ELSEVIER

Regulatory Peptides 67 (1996) 39–45

REGULATORY PEPTIDES

Cytokine production by human thymic epithelial cells: control by the immune recognition of the neurohypophysial self-antigen

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Received 8 February 1996; accepted 14 May 1996

Abstract

Oxytocin (OT) has been shown to be the dominant peptide of the neurohypophysial family expressed by thymic epithelial and nurse cells (TEC/TNC) in various species. Thymic OT is not secreted but, after translocation of a hybrid neurophysin/MHC class I protein, is integrated within the plasma membrane of TEC, thus allowing its presentation to pre-T cells. In order to further demonstrate that thymic OT behaves like a membrane antigen, we assessed the effect of mAbs to OT on cytokine productions by cultures enriched in human TEC. 75–85% pure TEC cultures were prepared from human thymic fragments. Using immunofluorescence and confocal microscopy, ir-OT, ir-interleukin-1β (IL-1β), ir-interleukin-6 (IL-6) and ir-leukemia inhibitory factor (LIF) could be detected in these TEC cultures. ir-OT was restricted to TEC, while some ir-IL-6 and ir-LIF were also seen in occasional fibroblasts. In basal conditions, ir-IL-6 and ir-LIF (but not ir-OT and ir-IL-1β) were detected in the supernatants of human TEC cultures. MAbs to OT induced a marked increase of ir-IL-6 and ir-LIF secretion in TEC cultures. No significant effect was observed using mAbs against vasopressin, mouse immunoglobulins, or control ascitic fluid controls. These data show that OT is fully processed and recognized by specific mAbs at the outer surface of TEC plasma membrane. They further support that thymic OT behaves as the self-antigen of the neurohypophysial family.

Keywords: Thymus; Neurohypophysial peptide family; Self-antigen; T-cell self-education

1. Introduction

The epithelial component of thymic stroma plays an important role in positive and negative selection of T lymphocytes, [1–7]. The thymic repertoire of neuroendocrine-related polypeptide precursors has been proposed to recapitulate at the molecular level the dual physiologic role played by the thymus in T-cell development [8–10]. With a special regard to the neurohypophysial peptide family, our group as well as others have shown that thymic epithelial and nurse cells (TEC/TNC) synthesize oxytocin (OT) and its precursor-associated protein, OT-neurophysin [11,12]. Thymic OT plays a dual role in T-cell selection. First, following its interaction with cognate receptors expressed on T cells [13,14], OT acts as a cryptocrine signal involved in an accessory pathway of T-cell positive selection [15,16]. Second, OT has been suggested to be the prototype neurohypophysial self-antigen presented to immature T cells by the thymic major histocompatibility complex (MHC) system.

Although ir-OT concentrations in the human thymus are much higher than those of ir-vasopressin (VP), both neurohypophysial (*proOT* and *proVP*) genes are transcribed in the human and murine thymus [17,18]. Thymic OT is not secreted but translocated in TEC plasma membrane in association with a membranal 55-kDa neurophysin/MHC class I hybrid protein [19]. A functional analogy was observed in the binding and transport of OT between neurophysin in the hypothalamo-neurohypophysial axis [20] and the neurophysin domain in the thymic microenvironment. Presenting OT in such a manner to pre-T cells is thought to lead to the central T-cell tolerance in neurohypophysial family members [21]. This model of T-cell education to the neurohypophysial self-antigen offers selective advantages such as avoiding MHC class I allelic

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PII S0167-0115(96)00105-X

restriction and presenting the overall conformational structure of the neurohypophysial peptide family.

TEC also express transcripts and proteins of various cytokines, such as interleukin 1 (IL-1) [22], interleukin 6 (IL-6) and leukemia inhibitory factor (LIF) [23] which were also found in the supernatants of TEC cultures. We previously showed ir-IL-1β to be colocated with ir-OT and its neurophysin in thymic sections, more specifically in TEC recognized with cytokeratin (CK) antibodies [24].

In the immune system, T-cell activation and cytokine production usually follow the recognition and binding of Abs to antigenic markers expressed on the surface of T-cell membranes. In an attempt to unravel the physiological significance of the colocalization of OT with other polypeptides in TEC, we investigated the effect of anti-OT monoclonal antibodies (mAbs) upon IL-1β, IL-6 and LIF secretion by cultured human TEC cultures.

