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Novel plasma extraction procedure and development of a specific enzyme-immunoassay of oxytocin: application to clinical and biological investigations of small cell carcinoma of the lung

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Paraneoplastic secretion of the lactation-inducing hormone oxytocin (OT) has been reported in about 30% of cases of small cell carcinoma of the lung (SCCL). In order to investigate the role of OT in the biology of SCCL tumours, a specific enzyme-immunoassay (EIA) for OT, which can be applied to both human plasma and culture medium, has been developed. OT EIA is performed on 96-well microtiter plates coated with a rabbit polyclonal antibody (Ab) anti-OT (O4). This antibody does not exhibit any significant cross-reactivity either with vasopressin (VP) or with vasotocin (VT). The immunological reaction involving Ab anti-OT is a competition between the tracer (biotinylated OT) and synthetic OT (standard curve) or OT present in biological samples. In order to limit interference induced by plasma proteins, plasma samples are filtrated by a one-step centrifugation on centricon YM-3 (cut-off 3000 Da). After plasma filtration, 90.7 ± 5.1 (SD) % (n = 22) immunoreactive (IR) OT is recovered. The sensitivity of OT EIA is 1 pmol/L, while intra- and inter-assay coefficients of variation (CV) are around 3.41% and 2.84%, respectively. In healthy volunteers, plasma IR OT is 7.28 ± 4.49 (SD) pmol/L (n = 32) with no gender difference. As shown by the data both from plasma of SCCL patients and from supernatants and cell contents of SCCL cell lines, this EIA procedure offers a novel, reproducible, specific and sensitive method for the measurement of IR OT.

Key words: Cancer; lung; neuropeptides; paraneoplastic secretion

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INTRODUCTION

Oxytocin (OT) is a non-peptide hormone of 1000 Da synthesized in magnocellular neurones of the hypothalamic supraoptic and paraventricular nucleus under the control of a unique gene (*OT*) [1]. *OT* traduction leads to a precursor molecule including a peptide signal, the OT peptide and the human OT-associated neurophysin (OT-NP, 10 kDa). This OT precursor is processed into OT and OT-NP. OT is specifically responsible for milk-ejection and $OT^{-/-}$ mice are unable to release milk in response to suckling [2]. OT is also known to take an active part

in parturition by stimulating contraction of the uterine myometrium (oxytocic activity). However, because of the redundancy of most birth-controlling mechanisms, parturition normally occurs in $OT^{-/-}$ mice [3]. In addition, OT has been shown to cause luteolysis by stimulation of endometrial prostaglandin secretion [4], to participate in male reproductive physiology [5] and to have a natriuretic effect through an indirect action upon the heart [6]. Through intrathymic *OT* expression and presentation by proteins encoded in the major histocompatibility complex, it has also been proposed that OT is implicated in the establishment of central T-cell tolerance of the neurohypophysial functions and in the control of T-cell development [7, 8]. OT also exerts a central influence on reproductive activities, and maternal, as well as affiliative behaviour [9, for review see 10]. We have previously shown that OT is present in acetone extracts of small cell carcinoma of the lung (SCCL), together with its associated OT-NP [11]. High concentrations of OT and OT-NP have been measured in plasma of about 30% of SCCL [12, 13]. This type of lung cancer is known to be associated with ectopic neuroendocrine secretion of vasopressin (VP), the other neurohypophysial hormone, which differs from OT by two amino acids only. Other kinds of tumours secrete neurohypophysial hormones, particularly breast cancer and thymic carcinoma [14–16, for a review see 17]. The incidence of such a paraneoplastic secretion is particularly important in SCCL, and is often associated with an elevation of VP-NP plasma levels [13]. The clinical expression of the VP ectopic secretion, known as the Schwartz-Bartter syndrome [18], leads to water intoxication with hyponatremia, and hypernatriuria. In addition to systemic endocrinal effects, several studies suggest that VP exerts a mitogenic effect on tumour cell growth through a paracrine/autocrine type of cell-to-cell signalling [19–21]. Until now, very little is known about the pathophysiological consequences of OT synthesis and secretion by SCCL on tumour development and patient survival. The primary objective of our investigations is to attempt to address that question [22]. In order to investigate the biological role played by OT in SCCL, we developed a specific and rapid method for the measurement of immunoreactive (IR) OT in plasma samples of patients with SCCL, as well as in culture media and cell contents of SCCL-derived cell lines. This paper describes the development of a specific enzyme-immunoassay (EIA) based on the use of rabbit anti-OT polyclonal antibodies (Ab) previously raised in our laboratory [23]. In addition, in this work we report on a rapid and efficient procedure for extraction of IR OT from human plasma samples.

