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Identification of Nuclear Triiodothyronine Receptors in the Thymic Epithelium*

DÉA MARIA SERRA VILLA-VERDE, MARIE-PAULE DEFRESNE†, MARCOS A. VANNIER-DOS-SANTOS, JEAN H. DUSSAULT, JACQUES BONIVER, AND WILSON SAVINO

Laboratory on Thymus Research, Department of Immunology, Institute Oswaldo Cruz (D.M.S.V.-V., W.S.), and the Laboratory of Ultrastructure and Electron Microscopy, Institute for Byophysics Carlos Chagas Filho, Federal University of Rio de Janeiro (M.A.V.), Rio de Janeiro, Brazil; the Laboratory of Pathological Anatomy and Cytopathology, University of Liege (D.M.S.V.-V., M.-P.D., J.B.), Liege, Belgium; and Unité de Recherche en Ontogénèse et Génétique Moléculaires, Université Laval (J.H.D.), Quebec, Canada

ABSTRACT

Thymic epithelial cell physiology is known to be under neuroendocrine control. In particular, thyroid hormones modulate thymic hormone secretion by thymic epithelial cells *in vivo* and *in vitro*, thus suggesting the existence of specific receptors for those hormones in this component of the thymic microenvironment. Yet, thyroid hormone-binding sites have previously been detected only in crude thymus fractions and lymphocytes. We, thus, decided to search for T_3 receptors in the thymic epithelium, by using an antinuclear T_3 receptor monoclonal antibody.

In situ immunohistochemical analysis of thymic frozen sections showed nuclear labeling of both lymphoid and nonlymphoid cells in the cortex and medulla. Moreover, *in vitro* studies using thymic epithelial cell lines and the so-called thymic nurse cells revealed a positive reaction in the chromatin, with nucleoli remaining negative. Immunoblot data clearly showed a single protein band of 57K reactive with the antinuclear T_3 receptor antibody in murine thymus extracts as well as in the thymic epithelial cell lines. Lastly, *in vitro* treatment of these cells with T_3 resulted in a transient, yet profound, down-modulation of the receptor.

In conclusion, our findings provide molecular evidence that the action of thyroid hormones on thymic epithelium occurs via the typical 57K nuclear T_3 receptors. (*Endocrinology* **131:** 1313–1320, 1992)

THE CONCEPT of the thymus as an endocrine gland is now largely established. Actually, several circulating biologically active peptides of thymic origin have been isolated, characterized, and obtained in synthetic form, such as thymulin, thymosin-α-1, and thymopoietin (see review in Ref. 1). These thymic hormones are produced by thymic epithelial cells (TEC) (2, 3) and were shown to mediate several steps in intra- and extrathymic T-cell differentiation (4, 5).

The control of thymic hormone secretion by TEC seems to be dependent on a complex network of events. Recent studies showed that thymulin itself exerts a controlling feedback effect on its own secretion by TEC *in vivo* and *in vitro* (6, 7). Additionally, the thymic epithelium is under a multifaceted neuroendocrine control, with thymic endocrine function, cytokeratin expression, extracellular matrix production, and cell proliferation being modulated by different hormones and neuropeptides (see reviews in Refs. 8 and 9).

Previous data demonstrated the intrathymic expression of receptors for different types of hormones, such as glucocorticoids (10) and sexual steroids (11–13), GH (14), and PRL (15). Moreover, specific binding sites for thyroid hormones have been detected in thymus fractions and thymocytes by using radiolabeled ligands bearing high specific activity, and their binding capacity was reported (16–19). Nonetheless, in such studies, whether TEC also expressed these receptors was not evaluated. Yet, one should expect to find them in TEC, since thyroid hormones influence thymulin secretion (20, 21) as well as TEC proliferation (22). Importantly, aneuthyroidic patients show abnormal levels of circulating thymulin, an effect that could be reversed by adequate clinical treatment (23).

