BRAIN, BEHAVIOR, AND IMMUNITY 5, 102–115 (1991)

Colocalization of Immunoreactive Oxytocin, Vasopressin and Interleukin-1 in Human Thymic Epithelial Neuroendocrine Cells

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Monoclonal antibodies to oxytocin (OT) and vasopressin (VP) revealed some positively staining stromal cells in the subcapsular cortex and in the medulla of the human thymus. We further demonstrated that these cells are a subset of epithelial endocrine cells and also contain immunoreactive interleukin-1 together with the neuropeptides. In addition, the thymic cells stained by monoclonal antibodies directed to the cyclic part of oxytocin or vasopressin also contained some immunoreactive neurophysins. These data support the concept of intrathymic synthesis of neurohypophyseal-like peptides fitting the hypothalamic model. However, we observed that, contrary to the situation in the brain, OT- and VP-like peptides colocalized in the same thymic cells. Furthermore, one monoclonal antibody, specific for the tail part of oxytocin, did not label thymic cells. Therefore, thymic neuropeptide(s) could be related to, but distinct from, authentic OT and VP. These observations suggest some molecular differences between hypothalamic and thymic oxytocin biosynthetic pathways which need to be further investigated.

INTRODUCTION

The neuropeptides oxytocin (OT) and vasopressin (VP) are under the control of two independent genes expressed in hypothalamic magnocellular neurons. They are synthesized as high-molecular-weight precursors which are then cleaved during axonal transport. Pro-oxytocin is a 16-kDa protein containing the OT sequence at its N-terminus which is separated from the oxytocin-associated neurophysin (NP-OT) by a Gly–Lys–Arg spacer sequence, and which contains an extra C-terminal amino acid (Land et al., 1983). Pro-vasopressin is a 20-kDa glycoprotein which contains the vasopressin sequence at the N-terminus separated from its associated neurophysin (NP-VP) by the same tripeptide spacer (Gly–Lys–Arg). In addition, Pro-VP contains a 39 amino acid terminal glycopeptide, which is separated from NP-VP by a single Arg spacer (Land, Schuetz, Schmale, & Richter, 1982). The hormones then are excreted directly into the blood, at the neurohypophyseal level. The nonapeptides, OT and VP, differ from each other by only 2 amino acids. However, in mammals, their physiological actions are quite different; OT stimulates milk ejection and uterine contractions, while VP is mainly involved in water metabolism homeostasis. It is now clear that these neuropeptides may have additional functions in the central nervous system as well as in a

0889-1591/91 $3.00

variety of peripheral tissues. At present, no biological property has been assigned to the neurophysins and to the VP-associated glycopeptide.

Traditionally, the hypothalamo-neurohypophyseal system has been considered the principal source of these circulating neuropeptides. However, the presence of neurohypophyseal hormones has been reported in a number of peripheral endocrine tissues such as ovary, testis, adrenal, and thymus (for review see Clements & Funder, 1986), but peripheral synthesis of OT was demonstrated definitively only for the ruminant corpus luteum (Ivell & Richter, 1984; Swann et al., 1984).

We have demonstrated that immunoreactive (ir) OT and its specific NP could be extracted from human thymuses, using radioimmunoassay, chromatography, and biological assay (Geenen et al., 1986). The further finding of positive dot blot hybridizations of thymic mRNA with OT and VP DNA probes constituted further evidence for *in situ* synthesis (Geenen et al., 1987), although this does not establish a conclusive demonstration of thymic OT and VP mRNA expression.

The thymus is a lymphoepithelial organ composed of lymphocytes, epithelial cells, and cells derived from the monocyte/macrophage lineage (Kendall, 1981). Lymphocytes are distributed in the cortical and medullary regions of the thymus within a network of epithelial cells endowed with T cell differentiating properties (Jordan & Robinson, 1981). Thymic epithelial cells are in intimate contact with thymocytes and produce thymic hormones that are thought to induce stages of T cell maturation. By using polyclonal antibodies, we previously localized some ir OT and ir VP neuropeptides in the subcapsular cortex (SCC) and in the medulla as well as in the lymphoepithelial complexes called thymic nurse cells (Geenen et al., 1988a). In the work reported here, we used a double immunofluorescence labeling technique and monoclonal antibodies (Mabs) to investigate the relative distribution of ir VP and ir OT containing cells and to further characterize them.