MATERIAL AND METHODS

Subjects and blood sampling

Blood samples from 32 healthy donors (15 males, 17 females), included as control subjects, and from 30 patients with SCCL at different clinical stages were collected on heparinized or EDTA tubes. Male donors were aged between 25 and 61 years, and females between 24 and 54 years. Plasma was separated by centrifugation at $2000 \times g$ (for 10 min at 4°C), then stored at -20°C until assay. In addition to OT EIA, VP radioimmunoassay (RIA, DPC Humbeek, Belgium) was performed on these plasma samples.

Reagents and cells

OT, VP, vasotocin (VT), bovine serum albumin (BSA fraction V, A-4503), biotin-aminocaproate N-hydroxysuccinimide ester (B-3295), dimethyl-sulfoxide (DMSO), and charcoal were obtained from Sigma (Germany). $(NH_4)_2SO_4$, Na_2HPO_4, KH_2PO_4, $NaHCO_3$, NaCl, KCl, glycerol, Tween 20 were obtained from Merck (Germany). DPBS (17-513F), foetal calf serum (FCS), RPMI 1640 were purchased from Bio Whittaker (Belgium). Other reagents included tetramethylbenzidine (TMB, BioSource, Belgium), H_2SO_4 (UCB, Belgium), streptavidin coupled with horseradish peroxidase (HRP, RPN 4401, Amersham Pharmacia, UK), goat anti-rabbit IgG (H-L goat n° 401962, Calbiochem, Germany), Dextran T70 (Pharmacia, Sweden) and [^{125}I]Tyr^2-OT (NEN, USA).

DMS79, H146 and H345 (SCCL cell lines) and Jurkat cells were purchased from American Type Culture Collection (ATCC, USA).

Cell-line cultures

Jurkat (10^6 cells/mL), DMS79 (10^6 cells/mL), H146 (2×10^6 cells/mL) and H345 (4×10^6

cells/mL) cell lines were cultured in 12-well plates. Cells were incubated in RPMI 1640 + 10% FCS (1 mL), sometimes supplemented as recommended by the ATCC, in a humidified atmosphere with 5% CO_2 at 37°C. Supernatants and cells were separated every 24 h until 120 h. Supernatants were directly frozen at − 20°C, while cells were lysed in 500 μL deionized water and frozen at − 20°C. OT EIA was then performed on supernatants and cell contents.

Rabbit anti-OT polyclonal Ab

Initially, 23 antisera were obtained from 7 rabbits subcutaneously injected with OT conjugated to thyroglobulin [23]. The anti-OT Ab, further named O4, used in the EIA procedure with direct anti-OT Ab coating, was obtained from antiserum n° 22. This antiserum, diluted at 1:10 000, shows a relative binding (B/Bo) of 42% when biotinylated-OT (1:10 000) is displaced by 10 nM unbiotinylated-OT. The dilution of antiserum n° 22 could be increased to 1:150 000 when the EIA procedure with anti-IgG Ab coating is used.

O4 was purified by precipitation with ammonium sulfate: antiserum n° 22 (1 mL) was added to $(NH_4)_2SO_4$ (1 mL) and shaken overnight at 4°C. After three successive washes with $(NH_4)_2SO_4$/phosphate buffer, the pellet containing O4 was dissolved in 1 mL phosphate buffer. As measured by Bradford assay, this solution contained 25 mg protein per mL.

Preparation of biotinylated OT

OT was biotinylated according to Prakash *et al.* [24]. OT (1 mL,1 mM) dissolved in phosphate buffer (PO_4^{3-} 0.05 M, pH 7.5) was mixed to biotinaminocaproate N-hydroxysuccinimide ester (50μL, 220 mmol/L) dissolved in DMSO. After 30 min incubation at room temperature, biotinylated-OT was separated from unbound OT by gel filtration on Sephadex G-25 (Pharmacia, Sweden). Elution was performed with phosphate buffer and 1 mL fractions were collected. The first peak (biotinylated-OT conjugate) was identified by optic density (OD) at 260 and 280 nm and stored at − 20°C after addition of BSA (1%) and an equal volume of glycerol, in order to prevent freezing.