Taken together, these findings prompted us to investigate the presence of thyroid hormone receptors in the epithelial component of the thymus. In this report we bring immunochemical evidence that TEC actually express nuclear T_3 receptors (NTR), bearing a molecular weight of 57K, that can be modulated *in vitro* by T_3.

Materials and Methods

Animals

Wistar rats and C57BL/Ka mice of both sexes, aged 1 day or 4–6 weeks, grown in the animal facility of Liege University (Liege, Belgium) and female C57BL/6 mice, aged 6–8 weeks, purchased from Sao Paulo University (Sao Paulo, Brazil) were used as sources of thymus or liver. Animals were killed after profound ether anesthesia; the specimens were immediately removed and embedded in mounting medium (Tissue-Tek O.C.T. compound, Miles Laboratories, Inc., Naperville, IL).

Chemicals

L-rT_3, *O*-phenylenediamine dihydrochloride, and 3,3′-diaminobezidine were provided by Sigma Chemical Co. (St. Louis, MO); H_2O_2 was

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Address all correspondence and requests for reprints to: Dr. Wilson Savino, Laboratory on Thymus Research, Department of Immunology, Institute Oswaldo Cruz-FIOCRUZ, Avenue Brasil 4365 Manguinhos, 21045 Rio de Janeiro-RJ, Brazil.

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† Senior Research Associate of the National Fund for Scientific Research (Belgium).

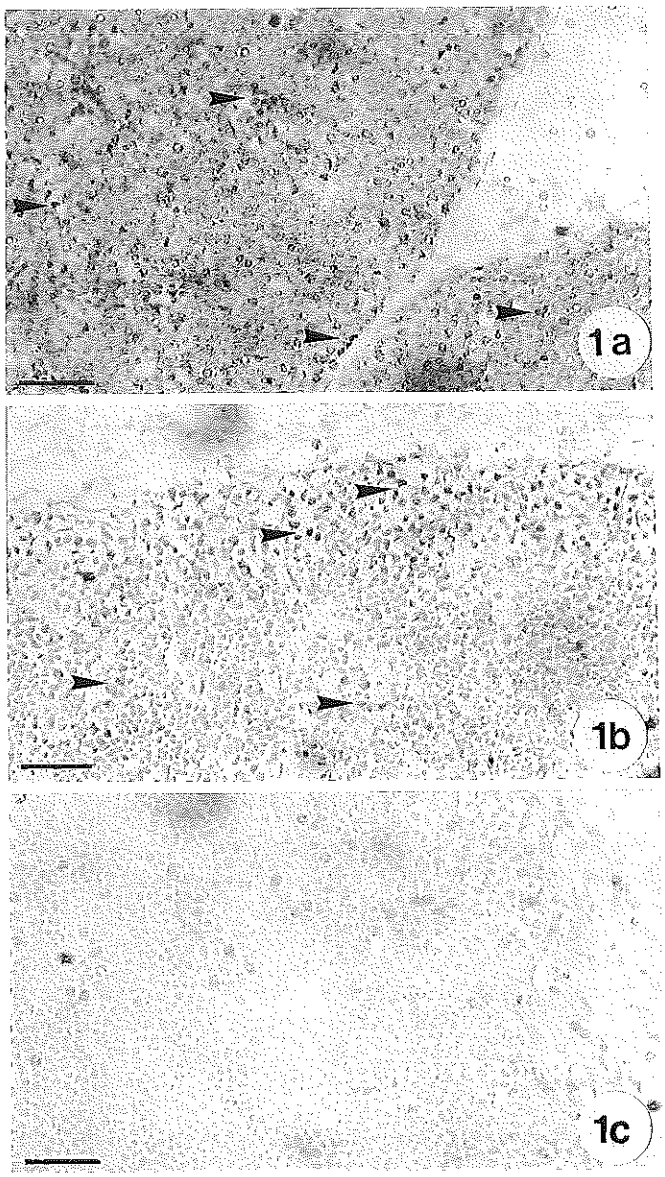


FIG. 1. Expression of NTR in the thymus of a 4-week-old rat (a) and a newborn mouse (b) revealed by the immunogold silver-staining technique. Note scattered positive cells in a, whereas staining is concentrated in subcapsular and outer cortex in b (*arrowheads*). c represents a newborn mouse thymus section that remained negative after exposition to an unrelated mouse MAb. Magnification, ×800. *Bar* = 12.5 μm.

purchased from Merck (Rio de Janeiro, Brazil). Streptavidin-biotinilated horseradish-peroxidase complex (streptavidin-PO) was obtained from Amersham International (Aylesbury, Bukinghamshire, United Kingdom).

