

The origin of ICT in human milk is not clarified by the present work even though the high concentration might indicate a local production.
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