EIA with direct anti-OT Ab O4 coating

The 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with anti-OT Ab O4 (200 μL, 5 μg/mL) precipitated with ammonium sulfate, as described above, then dissolved in coating buffer (0.1 M $NaHCO_3$, pH 8.4). The plates were washed three times with washing buffer (400 μL, PO_4^{3-} 0.01 M pH 7.5 + 0.05% Tween 20). Subsequent washes were performed in the same way (3 times with 400 μL washing buffer). Residual binding sites were saturated for 2 h at room temperature with buffer (300 μL, 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl) supplemented with BSA (1%). The following incubation steps were performed in assay buffer (buffer + 0.5% BSA, 0.1% Tween 20). After washing, unknown plasma or standard OT (200 μL, ranging from 1 to 1000 pmol/L) was added, plates were incubated overnight at 4°C, and then washed. Tracer (200 μL, biotinylated-OT, 1:10 000) was added for 30 min at room temperature and then washed. Streptavidin-HRP (200 μL, 1:6000) was added for 30 min at room temperature and after a final wash, the plates were incubated for 15 min at room temperature with TMB solution (200 μL, TMB 0.42 mM, 0.004% H_20_2(v/v) in 100 mM sodium acetate/citric acid, pH 4.9). The reaction was stopped by the addition of H_2SO_4 (50 μL, 0.9 M). Assay absorbency was measured using an automatic reader at 450 versus 650 nm.

EIA with anti-IgG coating [24]

The 96-well microtiter plates were coated overnight at 4°C with rabbit anti-IgG (200 μL, 5 μg/mL) dissolved in coating buffer. Residual binding sites were saturated with buffer supplemented with BSA 1% (300 μL) for 2 h at room temperature. The following incubation steps were performed in assay buffer. After washing, unknown plasma or standard OT (200 μL, ranging from 1 to 1000 pmol/L) was added simultaneously with anti-OT antiserum n° 22 (100 μL, 1:150 000). The plates were incubated overnight at 4°C. The procedure was ended as described above, using tracer, streptavidin-HRP, TMB solution and H_2SO_4.

OT plasma extraction by filtration

Unknown plasma or hormone-free human plasma (HFHP, treated overnight with 1%

charcoal, 0.1% dextran T70 at 4°C) (1 mL) was submitted to centrifugation (7500 × g, 4°C, 2 h) on centricon YM-3 (cut-off 3000 Da, Millipore, USA) previously saturated with DPBS + BSA 0.1% (200 μL). Only compounds with molecular weight lower than 3000 Da (such as OT) can pass through the filter.

For the assessment of the filtration, HFHP or plasma (1 mL) was supplemented with OT (10 μL) to obtain final concentrations of from10 to 500 pmol/L. After filtration on YM-3, OT of filtrates was measured with the OT EIA. Assay buffer and seven plasmas (1 mL) were supplemented with [^{125}I]Tyr^2-OT (100 μL, 3.85 mCi/L, 1.8 nmol/L). After filtration on YM-3, the radioactivity of each filtrate was measured on 100 μL using an automatic gamma counter (Perkin-Elmer, USA).

RESULTS

Standard curve

Different conditions were tested for the establishment of OT EIA standard curves, i.e. 1 to 1000 pmol/L of synthetic OT diluted in assay buffer, in charcoal-treated hormone-free human plasma (HFHP) and in culture medium. As shown in Fig. 1A, EIA standard curves obtained with direct O4 coating were similar and offer more sensitivity in assay buffer and culture medium than in HFHP. OD_{450} fell from 3.3 and 3.1 for OT diluted in assay buffer and culture medium, respectively, down to 2.0 for OT diluted in HFHP. Moreover, for the OT EIA standard curve in HFHP, a positive cooperativity and/or protein-matrix effect was observed between 0 and 10 pmol/L OT.

Since these preliminary results strongly suggested residual interferences from plasma proteins not eliminated by charcoal treatment, HFHP was further filtrated on centricon YM-3 (cut-off 3000 Da). Synthetic OT (from 1 to 1000 pmol/L) was then added to filtrated HFHP (HFHP/YM-3). As shown in Fig. 1A (anti-OT O4 coating EIA), the standard curve in HFHP/YM-3 was very close to the one diluted in assay buffer and the sensitivity of OT EIA was improved. This absence of interference was observed for both EIA procedures, but as shown in Fig. 1B, the sensitivity of OT EIA was also enhanced when using direct O4 coating compared to that with anti-IgG coating. Fig. 1C shows the definitive OT EIA standard curves performed with the procedure of O4 coating and with synthetic OT diluted either in assay buffer, or in HFHP/YM-3, or in culture medium.