Antibodies

The anti-NTR monoclonal antibody (MAb; clone G-8) was produced, as previously described (24), after fusioning mouse myeloma cells with splenocytes from a mouse immunized with NTR solubilized from purified rat liver nuclei. This reagent can recognize both α- and β-subtypes of T_3 receptors (Dussault, J. H., unpublished data). Goat antimouse immunoglobulin G (IgG) antibody was used coupled to colloidal gold particles (GAM-Gold) of 5 nm mean diameter for light microscopic immunogold silver staining or 10–15 nm for electron microscopy (Jans-

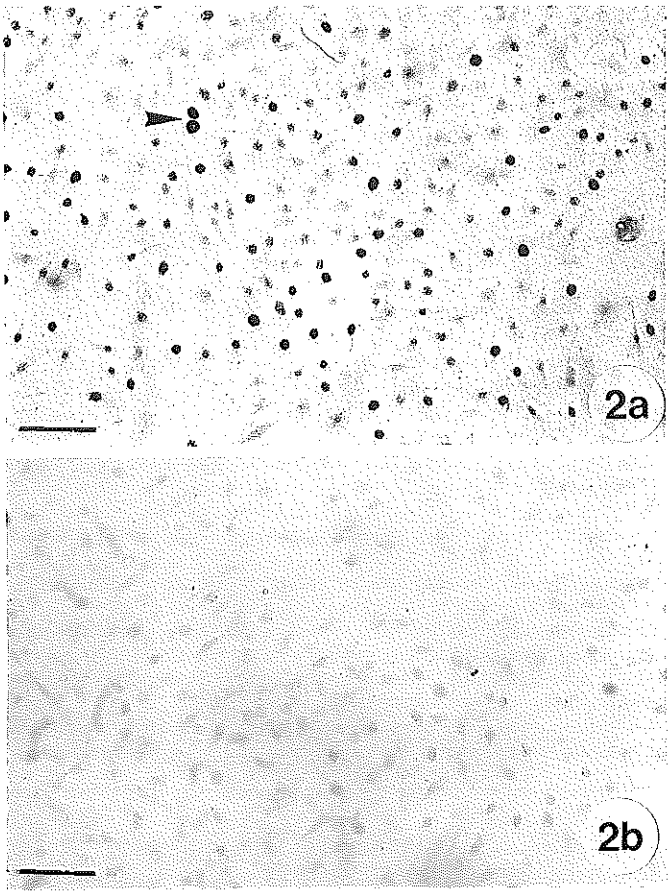


FIG. 2. NTR expression in the IT-76M1 mouse TEC line revealed by the immunogold silver-staining technique (a). The intensity of nuclear staining is variable among cells, but a very strong labeling is noted in TEC at late telophasis (*arrowhead*). b depicts an equivalent microscopic field that remained virtually negative after being exposed to an unrelated mouse MAb. Magnification, ×500. *Bar* = 20 μm.

sen Biotech N.V., Olen, Belgium). Biotin-coupled sheep antimouse Ig immune serum (SAM-biotin) was an Amersham product.

TEC lines

The mouse TEC line IT-76M1 (25) and the rat TEC line IT-45R1 (26) were developed and kindly provided by Dr. T. Itoh (Tohuku University, Sendai, Japan). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2×10^{-3} M L-glutamine, 10^{-3} M sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco Ltd., Paisley, Scotland) on 15-ml slide flasks (Nunc, Roskilde, Denmark) at 37 C in humidified atmosphere containing 5% CO_2.