2. Material and methods

2.1. Antibodies

Anti-human cytokeratin mAb MNF 116 (lot 33) and rabbit polyclonal Abs anti-cytokeratin A575 (lot 31D) were obtained from Dako (Denmark). O33 and O13 are anti-OT mAbs; O33 is directed against the cyclic part of OT and O13 is directed against the C-terminal part of OT [25]. BER 312 is an anti-VP mAb [26]. Anti-IL-6 mAb (clone 4B2) was obtained from Medgenix Diagnostics (Belgium). Anti-LIF is a specific antiserum kindly provided by A. Godard [27]. Second antibodies were fluorescein (FITC)-conjugated Affinipure goat anti-mouse IgG (lot 28970), hereafter referred to as second antibody A; FITC-conjugated Affinipure goat anti-mouse IgM (lot 17708), hereafter referred yo as second antibody B; and rhodamine (TRITC)-conjugated Affinipure $F(ab')_2$ fragment goat anti-rabbit IgG (H + L) (lot 27272), hereafter referred as second antibody C. All conjugated Abs were obtained from Jackson (Pennsylvania).

2.2. TEC cultures

Thymus fragments were obtained from children (6 months to 3 years old) undergoing corrective cardiovascular surgery for congenital cardiopathies. Surgical fragments were pre-cut and washed by sedimentation at $1 \times g$, twice for 5 min in HBSS (Flow, 19-101-49), then minced with scissors and washed again. The small fragments (< 2 mm) were then transferred in Eagle's minimum essential medium containing D-valine (Gibco) and supplemented with 2 mmol/l L-glutamine, 10 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS; Gibco), hereafter referred as complete TEC medium. D-Valine was used to reduce the growth of fibroblasts [28,29]. Fragments were then allowed to attach to T-75 flasks in the same medium in a humidified atmosphere at 37°C and 5% CO_2, and complete TEC medium was replaced every 3–4 days. On day 17, the explants and adherent cells were detached by treatment with Puck's-modified solution containing trypsin and EDTA (Gibco) and filtered through nylon gaze to eliminate fragment residues. Cells were counted and seeded at 6000 cells/well on 10 well/immunofluorescence microplates, and at 50000 cells/well in 12-well culture plates. The 10% FCS in complete medium was replaced at this point by 1% Ultroser G-steroid free (Gibco), hereafter designed as US-SF-TEC medium, for cytokine assays. Ultroser G was used in order to avoid possible artefacts reflecting the presence of cytokines in FCS, and to further reduce fibroblast growth [30].

2.3. Immunofluorescence procedures

Immunofluorescence microplates were kept for three days in FCS-TEC medium to allow plating of the TEC and then stained for immunofluorescence after a 7-min, −20°C methanol fixation [24]. Plates were first incubated for 30 min at room temperature with 10% normal goat serum to prevent non-specific binding of goat second antibody to mouse or rabbit immunoglobulins. Single or double immunostaining was performed with the following reagents: anti-human cytokeratins mAb MNF 116 diluted at 1:50 followed by second antibody A; anti-OT mAb O33, diluted 1:100 followed by second antibody B; anti-IL-6 mAb diluted 1:10 followed by second mAb A diluted 1:40; anti-LIF antiserum diluted 1:50, followed by second antibody C. In some experiments MNF 116 was replaced by rabbit polyclonal anti-cytokeratin A575 diluted 1:200 followed by second antibody C diluted 1:100. Extinction controls were performed with second antibody alone, or after the first non-related antibody. In the double immunostaining studies, the first step was always performed with first mAb followed by the appropriate second antibody prior to immunostaining with polyclonal antiserum. After the final wash, the plates were mounted with glycerol/gelatin medium.

2.4. Confocal microscopy

Immunostained cells were imaged using a Bio-Rad MRC 100 laser scanning confocal unit mounted on an Axiovert 135 Zeiss inverted microscope. Images were collected with an 40 × objective (aperture 1.3, plan Neofluar) or an oil immersion 63 × objective (aperture 1.4, plan Apochromat). An Argon-Krypton ion laser source was utilized for fluorescein (488 nm) and for rhodamine (568 nm). Kalman filtering was used to integrate the signal collected over four frames to decrease the background noise. Photographs were taken using a camera mounted on a FVM 1702 Lucius and Bauer film recorder.