METHODS

Tissue Treatment

Thymus fragments were obtained from children (ages 1 month to 12 years) undergoing corrective cardiovascular surgery for congenital cardiopathies. These fragments were embedded in Tissue-tek, immediately frozen in dry ice, and then stored at −70°C. Frozen sections (3–5 μm) were cut on a cryostat, air-dried, and postfixed, because we found that prefixation destroyed the thymic tissue morphology. We compared several fixatives generally used in immunohistochemical studies of thymus or in neuropeptides research. Thus we used either precipitative agents (methanol, acetone, ethanol/acetic acid) or cross-linking solutions (4% formaldehyde, Bouin's sublimate, picric acid/formaldehyde (PAF), or benzoquinone). The preservation of neurohypophyseal peptides immunoreactivity was found to depend on fixative. Precipitative agents produced a diffuse immunostaining, while formaldehyde and Bouin's sublimate resulted in complete loss of antigenicity. Conversely, we achieved sharp labeling with benzoquinone and PAF, a fixative described by Stefanini, De Martino, and Zamboni, (1967). Finally, we selected the latter because it produces a darker background for immunofluorescence studies and is easier to handle.

Antibodies

Monoclonal antibodies to oxytocin have been characterized previously (Burgeon et al., in press). O13 was found to be very specific for OT using three different techniques and is directed to the C-terminal tripeptide. O22 and O33 probably recognize tyrosine-containing epitope(s) also shared by vasopressin and other related nonapeptides.

We previously demonstrated that the Mab BER-312 is directed to the ring moiety of the vasopressin molecule and does not react with OT (Robert, Léon-Henri, Chapleur-Chateau, Girr, & Burlet, 1985).

The rabbit antiserum to bovine neurophysins (A/5/III) previously was shown to recognize the central part of the molecule that is common to OT-NP and VP-NP and is well conserved through mammals (Legros, 1975).

We used commercially available antibodies to human cytokeratins (Immunotech, Marseille, France). Rabbit antiserum directed against human cytokeratins (A-575, Dako, Copenhagen, Denmark) was used at 1/400 dilution. The monoclonal antibody KL1, when applied to human epidermis, colors the suprabasal (but not the basal) cell layers and it recognizes the 55- to 57- and 63- to 67-kDa epidermal keratins, determined by immunoblot technique (Viac, Reano, Brochier, Staquet, & Thivolet, 1983). KL4 stains all the human epidermal cell layers on skin sections and binds to the main epidermal keratins (50 to 67 kDa), determined by immunoblot assay (Haftek, Staquet, Viac, Schmitt, & Thivolet, 1984).

A2B5 (ATCC HB-29) is a murine Mab that binds to a complex neuronal GQ ganglioside expressed on the cell surface of neurons, neural crest-derived cells, and peptide-secreting endocrine cells (Eisenbarth, Walsh, & Nirenberg, 1979). It previously was shown to react with a subset of thymic epithelial cells (Haynes, Shimizu, & Eisenbarth, 1983). This mouse hybridoma was grown in our lab and used as undiluted supernatant.

Antibodies to IL-1β were produced by Medgenix (Fleurus, Belgium). The rabbit antiserum (L-62) did not cross-react with other cytokines (Reuter et al., 1988). Mab 1C3 was obtained from Balb/c mice immunized against human recombinant IL-1β.

All Mabs shared IgG_1 isotype except O33 and A2B5 that were typed as IgM.

Immunofluorescence Procedure

Sections were rinsed (3 × 5 min) in washing buffer (Tris–NaCl buffer 0.05 *M*, pH 7.6, containing 0.05% Tween 20 and 0.02% NaN_3). They were incubated for 30 min, at room temperature, with 10% normal goat serum (NGS). The antibodies were all diluted in 0.1 *M* Tris–HCl, pH 7.6, containing 0.02% NaN_3. First-step antibodies were incubated overnight at +4°C (anti-neuropeptides) or 30 min at room temperature.

Sections were washed (3 × 5 min) in washing buffer. Fluorescein- or rhodamine-conjugated goat antisera to mouse IgG_1 or IgM (Fc specific) or to rabbit Ig (Nordic, Tilburg, The Netherlands), diluted 1:40 and 1:100, were used as second-step reagent for 30 min at room temperature. After three washings, the

sections were mounted with glycerol/gelatin medium. The double staining procedure involved sequential incubations of tissues sections.

Fluorescence of tissue sections was observed with a Leitz Orthoplan fluorescence microscope using an epi-illumination system and X10 or X50 water-immersion objectives. Photographs were taken using Ektar 1000 film and exposure times of 20–40 s.

Controls

No specific staining was observed in the thymus when dilution buffer, normal rabbit serum (NRS, Dako, Copenhagen, Denmark), or anti-BSA or anti-OVA control ascites (IgG_1 or IgM) was used in place of the primary antibodies.