Assay performance

As shown in Fig. 1D, anti-OT O4 Ab selected in EIA is specific for OT and did not exhibit any cross-reactivity with either VP or VT at concentrations up to 10 nmol/L. The detection limit (2 SD from zero) of the OT EIA was 1 pmol/L. The inter-assay and intra-assay coefficient of variation (CV) has been determined, on standard curves, for three various OT concentrations, respectively 10 pmol/L, 100 pmol/L and 5000 pmol/L. The inter-assay CV was 1.83% for a sample B/Bo mean of 89.01 ± 1.63 (SD) % (n = 15), 2.02% for a sample B/Bo mean of 65.30 ± 1.32 (SD) % (n = 15) and 4.68% for a sample B/Bo mean of 41.44 ± 1.94 (SD) % (n = 15). The intra-assay CV was 3.15% for a sample B/Bo mean of 87.4 ± 2.7 (SD) % (n = 8), 3.11% for a sample B/Bo mean of 68.1 ± 2.1 (SD) % (n = 8) and 3.97% for a sample B/Bo mean of 40.5 ± 1.6 (SD)% (n = 8).

YM-3 filtration

To assess centrifugation and filtration efficiency, OT was added to HFHP at various concentrations before filtration (Table I). The mean of OT recovery was 89.4 ± 1.2 (SD)% (n = 5). The same experiment was performed on two plasma samples from SCCL patients (Table II). Moreover, in this experiment, plasma centrifugations were stopped after half the total time, filtrates were collected, centrifugation was continued until the end of the filtration and filtrates from the second part of the centrifugation were collected. This test established the absence of any significant difference between OT values obtained from the filtrate collected at half-time centrifugation or filtrates collected at the end of the centrifugation procedure. Indeed, the mean of OT recovery was 91.9 ± 3.1 (SD)% (n = 4) for filtrates from the first half-time filtration, and 89.1 ± 6.4 (SD)% (n = 4) for filtrates from the second half-time filtration. Assay buffer and seven plasma samples from patients were supplemented with [^{125}I]Tyr^2-OT (Table III). The mean of [^{125}I]Tyr^2-OT recovery after filtration was 91.8 ± 5.1 (SD) % (n = 7) compared with the