Preparation of thymic nurse cells (TNC)

TNC were isolated from C57BL/6 female mice, aged 4–5 weeks, as previously described (27, 28). When freshly isolated, these structures comprise 1 epithelial cell surrounding 20–200 thymocytes. Once obtained, TNC were plated in tissue culture dishes (Nunc) in complete RPMI medium for 48–72 h. TNC-derived epithelial cultures were then washed, fixed, and processed for immunocytochemistry.

Immunocytochemical techniques

Five-micron thick thymus frozen sections as well as TEC cultures were fixed with 3% (wt/vol) paraformaldehyde in PBS, pH 7.2, for 10

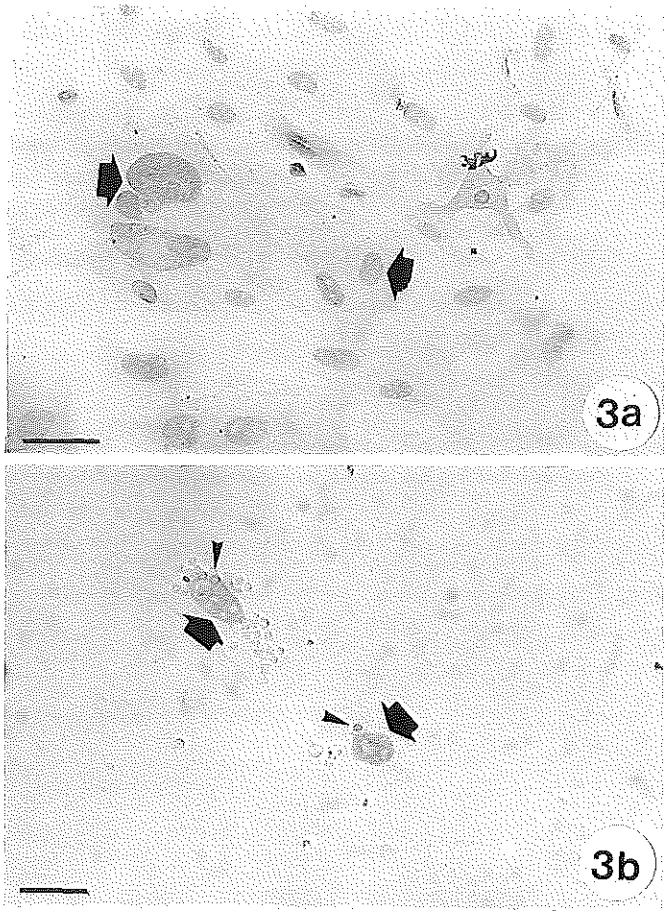


FIG. 3. Expression of NTR in TNC complexes. After a 24- to 48-h period of culture, thymocytes are spontaneously released from complexes. In a, a lymphocyte-free day 6 TNC culture is depicted. Epithelial cells express NTR (*large arrows*) with negative nucleoli. Cytoplasmic profiles can also be seen, remaining negative. In b, positive staining is also noted in some thymocytes that lie over epithelial cell cytoplasm (*arrowheads*) of a 48-h TNC culture. Immunoperoxidase technique. Magnification, ×800. *Bar* = 12.5 μm.

min at room temperature. Slides were rinsed in PBS and incubated for 2 h with 1:50 diluted normal goat serum, followed by 1-h incubation with anti-NTR MAb diluted 1:50. Specimens were washed in PBS three times and subjected to the GAM-Gold (dilution 1:10) for 1 h. Gold labeling was revealed by 10-min incubation with IntenSE M silver solution (Janssen Biotech N.V.) in the dark. Slides were then extensively rinsed in tap water and mounted for light microscopy. Controls included the omission of the anti-NTR MAb or its replacement by an unrelated mouse MAb.

For immunostaining of TEC cultures, including TNC, antibodies were diluted in 0.02% Triton X-100 solution in PBS, pH 7.2. Control specimens were treated with normal goat serum alone or followed by the second step antibody, both being revealed by the silver solution. NTR-positive cells in thymus sections and TEC cultures were expressed as percentages after counting at least 1000 cells.