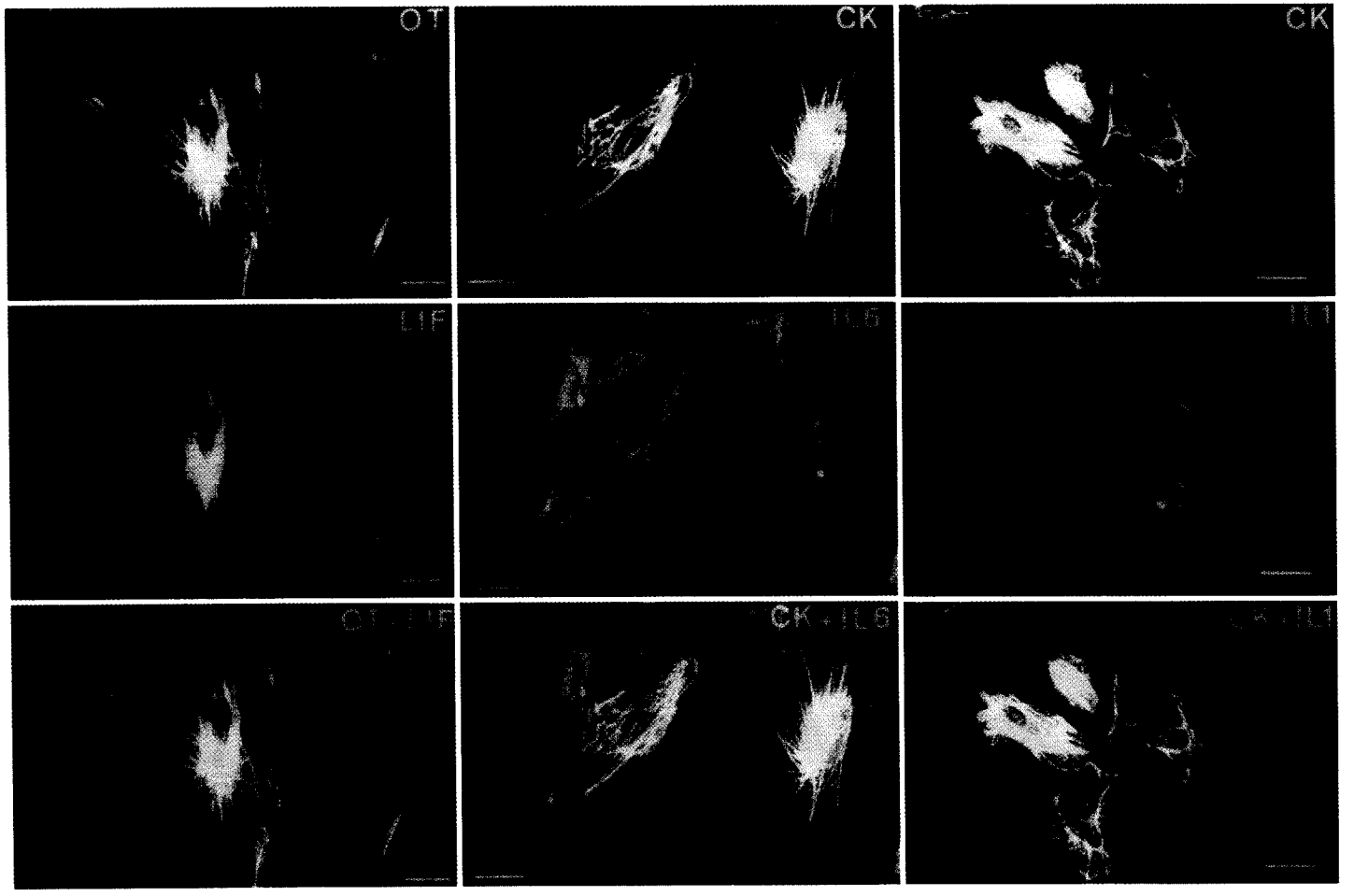

Fig. 1. Colocalization of ir-OT, ir-LIF, ir-IL6 and ir-IL1ß in human TEC after 21-day culture. First column, immunofluorescence staining with anti-OT (green) mAb O33 and anti-LIF (red) antiserum. The lower frame is a superimposed image of OT and LIF immunostaining. Yellow-orange indicates an overlap of ir-OT and ir-LIF. Note the positive staining with anti-LIF of fibroblast-like cells that are very weakly labeled with anti-OT. Second column, immunofluorescence with anti-cytokeratin (CK) polyclonal A575 (green) and anti-IL6 (red). Superimposed image shows ir-IL6 in TEC, but also in CK negative cells. Third column, immunofluorescence staining of TEC cultures with anti-CK mAb MNF 116 (green) and mAb anti-IL1ß (red). Superimposition shows that the major part of the CK network is associated with ir-IL1β. Bar is 10 μm.

2.5. Cytokine assays

One ml of US-SF-TEC medium, with or without additives, was added on days 17, 21, 24 and 28 and supernatants collected for assay on days 21, 24, 28 and 31. Each experiment was repeated at least three times in duplicate. Collected medium was stored at −20°C for cytokine measurements. Additives were anti-OT IgM O33 ascitic fluid, anti-OT IgG O13 ascitic fluid, anti-VP BER-312 ascitic fluid, mouse ascitic fluid depleted of IgG by three fast-flow protein-A affinity absorption, and 5 mg/ml mouse IgG (Sigma; lot 38F-8912), each at 1% (v/v).

Cytokine levels were measured using IL-1β, IL-6 and LIF EASIAs (Medgenix Diagnostics) according to published methods [31,32]

2.6. Statistical analyses