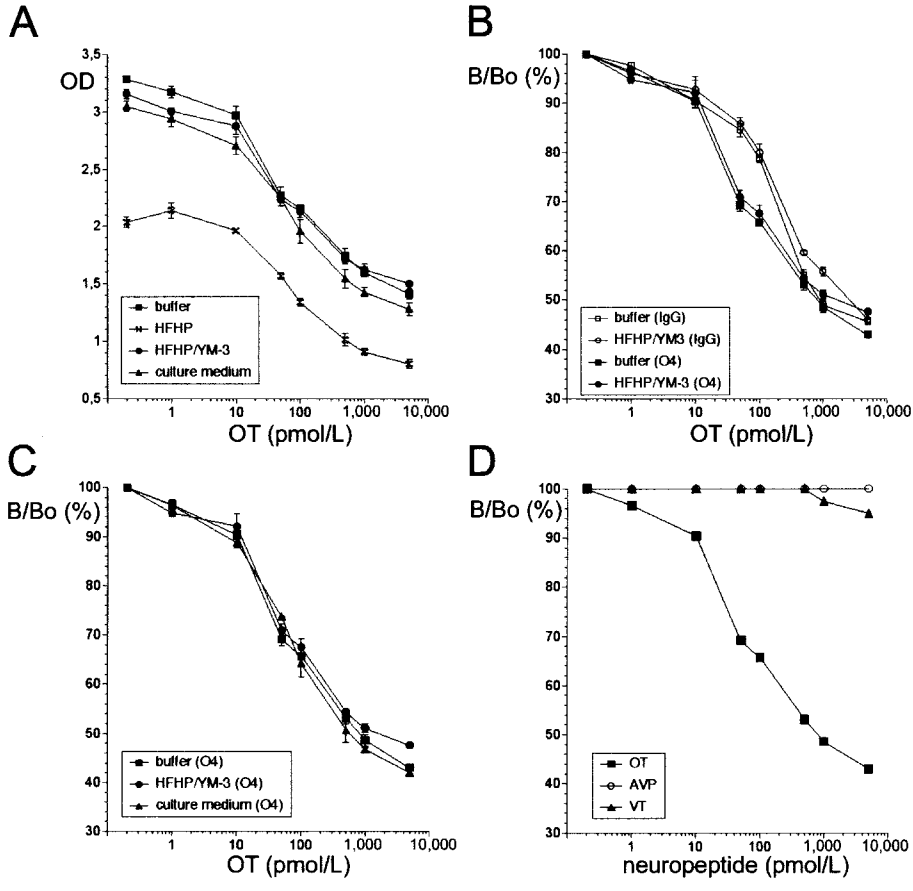

FIG. 1. OT EIA standard curves. A. Comparison of standard curve sensitivity as a function of OD: in assay buffer (buffer, ■), in hormone-free human plasma filtrated on centricon YM-3 (HFHP/YM-3, ●), in culture medium (▲) and in hormone-free human plasma (HFHP, X). Assays were conducted with the procedure using direct O4 coating. B. Comparison between standard curves obtained in assay buffer (■, □) and in hormone-free human plasma filtrated on centricon YM-3 (HFHP/YM-3, ●, ○), using the procedure with direct O4 coating (■, ●) or with IgG coating (□, ○). Curves are plotted as a function of %B/Bo. C. Standard curves are plotted as a function of %B/Bo and were conducted with the procedure using direct O4 coating in assay buffer (buffer, ■), in hormone-free human plasma filtrated on centricon YM-3 (HFHP/YM-3, ●) and in culture medium (▲). All values are expressed as means ± SD. D. Cross-reactivity of O4 with various neurohypophysial-related peptides: OT (■), VP (○), VT (▲). The assay was performed with the procedure using direct O4 coating.

value obtained in buffer. The linearity of the EIA was tested by measurements of OT in serial dilutions (1:2, 1:5 and 1:10) of plasma from three patients suffering from SCCL (Table IV). OT concentrations were proportional at each dilution, the mean of proportionality being 91.5 ± 6.8 (SD)% (n = 9). Moreover, similar standard curves were observed when OT was added to HFHP before or after YM-3 filtration (data not shown). This filtration method allows up to 90.7 ± 5.1 (SD)% (n = 22), considering the mean of these assessment experiments.

TABLE I. YM-3 filtration assessment: oxytocin (OT) recovery in hormone-free human plasma (HFHP).

OT (pmol/L) added before filtration	HFHP, OT recovery (pmol/L)
10	9 (90%)
50	44 (88%)
100	89 (89%)
250	228 (91%)
500	445 (89%)

TABLE II. YM-3 filtration assessment: oxytocin (OT) recovery in plasma with a stop at half-time of total filtration.

OT (pmol/L) added before filtration	Plasma1, OT recovery (pmol/L)		Plasma 2, OT recovery (pmol/L)	
	1st half time	2nd half time	1st half time	2nd half time
0	8.4	8.2	7	7.5
200	185 (92.5)	160 (80)	175 (87.5)	180 (90)
500	471 (94.2)	455 (91)	466 (93.7)	476 (95.2)

Columns headed 1st half time show values of IR OT measured in filtrates collected after a half-time filtration. Columns headed 2nd half time show values of IR OT measured in filtrates collected after the second half part of filtration of the same initial plasma. Numbers in parentheses represent the percentage of OT recovery.

TABLE III. YM-3 filtration assessment: [^{125}I]Tyr^2-OT recovery in plasma.

	Recovery of [^{125}I]Tyr^2-OT ± SD (cpm)
Buffer	55 810 ± 815 (100)
Plasma 3	49 582 ± 442 (88.8)
Plasma 4	54 473 ± 273 (97.6)
Plasma 5	56 005 ± 898 (100.3)
Plasma 6	49 925 ± 419 (89.5)
Plasma 7	49 848 ± 817 (89.3)
Plasma 8	48 088 ± 642 (86.2)
Plasma 9	50 735 ± 306 (90.9)

Numbers in parentheses represent the percentage of [^{125}I]Tyr^2-OT recovery in plasma, compared with buffer.

IR OT and VP concentrations in plasma of control subjects and patients with SCCL

Plasma IR OT concentration in control subjects was 7.28 ± 4.49 (SD) pmol/L (n = 32). There was no significant gender difference: 6.78 ± 4.06 (SD) pmol/L for males (n = 15), and 7.73 ± 4.92 (SD) pmol/L for females (n = 17). From the 30 patients with SCCL, three groups of patients could be distinguished on the basis of IR OT and VP plasma values: a group (n = 12, 40%) with elevated plasma IR OT concentrations (> 11.77 pmol/L, corresponding to the mean in control subjects + SD); a group (n = 13, 43.3%) with elevated IR VP plasma concentrations (> 5 pmol/L, upper limit for normal VP value used at the University Hospital (CHU) of Liège); and a group (n = 10, 33.3%) with normal OT and VP plasma concentrations. Both IR OT and VP plasma concentrations were elevated in 16.7% (n = 5) of SCCL cases (Fig. 2). A very high IR OT concentration of 980 pmol/L was exhibited by patient B. IR OT concentrations of patients D, J, K, L were situated between 12 and 12.5 pmol/L. The highest IR VP value obtained in this study is 73 pmol/L (patient P).