For immunoelectron microscopy, freshly isolated TNC, TEC cultures, and thymus fragments were fixed for 60 min in a solution containing 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodilate buffer, pH 7.2. Specimens were then washed twice in the buffer, dehydrated in methanol, and embedded in Lowicryl K4M at −20 C under UV light (29). Thin sections were collected on 300-mesh nickel grids and incubated for 2 h with anti-NTR MAb diluted 1:20 in PBS-BSA (0.1%). Grids were then washed three times and submitted for 2 h to GAM-gold antibody (1:20 dilution). Control grids were incubated in the presence of PBS-BSA (0.1%) and the second antibody. Lastly, specimens were stained with uranyl acetate and lead citrate and observed under a Zeiss 900 transmission electron microscope (New York, NY).

Immunoblotting

Extracts containing 2-mercaptoethanol were obtained from C57BL/6 thymus and the two TEC lines, as previously described (30). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 0.8-mm thick gels, with Tris-glycine as running buffer. Once resolved, protein bands were transferred onto nitrocellulose sheets. After checking transfer with 0.2% Ponceau red (in 3% trichloroacetic acid), nonspecific protein-binding sites of nitrocellulose paper were saturated for 30 min with a PBS solution containing 10% FCS and 0.2% Tween-20. Lanes were then incubated with anti-NTR MAb (diluted 1:50) overnight at 4 C, washed in FCS-Tween-PBS for 30 min, submitted to SAM-biotin (diluted 1:250) for 2 h, washed again, and finally incubated with streptavidin-PO (1:250 dilution) for 2 h. Enzyme activity was revealed with 0.03% H_2O_2 in the presence of 0.25 mg/ml 3,3′-diaminobenzidine previously dissolved in citrate-phosfate buffer, pH 5.0.

Modulation of NTR expression

In an attempt to modulate T_3 receptor expression in TEC cultures, the mouse TEC line was grown in the presence of T_3 (10^{-6} or 10^{-8} M), added 24 h after plating, for various times ranging from 15 min to 48 h. Cells were then washed, fixed as described above, and submitted to immunoperoxidase or enzyme-linked immunosorbent (ELISA) assays.

Immunoenzymatic assay (ELISA)

Semiquantitative evaluation of NTR was carried out as previously described (31), using an ELISA directly applied onto growing TEC. Nonspecific binding sites of 96-well microplates were initially blocked with a 1% BSA solution in PBS overnight at 4 C. Wells were incubated in triplicate with the anti-NTR MAb (1:300) for 1 h, washed in PBS three times, and submitted to the SAM-biotinilated antibody (diluted 1:500), followed by the streptavidin-PO complex in the same dilution. Enzyme activity was developed with *O*-phenylenediamine dihydrochloride and quantitated using a Titertek Multiskan MCC/340 ELISA reader.