Preadsorption of O33 and BER-312 monoclonal antibodies with homologous synthetic peptides, OT and VP, respectively, coupled to CNBr-activated Sepharose-4B (Pharmacia, Sweden), abolished the immunostaining.

We also checked that our Mabs to neuropeptides did not detect a cross-reacting antigen in epidermal epithelium. Cutaneous biopsy fragments were fixed with 10% formol, dehydrated, and included in paraffin before 5-μm sections were prepared. Immunohistological staining was performed with a kit from Monosan using the alkaline phosphatase anti-alkaline phosphatase technique with fast red as substrate.

RESULTS

Distribution of Neurohypophyseal-like Peptides in Human Thymus

Using an immunofluorescence technique and monoclonal antibodies, we confirmed the presence of ir OT in human thymus. A Mab to OT (O33) revealed two immunoreactive areas (Fig. 1a). In the SCC, this antibody mainly stained a monolayer of flattened cells lining the inner surface of the thymic capsule and lobular septae. In the medulla (M), it stained a dense reticular network of stellate cells with voluminous cytoplasm and short processes; these cells surrounded Hassall's corpuscles (HC), but the central area of HC was generally unreactive (Figs. 1c, 2a, 2c, and 3c). In the inner cortex, very few scattered stellate cells were labeled. We only observed a cytoplasmic labeling of the stromal component of human thymus; thymocytes were never visualized.

Among the Mabs to VP used, we found that BER-312 was the brightest, although it labeled thymus sections less intensely than O33, in accordance with lower thymic content of ir VP. The pattern of reactivity of BER-312 in human thymus was similar to that obtained with O33 (Fig. 1b).

Antibody A/5/III, a rabbit serum anti-neurophysin, also produced similar staining (Fig. 1d).

Identification of OT-like Positive Cells as Epithelial Cells

In a previous work, based on serial sections which showed parallel staining patterns, we presumed that the stromal cells containing ir neuropeptides were part of the thymic epithelium (Moll, Lane, Robert, Geenen, & Legros, 1988). Now,

using a double immunofluorescence technique, we found that these O33+ cells also were labeled by an antiserum to keratins, thus demonstrating their epithelial nature (results not shown). Two Mabs to human cytokeratins, KL1 and KL4, also stained O33+ cells (Fig. 2). It should be noted that, on human thymus, KL1 stained the whole epithelial network (subcapsular cortex, inner cortex, medulla, and Hassall's bodies) while KL4 stained mainly the epithelial cells from SCC, M, and HC. Cortical epithelial cells, which were brightly labeled by KL1 but less stained by KL4, were O33−.

In summary, in human thymic microenvironment, cells containing some neurohypophyseal-like peptides correspond to a subset of epithelial cells expressing KL1- and KL4-defined cytokeratins.

Identification of NP-like Positive Cells as Endocrine Cells

Haynes et al. described a similar pattern of reactivity for the monoclonal antibody A2B5 that correlates with the endocrine function of the thymus (Haynes et al., 1983). Therefore we used this Mab, together with the rabbit serum directed to neurophysins, in a double immunofluorescence technique. The ir NP-containing cells were always A2B5+ (Figs. 3a and 3b); however, we occasionally observed that a few cells were labeled only by A2B5.

Thus it appears that those epithelial cells containing ir neuropeptides are part of the thymic endocrine microenvironment defined by A2B5.

Colocalization of OT-like and Interleukin-1 Peptides

It was previously demonstrated that human thymic epithelial cells produce interleukin-1 (Le, Tuck, Dinarello, Haynes, & Singer, 1987).

Using a double immunofluorescence assay with L-62, a rabbit antiserum raised against IL-1β, we observed that a subpopulation of O33+ cells also were stained by this serum (results not shown). However, the staining patterns were different for the two markers; some IL-1+ cells (possibly macrophages/dendritic cells) did not react with O33, particularly in the medulla and interlobular septae.

When testing Mabs to IL-1, we observed that 1C3 stained the subcapsular cortex and the medulla, but did not label the inner cortex or Hassall's corpuscles. The staining appeared to be restricted to the cytoplasm. We found that all O33+ cells, except HC, also were stained by 1C3 (Figs. 3c and 3d), again suggesting that a subset of epithelial cells can produce both IL-1 and neurohypophyseal-like peptides.

Colocalization of OT-, VP-, and NP-like Peptides

By using double immunofluorescence labeling, we found that the same cells express together ir OT and ir NP (Figs. 1c and 1d). This would argue for a local synthesis of oxytocin by thymic epithelial cells.

Using the antiserum to NP and BER-312, we also were able to colocalize ir NP and ir VP in the same thymic epithelial cells (results not shown).

Surprisingly, we also found that the same cells could contain some ir VP together with ir OT (Figs. 1a and 1b).