IR OT concentration in culture medium and cell content of SCCL cell lines

IR OT was assayed in culture supernatants as well as in cell contents of DMS79, H146 and H345 SCCL cell lines. As shown in Fig. 3, IR OT concentrations stood in the same order of magnitude in DMS79 and H146 cells. For these two SCCL cell lines, the relative cellular fraction was significantly higher than the supernatant fraction. Values of IR OT, obtained in supernatants of these three cell lines, ranged from 5 to 40 pmol/L, demonstrating the existence of an OT secretion by the SCCL cell lines studied.

DISCUSSION

Our main objective was the development of a rapid and specific EIA for measurement of IR OT in plasma, in culture media and in cell contents of SCCL cell lines. The present OT EIA offers the advantage of being readily applied to these conditions. The OT EIA detection limit is 1 pmol/L, an order of magnitude very similar to other commercially available methods for measurement of IR OT, moreover this limit is below the values observed in control subjects 7.28 ± 4.49 (SD) pmol/L (n = 32). The inter- and intra-assay CVs are situated in a range lower than 5%, which ensures a good reproducibility and reliablility to the OT EIA. When directly applied to plasma, some interference by plasma proteins was suggested by the observation of a positive cooperativity and/or protein-matrix effect. To avoid such interference, an extraction

TABLE IV. YM-3 filtration assessment: oxytocin (OT) recovery in diluted plasma.

Plasma dilutions before filtration	Plasma 7, OT (pmol/L)	Plasma 10, OT (pmol/L)	Plasma 11, OT (pmol/L)
1:1	980	74	45
1:2	500 (102)	31 (83.8)	19 (84.4)
1:5	196 (100)	13 (87.8)	7.9 (87.8)
1:10	96 (98)	6.7 (90.5)	4 (88.9)

Numbers in parentheses represent the OT proportionality (%) obtained after dilution compared to plasma without dilution.

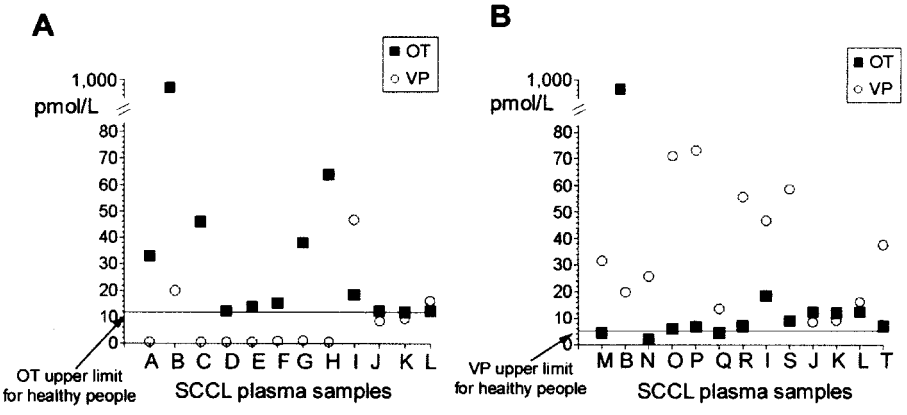

FIG. 2. IR OT (■) and VP (○) concentrations (pmol/L) in plasma of patients with SCCL. A. OT and VP concentrations in patients with elevated OT plasma concentrations. B. OT and VP concentrations in patients with elevated VP plasma concentrations.