Values obtained from multiple experiments are reported as percentage (%) of basal value at each time point. Significance levels were determined by Mann-Whitney *U*-test.

3. Results

3.1. Cytokeratin (CK), ir-OT, ir-LIF, ir-IL-6, ir-IL-1β immunofluorescence in human TEC after 21-day culture (Fig. 1)

The staining obtained with anti-CK mAb demonstrates the dominant epithelial phenotype of cultured TEC. Counting of mAb-stained cells versus total cultured cells showed that 75–85% cells were authentic TEC (CK-positive) at day 21, while 15–25% exhibited a fibroblast-like morphology. All cultured cells were stained with anti-LIF antiserum, with a slightly different pattern according to the cell of origin: bright filaments were stained in epithelial cell, while a speckled and diffuse staining pattern was observed in fibroblast-like cells (Fig. 1, first column). Ir-OT was found in very close association with the CK network. Epithelial cells appeared to be brightly stained with anti-IL-6, while anti-IL-6 staining was weaker in CK-negative cells (Fig. 1, second column). Ir-IL-1β was also detected in the cytoplasm of cultured human TEC associated with the major part of the CK network.

TEC and fibroblast-like cells were found to contain both ir-IL-6 and ir-LIF, while ir-OT was almost exclusively detected in epithelial (CK-positive) cells. Moreover, the combined use of confocal microscopy and immunofluorescence showed that anti-LIF and anti-OT outlined a common filament network in epithelial cells, while LIF immunoreactivity was much more less marked in fibroblast-like cells.