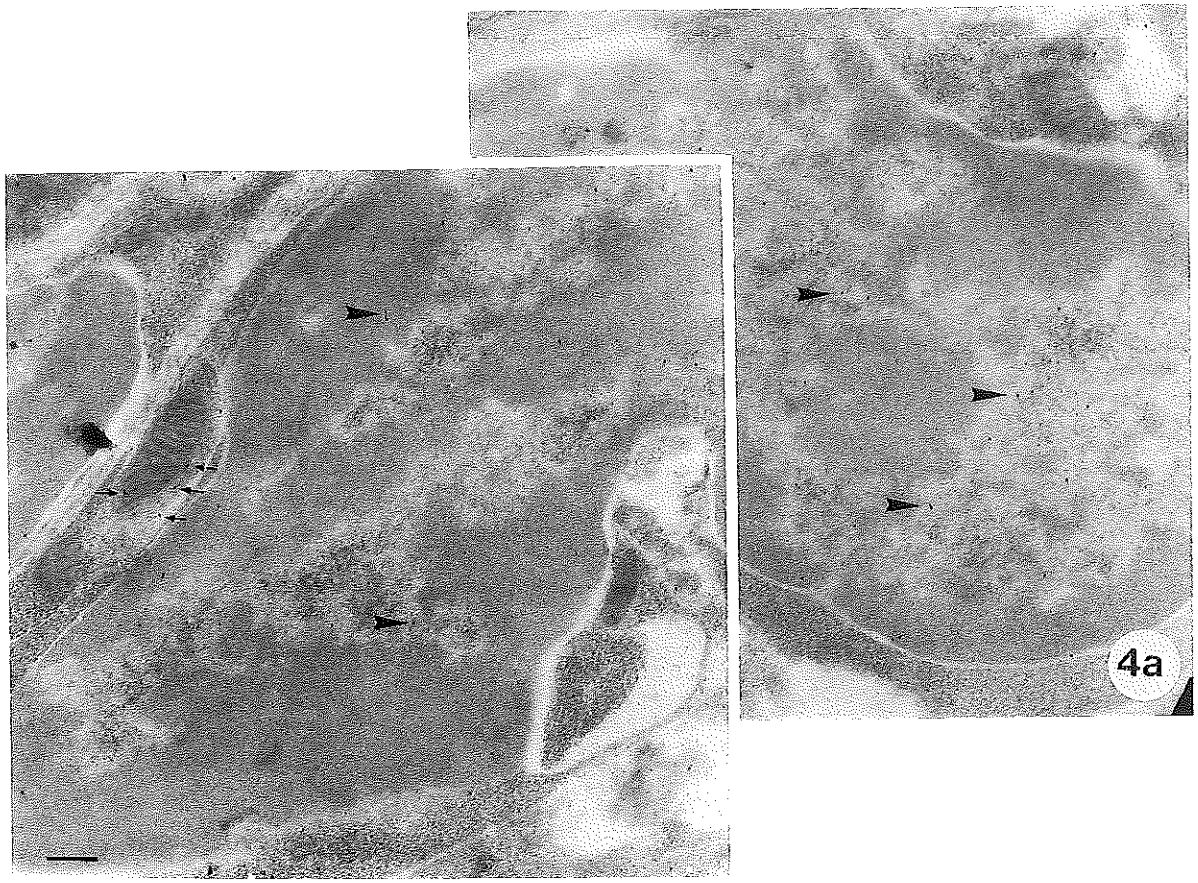
Statistical analysis

Differences in NTR expression by TEC were assessed by Student's *t* test.