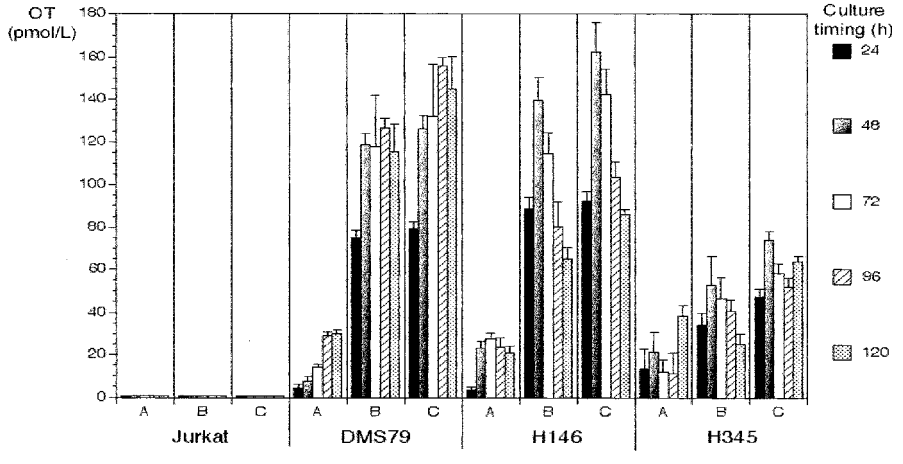

FIG. 3. IR OT concentration in SCCL cells cultures. IR OT concentration was measured in supernatants (A) and cell contents (B) of SCCL cell lines DMS 79, H146 and H345. The total OT production (C) was calculated from A + B. No production could be detected in Jurkat cell cultures (negative control).

procedure was developed using a simple filtration of the plasma by centrifugation on centricon YM-3. This plasma filtration is particularly efficient and offers the advantage of being easily performed in one step. The various experiments implemented to assess this filtration, i.e. the addition of OT or [^{125}I]Tyr^{2}-OT to buffer, HFHP or plasma from SCCL patients, allow us to estimate the mean of OT recovery at 90.7 ± 5.1 (SD)% (n = 22). Moreover, this recovery is homogenous during the all time filtration. This procedure also precludes the time-consuming drawback of the usual extractions requiring several procedures such as elution with organic solvent on the C_{18} column, evaporation and lyophilization [25, 26]. Therefore, we consider this filtration on YM-3 to be particularly simple and convenient in isolating a peptide such as OT from interference by other plasma proteins.

The EIA procedure using an anti-IgG Ab coating as described by Parkash *et al.* [24] was adapted to our conditions and compared with our EIA with direct anti-OT O4 coating. Standard curves in HFHP/YM-3 using both EIA procedures show identical absence of interference. However, the EIA procedure with direct O4 coating exhibits a better sensitivity in our experimental conditions and avoids a time-consuming step. It is also important to note that standard curves in HFHP/YM-3, in culture medium and in assay buffer are equivalent, so that future standard reference curves can be established in one of these conditions depending on the nature of the biological fluid where IR OT has to be measured.

The IR OT plasma concentration measured in healthy blood donors was 7.28 ± 4.49 (SD) pmol/L (n = 32), which is in close accordance with values reported by previous authors [27, 28]. Among the 30 patients suffering from SCCL reported here, 66.7% exhibited elevated plasma values of neuropeptides, which corresponds to data reported by North *et al.* [13]. Using our OT EIA, we observed elevated IR OT plasma concentrations from 12 to 980 pmol/L in these patients. In this population of SCCL patients presenting high plasma levels of neuropeptides, the distribution of elevated OT is 40%, elevated VP 43.3% and both elevated hormones 16.7%, which is in good accordance with our previous data [12] and with data reported by North *et al.* [13]. IR OT concentration in supernatants (5 to 40 pmol/L) and cell contents (25 to 155 pmol/L) of SCCL cell lines could even be measured early at the beginning of the cultures, when they are low. This allows us to demonstrate that OT could be secreted by SCCL cell lines.

In conclusion, this OT EIA using a stable biotinylated-OT as a tracer and the associated plasma filtration procedure offers a reproducible, specific and sensitive method that precludes the usual time-consuming plasma extraction requirement. The measurement of elevated IR OT concentrations in plasma of SCCL patients and in supernatants and cell contents of SCCL cell lines suggests that paraneoplastic secretion of OT also occurs in this pathological condition. Using this method, the aim of our future studies will be to decipher the significance of this paraneoplastic secretion in the biology of SCCL and other cancers.

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REFERENCES

1 Mohr E, Meyerhof W, Richter D. Vasopressin and oxytocin: molecular biology and evolution of the peptides hormones and their receptors. Vitam Horm 1995; 51: 235–66.
2 Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, Matzuk MM. Oxytocin is required for nursing but is not essential for parturition or reproductive behaviour. Proc Natl Acad Sci USA 1996, Oct 15, 93: 11699–704.
3 Russell JA, Leng G. Sex, parturition and motherhood without oxytocin? J Endocrinol 1998; 157: 343–59.
4 Flint APF, Riley PR, Kaluz S, Stewart HJ, Abayasekara DRE. The sheep endometrial oxytocin receptor. Adv Exp Med Biol 1995; 395: 281–94.
5 Nicholson HD, Jenkin L. Oxytocin and prostatic function. Adv Exp Med Biol 1995; 395: 529–38.