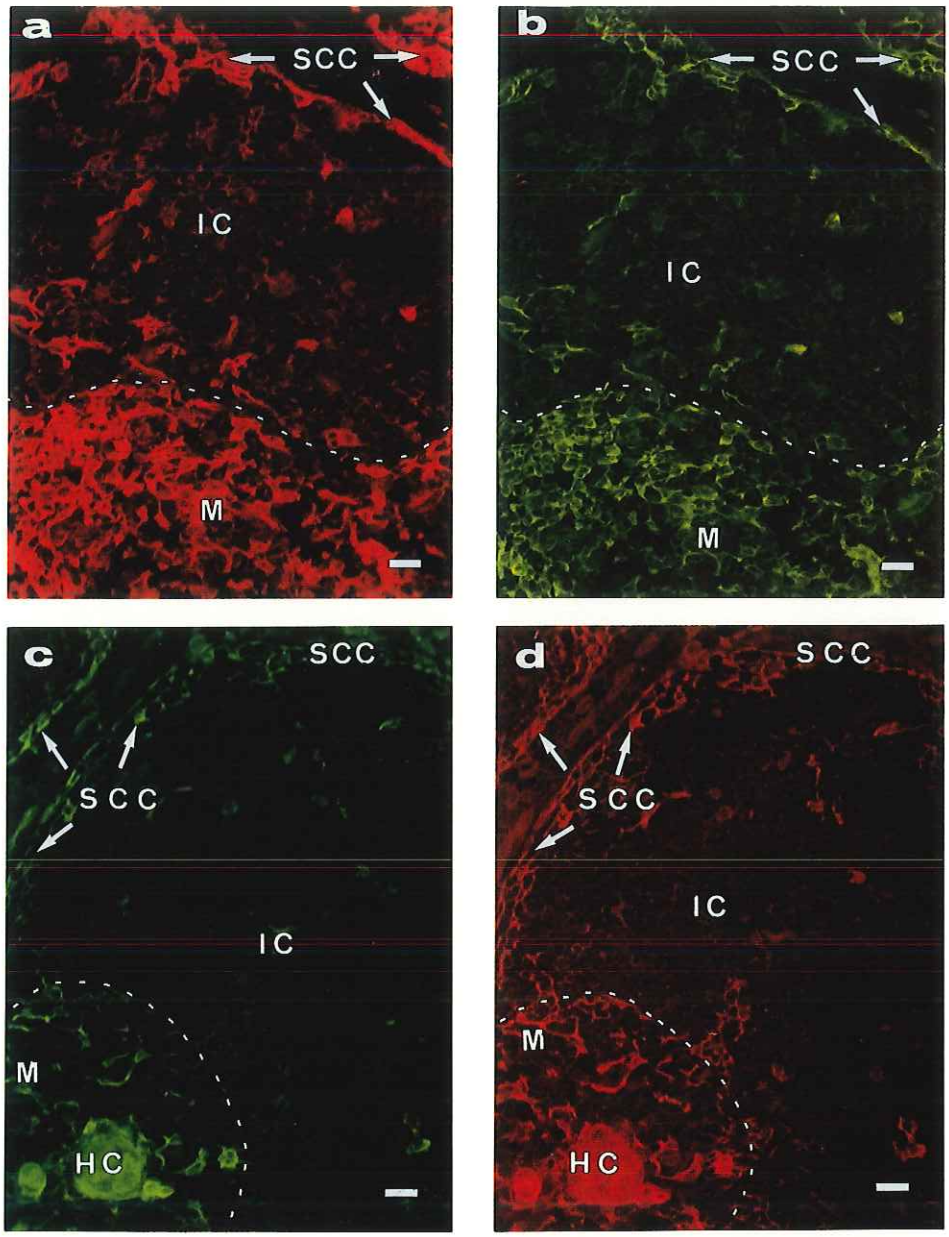

FIG. 1. Detection of ir neurohypophyseal peptides in human thymus by double immunofluorescence labeling: (a) using monoclonal anti-OT antibody, O33 (1/80), and TRITC goat anti-mouse IgM (1/40); (b) using monoclonal anti-VP antibody, BER-312 (1/8), and FITC goat anti-mouse IgG_1 (1/40); (c) using monoclonal anti-OT antibody, O33 (1/80), and FITC goat anti-mouse IgM (1/40); (d) using polyclonal anti-NP rabbit serum, A/5/III (1/800), and TRITC goat anti-rabbit Ig (1/100). SCC, subcapsular cortex; IC, inner cortex; M, medulla; HC, Hassall's corpuscle; dotted line, approximate cortico-medullary junction. Scale bar = 20 μm.