Results

Detection of NTR in TEC and thymocytes

Murine young adult thymus frozen sections stained with the anti-NTR MAb exhibited a nuclear positive reaction in both lymphocytes and microenvironmental cells in the cortex and medulla of thymic lobules (Fig. 1a). This pattern of labeling was similar to that observed in hepatocytes, herein applied as positive controls of the immunohistochemical reaction (not shown). In fact, for both rat and mouse adult thymuses, the percentages of NTR-positive cells were 32% (range, 28.5–35.5%) for cortex and 40% (range, 35–44%) for medulla. Interestingly, neonatal mouse thymus sections showed intense nuclear labeling in lymphocytes and microenvironmental cells concentrated in the subcapsular and outer cortex (with 90% of the positive cells in this region), although the deep cortex and medulla exhibited 75% positive cells of weaker staining (ranges varying, respectively, from 87–94% and 65–84%; Fig. 1b).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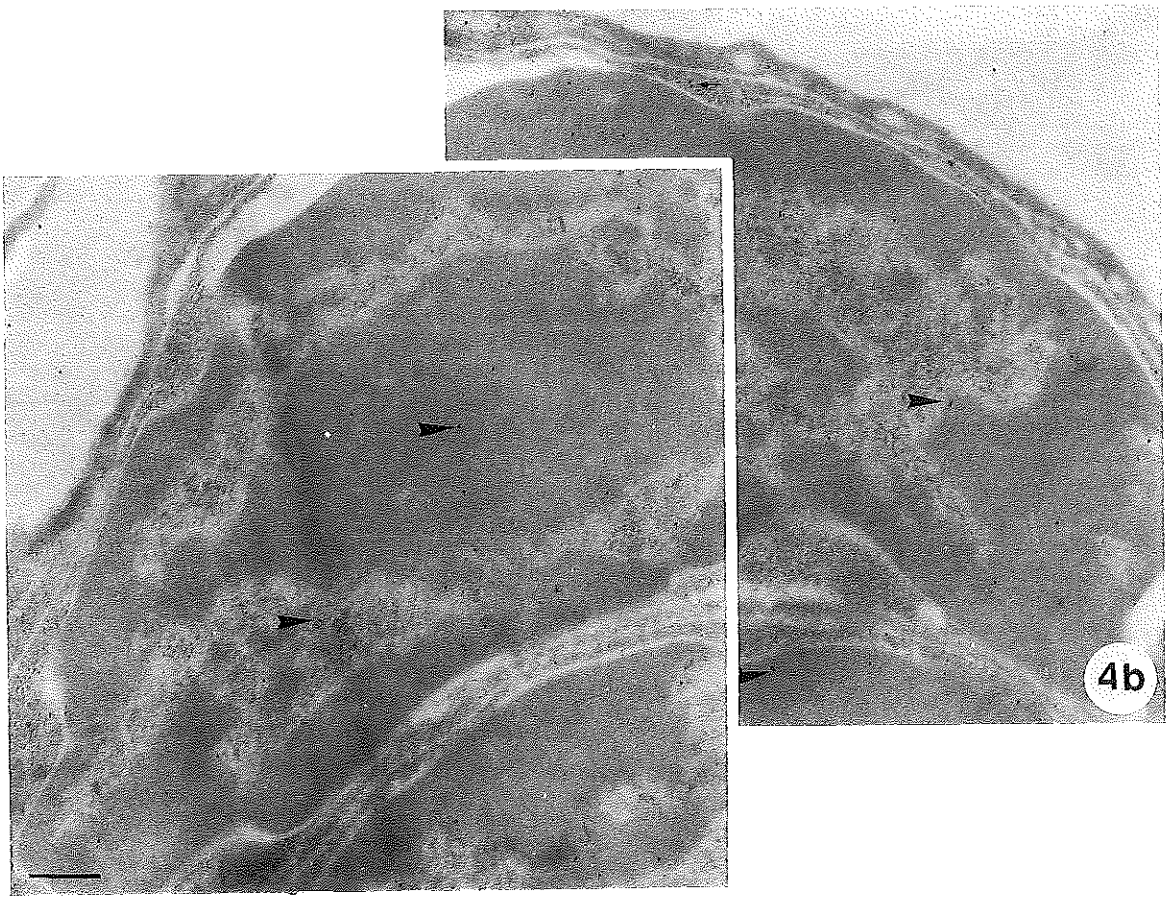
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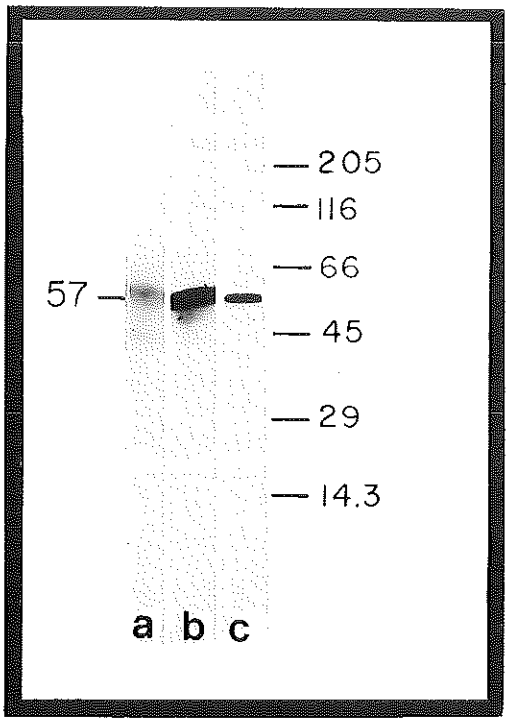

FIG. 5. Immunoblotting for detection of NTR in extracts from mouse thymus (b) and the TEC line (c). A single 57K protein band is observed in both extracts, whereas a weaker 45K protein band is also present in liver (a). Mol wt markers are indicated ($\times 10^{-3}$).