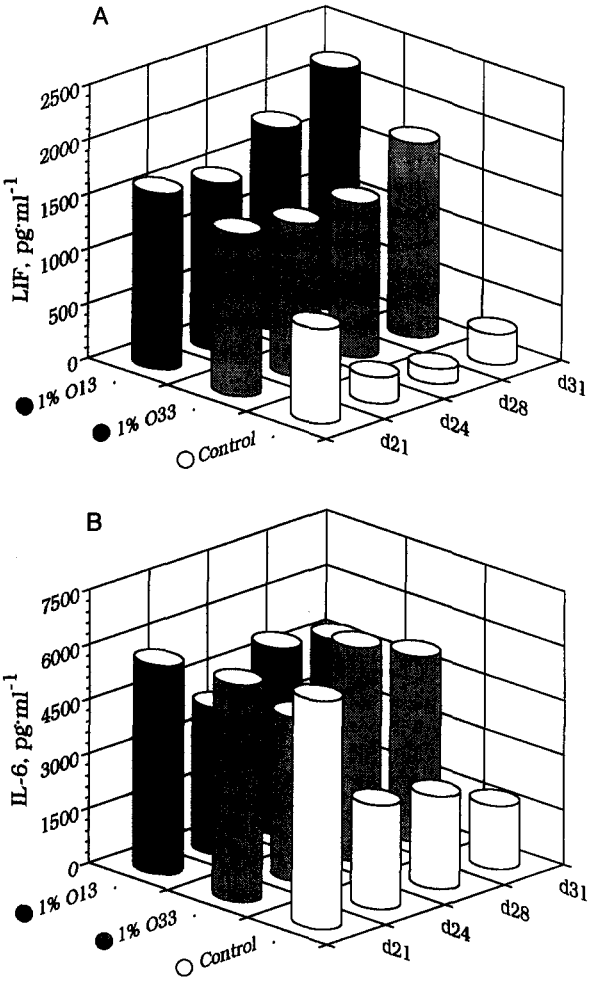

Fig. 2. Production of LIF and IL6 by human TEC: influence of mAb to OT O13 and O33. TEC supernatants tested for LIF (A) and IL6 (B) content at time indicated, from day 21 to day 31. White columns are for control culture, grey are for 1% O33 added-culture and black are for 1% O13. Each column is the mean of quadruplicate measurements, with c.v. less than 5%.

3.2. Production of IL-6 and LIF by human TEC: Influence of mAbs to neurohypophysial peptides

IL-1β was not detected in our experimental conditions, even in 5-fold concentrated medium, in either basal or mAb-stimulated TEC supernatants. As the detection limit of the IL-1β EASIA is 50 pg/ml, IL-1β production by 50000 cultured TEC is under 10 pg/ml. On the other hand, under basal conditions cultured TEC produce 500–1000 pg/ml ir-LIF, as well as 6–40 ng/ml ir-IL-6. LIF production showed a pronounced and sustained increase over basal when anti-OT O33 and O13 mAbs were added to TEC cultures (Fig. 2A). IL-6 production was sustained by the addition of anti-OT mAbs, in contrast with the rapid decrease under basal conditions (Fig. 2B).

3.3. Controls

LIF and IL-6 production by human TEC were not affected by anti-VP mAb BER-312, by IgG-starved ascitic

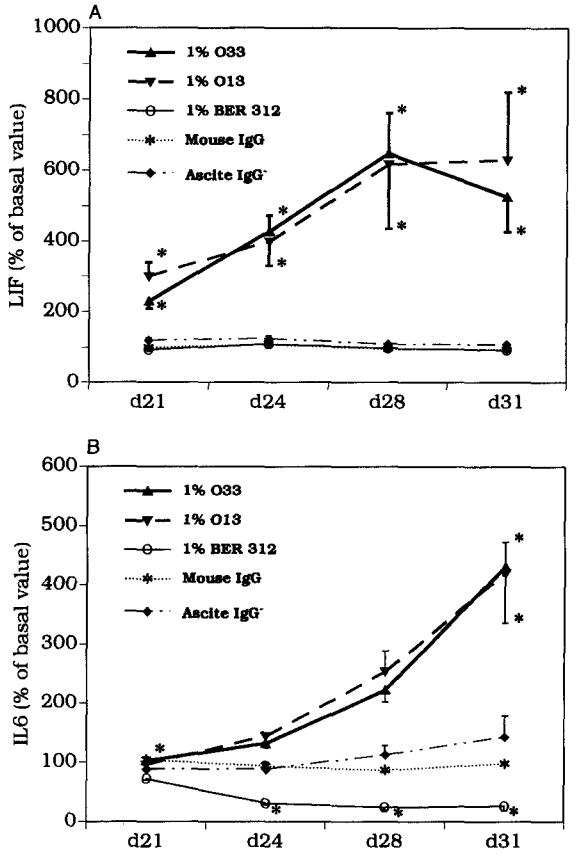

Fig. 3. Production of LIF and IL6 by human TEC: control experiments. Only mAbs to OT (O13 and O33) induced a marked increase of both LIF (A) and IL6 (B) production by human TEC. Anti-VP mAb BER-312, murine IgG, or murine ascitic fluid without IgG had no significant effect upon IL6. Anti-VP induced a significant inhibition of IL6 production. Each point is the mean of four experiments ± SD in triplicate. * $P < 0.001$, by Mann-Whitney U-test.

fluid, or by mouse IgG (Fig. 3A). In terms of IL-6 production, a slight but significant inhibitory effect of anti-VP BER-312 was observed (Fig. 3B). There was no significant difference between the positive effects of anti-OT O13 and O33.