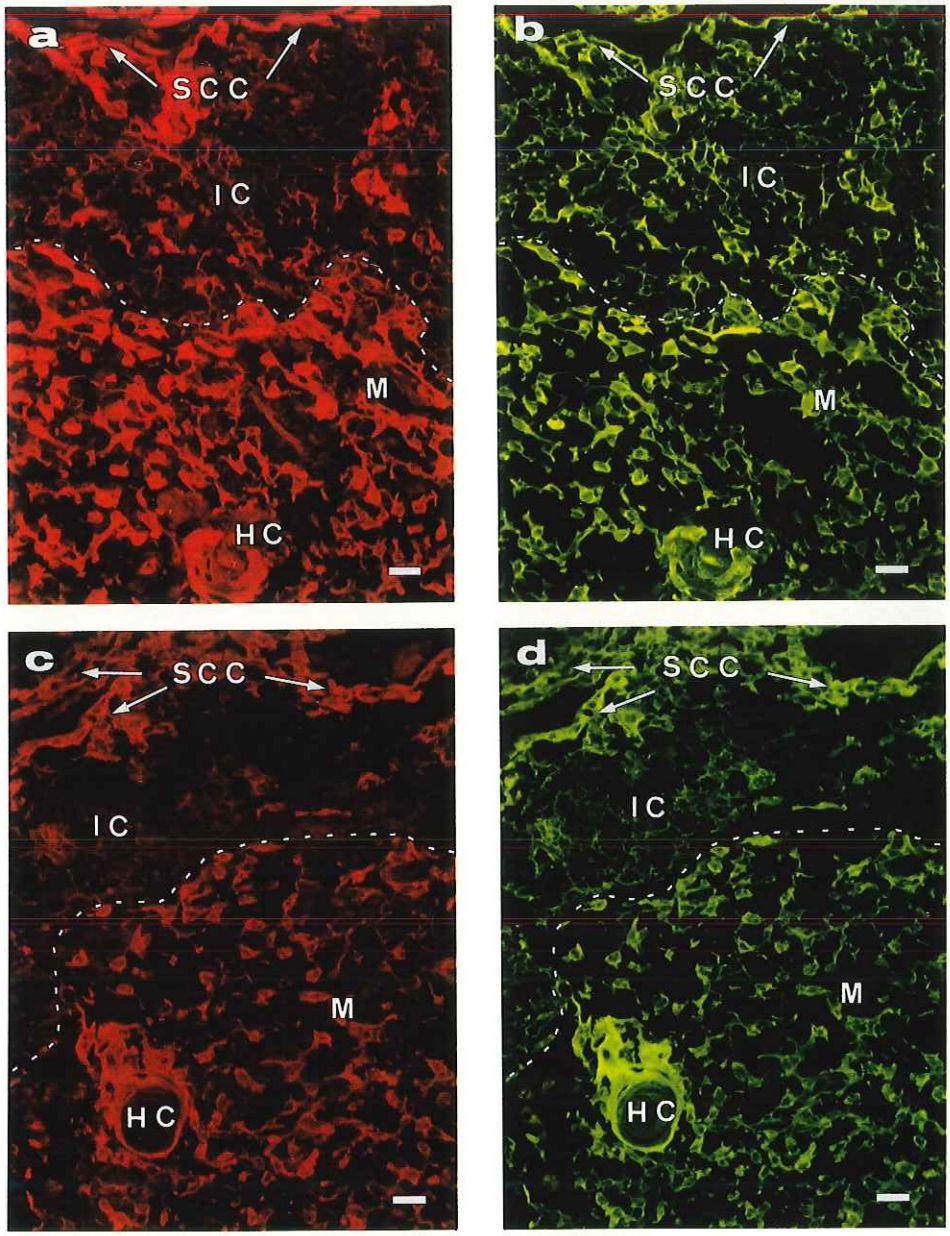

FIG. 2. Colocalization of ir neurohypophyseal peptides and keratins in human thymus by double immunofluorescence labeling: (a and c) using monoclonal anti-OT antibody, O33 (1/80), and TRITC goat anti-mouse IgM (1/40); (b) using monoclonal anti-keratin antibody, KL1 (1/80), and FITC goat anti-mouse IgG_1 (1/40); (d) using monoclonal anti-keratin antibody, KL4 (1/80), and FITC goat anti-mouse IgG_1 (1/40). SCC, subcapsular cortex; IC, inner cortex; M, medulla; HC, Hassall's corpuscle; dotted line, approximate cortico-medullary junction. Scale bar = 20 μm.