6 Jankowski M, Hajjar F, Kawas SA, Mukaddamdaher S, Hoffman G, McCann SM, Gutlowska J. Rat heart: a site of oxytocin production and action. Proc Natl Acad Sci USA 1998; 95: 14558–63.
7 Martens H, Goxe B, Geenen V. The thymic repertoire of neuroendocrine self-antigens: physiological implications in T-cell life and death. Immunol Today 1996; 17: 312–7.
8 Geenen V, Kecha O, Martens H. Thymic expression of neuroendocrine self-peptides precursors: role in T-cell survival and self-tolerance. J Neuroendocrinol 1998; 10: 811–22.
9 Insel TT, Winslow JT, Wang Z, Young LJ. Oxytocin, vasopressin, and the neuroendocrine basis of pair bond formation. Adv Exp Med Biol 1998; 449: 215–24.
10 Evans JJ. Oxytocin in the human—regulation of derivations and destinations. Eur J Endocrinol 1997; 137: 559–71.
11 Legros JJ. The radioimmunoassay of human neurophysins: contribution to the understanding of the physiopathology of neurohypophysial function. Ann NY Acad Sci 1975; 248: 281–303.
12 Legros JJ, Geenen V, Carvelli T, Martens H, Andre M, Corhay JL, Radermecker M, Zangerle PF, Sassolas G, Gharib C, Vantyghem M, Lefebvre J. Neurophysins as markers of vasopressin and oxytocin release. Horm Res 1990; 34: 151–155.
13 North WG, Friedman AS, Yu X-M. Tumor biosynthesis of vasopressin and oxytocin. Ann NY Acad Sci 1993; 689: 107–21.
14 North WG, Pai S, Friedman A, Yu X, Fay M, Memoli V. Vasopressin gene-related products are markers of human breast cancer. Breast Cancer Res Treat 1995; 34: 229–35.
15 Friedman AS, Memoli VA, North WG. Vasopressin and oxytocin production by non-neuroendocrine lung carcinomas: an apparent low incidence of gene expression. Cancer Lett 1993; 78: 79–85.
16 Ferlito A, Rinaldo A, Devaney KO. Syndrome of inappropriate antidiuretic hormone secretion associated with head and neck cancers: review of literature. Ann Otol Rhinol Laryngol 1997; 106: 878–83.
17 North WG. Gene regulation of vasopressin and vasopressin receptors in cancer. Exp Physiol 2000; 85S: 27S–40S.
18 Bartter FC, Schwartz WB. The syndrome of inappropriate secretion of antidiuretic hormone. Am J Med 1967; 42: 790–806.
19 North WG, Fay MJ, Longo K, Du J. Functional vasopressin V1 type receptors are present in variant as well as classical forms of small-cell carcinoma. Peptides 1997; 18: 985–93.
20 Carter DA, Fai CK, Murphy D. Neurohypophyseal peptides as regulators of growth and development. J Mol Neurosci 1993; 4: 11–9.
21 Woll PJ, Rozengurt E. Multiple neuropeptides mobilise calcium in small cell lung cancer: effects of vasopressin, bradykinin, cholecystokinin, galanin and neurotensin. Biochem Biophys Res Commun 1989; 164: 66–73.
22 Péqueux C, Breton C, Hagelstein MT, Hendrick JC, Geenen V, Legros JJ. Oxytocin receptor in small cell carcinoma of human lung cell lines. Pflügers Arch—Eur J Physiol 2000, 440: R214. Abstr 7.
23 Geenen V, Legros JJ, Hazee-Hagelstein MT, Louis-Kohn F, Lecomte-Yerna MJ, Demoulin A, Franchimont P. Release of immunoreactive oxytocin and neurophysin I by cultured bovine granulosa cells. Acta Endocrinol 1985; 110: 263–70.
24 Prakash BS, Metten M, Schams D, Wuttke W. Development of a sensitive enzymeimmunoassay for oxytocin determination in bovine plasma. Anim Reprod Sci 1998; 51: 185–94.
25 Porstmann T, Kiessig ST. Enzyme immunoassay techniques: an overview. J Immunol Methods 1992; 150: 5–21.
26 Avrameas S. Amplification systems in immunoenzymatic techniques. J Immunol Methods 1992; 150: 23–32.
27 Samson WK. The posterior pituitary and water metabolism. In: Griffin JE, Ojeda SR, editors. Textbook of endocrine physiology. 2nd ed. Oxford: Oxford University Press, 1992: 106–17.
28 Silber M, Almkvist O, Larson B, Uvnäs-Moberg K. Temporary peripartal impairment in memory and attention and its possible relation to oxytocin concentration. Life Sci 1990; 47: 57–65.

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