4. Discussion

The co-localization of ir-OT, IL-1β, IL-6 and LIF in cultured human TEC has been shown by combined immunofluorescence and confocal microscopy. OT and LIF staining in epithelial cells was closely related to the cytokeratin network on double immunofluorescence with anti-cytokeratin MNF 116 mAb. This finding suggests that the cytoskeleton of human TEC could be implicated in the processes of membrane translocation and secretion for this cell type. The results also are in close accord with previous ultrastructural analyses showing ir-OT to be diffusely located in the cytoplasm of murine TEC [12]. Cultured human TEC thus express both OT and cytokines, and the colocalization demonstrated confirms previous observations on human thymic sections [24]. Anti-OT staining of cultured TEC adds further evidence that OT is the dominant neurohypophysial-related peptide expressed in thymic epithelium.

A particular finding of the present study is that anti-OT mAbs strongly stimulate LIF and IL-6 secretion by cultured human TEC. The absence of EGF in US-SF might explain why ir-IL-6 is below detection limits under basal conditions, since biologically active and ir-IL-6 have been detected in human TEC culture in EGF-supplemented serum-free media, but not in DMEM or other EGF-deficient media [33]. Moreover, this cytokine has been shown to be regulated by EGF at a post-transcriptional level [34]. Colic et al reported IL-1β in the supernatants from rat TEC cultures [35], but Shoham et al found IL-1β below detection limits in murine and human TEC cultures [36,37]. Thus, species differences or culture methods could explain some of the discrepancies in the data previously reported.

The specificity of TEC activation by anti-OT mAbs has been carefully established by a variety of control experiments. It is not clearly due to a non-specific effect of Ig or ascitic fluid, nor did the anti-VP mAb exert any significant effect on LIF production, whereas it slightly inhibited ir-IL-6 production. As OT and VP differ by only two amino acids, the specific increase of cytokine production by TEC upon antigen recognition confirms that OT is the dominant self-antigen of the neurohypophysial family expressed by TEC. Both O33 and O13 stimulate cytokine production by TEC, although O13 was previously shown not to stain human thymic sections [24,38]. In processing of the OT-precursor, OT must be cleaved from neurophysin before ^{9}gly-OT amidation. The absence of TEC labelling by O13 may thus reflect incomplete processing of the OT precursor within the TEC cytoplasm. In contrast, the biological effects observed with O13 argue for presentation of fully-processed OT at the level of the TEC membranes.

The marked stimulating effect of anti-OT mAbs on TEC cytokine production may be explained by induction following immune recognition of the neurohypophysial-related antigen presented at the level of the TEC membrane. Altogether, these data strongly support a membrane localization of OT. The positive effects observed with two OT mAbs directed against different epitopes of the OT molecule strongly suggest that a fully processed OT is presented at the TEC plasma membrane. Somehow like the transduction following the binding of a signal to its receptor, the immunological recognition of OT leads to TEC activation reflected here by the secretion of IL-6 and LIF. It will be also of interest to determine whether Abs against other thymic self-antigens such as neurokinin A [39] and insulin-like growth factor II [40,41] are also able to affect TEC secretory activity. At the present stage, this study supports our model on the dual physiological role played

in T-cell differentiation by the thymic repertoire of neuroendocrine-related polypeptides [8,9].

Acknowledgements

V.G. is a Research Associate and Brigitte Malgrange is Senior Research Assistant of the National Fund of Scientific Research (Belgium). We thank Prof. J. Urbain (Laboratory of Physiology, Free University of Brussels, Rhode-Saint-Genèse, Belgium) who provided us with hybridomas producing anti-OT mAbs. These studies were supported by the Research Fund of Liège University Medical School, by the Fondation Léon Frédéricq of the Liège University Hospital, by the Fund for Scientific Medical Research of Belgium (grant No. 3.4562.90; grants Télévie No. 7.4611.91, 7.4548.93 and 7.4532.95), by the Association contre le Cancer (Belgium), and by the European Science Foundation (Strasbourg).

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