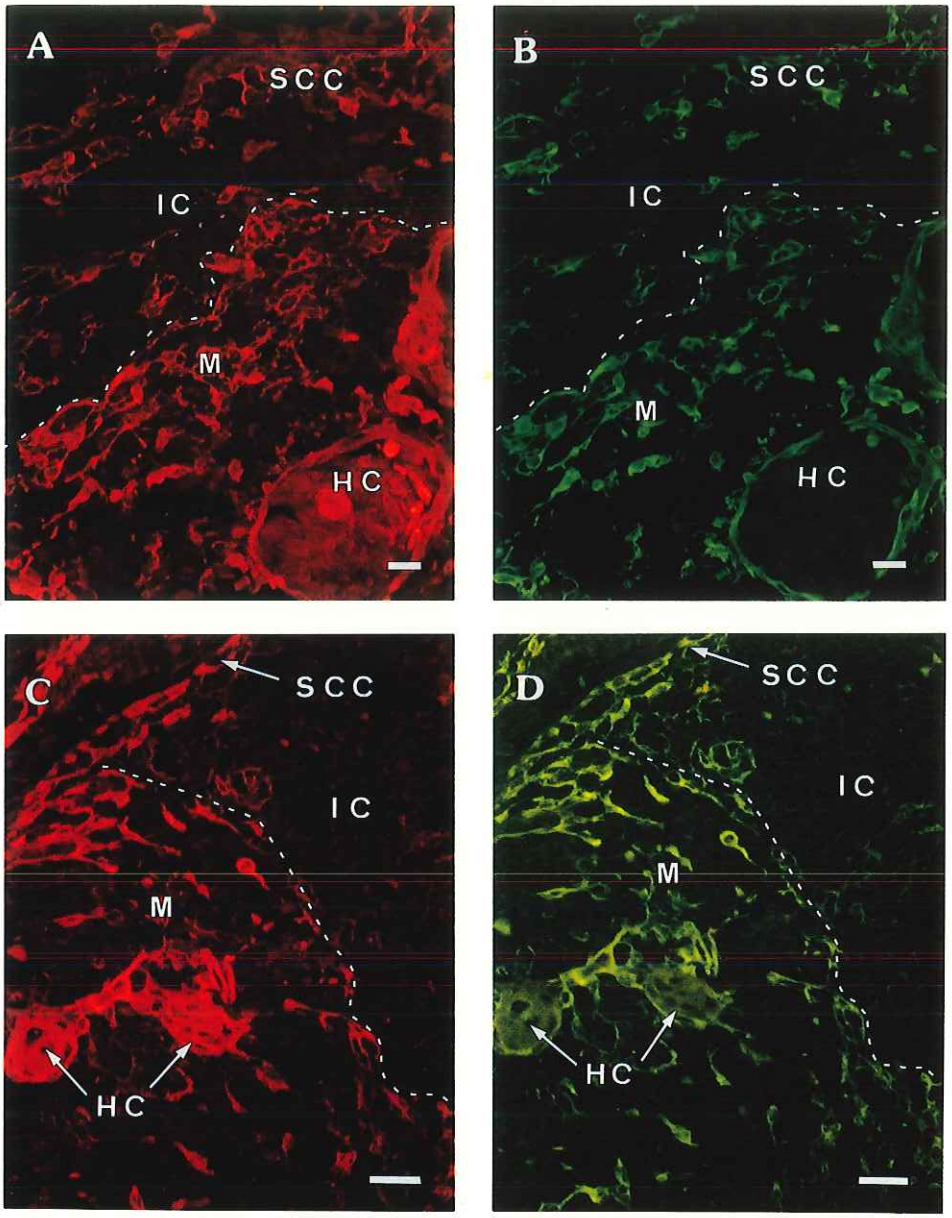

FIG. 3. Colocalization of ir neurohypophyseal peptides with GQ ganglioside in human thymus by double immunofluorescence labeling: (a) using polyclonal anti-NP rabbit serum, A/5/III (1/800), and TRITC goat anti-rabbit Ig (1/100); (b) Using monoclonal anti-GQ ganglioside antibody, A2B5 (undiluted supernatant), and FITC goat anti-mouse IgM (1/40). Colocalization of ir OT with ir IL-1β in human thymus by double immunofluorescence labeling; (c) using monoclonal anti-OT antibody, O33 (1/80), and TRITC goat anti-mouse IgM (1/40); (d) using monoclonal anti-IL-1β antibody, 1C3 (1/20), and FITC goat anti-mouse IgG_1 (1/40). SCC, subcapsular cortex; IC, inner cortex; M, medulla; HC, Hassall's corpuscle; dotted line, approximate cortico-medullary junction. Scale bar = 20 μm.

DISCUSSION

In this study we used Mabs to oxytocin and vasopressin as well as polyclonal antibodies to neurophysins to localize the human thymic cells containing some immunoreactive neurohypophyseal peptides. We found these peptides in a monolayer of flattened cells lining the inner surface of the thymic capsule and lobular septae and in medullary thymic epithelium. In double labeling experiments, we characterized these cells as a subset of epithelial cells expressing KL1- and KL4-defined cytokeratins.

Thymic epithelium appears to be heterogeneous and four subsets of epithelial cells have been described in the subcapsular cortex, the inner cortex, the medulla, and Hassall's bodies. It is possible to characterize the different zones of the thymic microenvironment with regard to their reactivity with reagents against keratins (Laster, Itoh, Palker, & Haynes, 1986; Nicolas, Reano, Kaiserlian, & Thivolet, 1986; Savino & Dardenne, 1988). In the thymus of five mammalian species, KL1 labels exclusively a small subset of medullary epithelial cells (or Hassall's corpuscles) characterized by its contents in high-molecular-weight keratins (Nicolas, Reano, Kaiserlian, & Thivolet, 1989). In contrast, KL4 reacts with the whole thymic epithelium of mouse, rat, and rabbit, but only with SCC, M, and HC in guinea-pig and pig. In our hands, in human thymus, KL1 labeled the whole epithelial network while KL4 stained mainly SCC, M, and HC subsets.

We further demonstrated that these epithelial thymic cells containing immunoreactive neurohypophyseal peptides exhibit the characteristics of A2B5 defined endocrine cells. This subset of epithelial cells within the thymic microenvironment contains a variety of thymic hormones that are postulated to induce certain stages of T cell maturation (Haynes et al., 1983).

We also observed that interleukin-1 immunoreactivity colocalized with OT-like peptides. IL-1 was first discovered as endogenous pyrogen and later as a macrophage product that stimulates thymocyte proliferation, activates B lymphocytes, and mediates a wide range of inflammatory effects. In addition, IL-1 exerts a number of central effects including the modulation of slow wave sleep, food intake, analgesia, corticotrophin-releasing factor secretion, and astroglial growth (Dinarello, 1989). Recently, IL-1β was reported in neurons in human (Breder, Dinarello, & Saper, 1988) and rat brain (Lechan et al., 1990). The presence of IL-1β immunoreactivity in the hypothalamic neurohypophyseal system, including perikarya in magnocellular neurons of the paraventricular nucleus, suggests that this cytokine may modulate VP/OT secretion in the brain. The simultaneous detection of neurohypophyseal-like peptides and IL-1β immunoreactivity in a subset of thymic epithelial cells could also indicate that IL-1 may regulate OT/VP release in human thymus. However, the physiological significance of the presumed interactions between thymic neuropeptides and IL-1β remains to be further explored.

Nevertheless, our data confirm previous observations by Le et al. (1987) of the presence of IL-1 in epithelial cells in human thymic sections. However, we did not localize immunoreactive IL-1 in cortical epithelial cells. Such a discrepancy could be ascribed to the use of different antibodies to IL-1.

The discovery of ir NP together with ir OT and ir VP peptides suggests that thymic epithelial cells, like hypothalamic neurones, synthesize OT and VP precursors that are then cleaved to produce the hormones.

The coexistence of OT and VP in stromal thymic cells is in sharp contrast to the situation observed in the hypothalamo-neurohypophyseal system, where VP- and OT-containing neurons are separate (Vandesande & Dierickx, 1975) except for a few neurons (Baldino, O'Kane, Fitzpatrick-McElligott, & Wolfson, 1988; Kiyama & Emson, 1990). Nevertheless, such a colocalization has been reported at the periphery in dorsal root ganglia (Kai-Kai, Anderton, & Keen, 1986) and in adrenal medulla (Hawthorn, Nussey, Henderson, & Jenkins 1987).

However, the presence of a material in human epithelial thymic cells which exhibits immunological similarities to OT, and to a lesser extent to VP, does not substantiate that we are dealing with authentic OT and VP. Some molecular differences between hypothalamic and thymic nonapeptides are obvious, since our Mabs all recognized OT or VP in brain slices (Burgeon et al., in press; Robert et al., 1985) while human thymus sections were only brightly stained by O33.

This Mab probably binds to an epitope which is also shared by other natural neurohypophyseal peptides, because it recognizes isotocin and vasopressin, labels a few structures in the suprachiasmatic nucleus (claimed to contain only VP), and is absorbed on both synthetic OT and VP. Because it does not bind to ^{125}I-OT which is modified at position 2 (Tyr), we presume that it recognizes the N-terminus of the molecule.

The nonreactivity of O13 is particularly remarkable as this Mab was shown to be highly specific for OT and to recognize more precisely the C-terminus of the molecule. Since it does not stain thymus sections, it could be that the tail part of OT differs in the thymus and in the brain.

Therefore, it seems that we are dealing with a peptide(s) related to, but distinct from, authentic OT and VP. For instance, vasotocin (VT) is a natural nonapeptide which has the particularity of being formed by the hexapeptidic cyclic moiety of OT and the tripeptidic terminal part of VP, thus sharing cross-reactive determinants with both OT and VP. It is usually found in nonmammalian vertebrates, but some VT-like peptide has been reported in fetal and adult mammalian pineal gland (Nieuwenhuis, 1984), human fetal posterior pituitary (Smith & McIntosh, 1983), ovine fetal blood (Ervin, Leake, Ross, Calvario, & Fisher, 1985), and human newborn plasma (Ervin, et al., 1988a). However, Liu, Poulter, Neacsu, and Burbach (1988) excluded the presence of authentic VT, but isolated a novel form of OT from bovine pineal gland. Chromatographic and immunological properties of the purified peptides identified it as N^{α}-acetyl-OT. In ovine fetal thymus, Ervin et al. (1988b) found that VT-like levels were 8–10 times higher than OT- and VP-like contents. The assumption that a VT-like molecule also is expressed in postnatal human thymus would explain why O33, which seems to be directed to the cyclic part, shared by both OT and VT, labels thymus sections, while O13, which recognizes very specifically the C-terminal moiety of OT, does not.

The question of a variant gene(s) for OT and VP, expressed only in peripheral organs, also should be addressed. The Brattleboro rat has an autosomal recessive defect, elicited by a single nucleotide deletion, characterized by an absence of

hypothalamic neurohypophyseal VP and a resultant diabetes insipidus. As ir neurohypophyseal peptides were detected in testis, adrenal, and thymus of Brattleboro rats (reviewed in Clements & Funder, 1986), it can be postulated that a variant gene is being expressed in these peripheral tissues. Recently, expression of high-molecular-weight forms of propressophysin was reported in bovine pituitary gland (Rosenbaum, Zimmerman, & Nilaver, 1990), and rat placentas and testis (Lefebvre, Paradis & Zingg, 1990). VP gene-derived RNAs with a structure different from those of hypthalamus also were identified in the rat testis (Murphy, Hwee Luan, Ngee Chih & Carter, 1990).

Instead of a gene disparity, we can also postulate that post-translational cleavage of OT and VP prohormones could be different in the thymus and the hypothalamus, thus resulting in modified peptides. Forms of VP/OT with extended sequence at the N-terminus have been reported (Gitelman, Klaper, Alderman, & Blythe, 1980), as well as incompletely processed intermediates of biosynthesis with extensions at the C-terminus (Altstein, Whitnall, House, Key, & Gainer, 1988; Amico, 1988).

After their release at axonal terminals in the brain, VP and OT were reported to undergo further metabolic processing involving either N-terminal or C-terminal cleavages (Burbach, 1986). Therefore, O33 could preferentially detect OT/VP metabolic fragments accumulating in the thymus as a result of specific enzymatic context. The elucidation of the precise biochemical nature of a thymic immunoreactive neurohypophyseal peptide(s) is currently under investigation.

The physiological action of OT- and VP-like peptides within the thymus remains to be defined. A first step toward a clarification of this enigma was achieved by the demonstration of specific receptors for OT and VP on rat thymic membranes (Elands, Resink, & De Kloet, 1988), thymocytes (Elands, Resink, & De Kloet, 1990), and an immature T-cell line (Geenen et al., 1988b). The coexistence of neuropeptide signals and receptors in the thymus suggests that these agents could exert paracrine/autocrine functions on T cell ontogeny.

ACKNOWLEDGMENTS

The authors thank Drs. J. Arrese Estrada and G. Piérard (Dermatopathology, Liège) for skin controls. Human thymuses were kindly provided by Dr. D. Amerin (CHU, Nancy) and Pr. G. Primo (Hopital des Enfants Malades, Bruxelles). This work was supported by an accord of scientific cooperation between CGRI (Belgium) and INSERM (France) and by a grant (87/41) from the European Scientific Foundation. We are also indebted to the Fondation pour la Recherche Médicale Française and the Association Française contre les Myopathies (AFM). V. Geenen and J. Schoenen are Research Associate and Senior Research Associate of the National Fund for Scientific Research (Belgium). Part of this work was supported by grant (3.4562.90) from the Fund for Medical Scientific Research and by grant from the Special Research fund of the University of Liège (Belgium).

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Received July 30, 1990