In vitro studies using the murine TEC lines definitely revealed intranuclear labeling. In these cultured TEC, the intensity of nuclear staining was variable among cells. The percentage of positive nuclei was about 94% for the rat TEC line and 81.5% for the mouse line (with ranges, respectively, from 90–96.5% and 71.5–84.5%). Interestingly, nuclei of TEC undergoing late telophasis exhibited very strong labeling (Fig. 2).

Since we found cells expressing the T_3 nuclear receptor in the subcapsular and outer thymic cortex, we investigated the presence of NTR in TNC, which are presumed to be located in this region (28, 32, 33). In fact, as depicted in Fig. 3, TNC-derived epithelial cells were clearly stained with the anti-NTR MAb, with the nucleolus region remaining negative. Positive staining could also be noted in some TNC-contained thymocytes (Fig. 3b).

It should be noted that thymus or liver frozen sections and TEC cultures exposed to an unrelated MAb used at equivalent Ig concentrations showed no staining (Figs. 1c and 2b).

In addition to light microscopy, immunostaining of ultrathin sections from TNC or TEC line cultures with the anti-NTR MAb and further analyzed at the ultrastructural level consistently revealed nuclear labeling in both thymocytes and TEC. In this latter cell type, positive staining was predominantly found in the euchromatin region (Fig. 4a), whereas thymocytes also showed labeling in the heterochromatin (Fig. 4b).

Mol wt of thymic NTR

Immunoblot analysis of murine thymic extracts using the anti-NTR MAb consistently revealed a single protein band, with an apparent mol wt of 57,000 (57K), a pattern also observed when extracts from both murine TEC lines were applied. An additional weaker 45K protein could be observed in liver extracts, as previously described (24). These results are summarized in Fig. 5.

In vitro modulation of NTR expression

To determine whether these 57K T_3 receptors were functional in terms of recognizing the respective ligand, cultures of the mouse TEC line were treated with T_3 (10^{-6} or 10^{-8} M) and analyzed at various times for immunochemical detection of the NTR. As assessed by blind analyses by two different observers, no change could be observed in NTR immunostaining 15 min after T_3 treatment. However, after 1-h incubation with the hormone, we observed a clear-cut fading in NTR labeling, which could still be seen 5 h posttreatment. By 8 h, NTR immunoreactivity reappeared. Nonetheless, a labeling intensity comparable to that in control cultures was obtained only 48 h after the addition of T_3 to cultures. These immunocytochemical data, depicted in Fig. 6, were further confirmed by ELISA semiquantitative evaluation (Fig. 7).

Discussion

In this report we show that both *in vivo* and *in vitro*, microenvironmental as well as lymphoid cell components of the thymus bear NTR. This could be demonstrated with the use of an anti-NTR MAb, originally produced against rat purified liver-derived NTR, which also recognizes NTR in a variety of rat tissues, namely pituitary, kidney, spleen, heart, and lung (24, 34).

In respect to the thymus, the presence of specific binding sites for T_3 as well as T_4 was demonstrated previously by using radiolabeled ligands (16–18). These receptors were essentially located in nuclear nonhistone proteins (18), although cytoplasmic (16) and plasma membrane (17) binding sites for T_3 have also been described in rat thymocytes. Nonetheless, the precise intrathymic localization of T_3 receptors, which is relevant for a better understanding of thyroid hormone influences on the organ, was not defined. The combined *in situ* and *in vitro* immunocytochemical approaches allowed us to demonstrate that not only thymocytes but also TEC bear NTR.

In regard to the composition of the thymic T_3 receptor, immunoblot studies indicate that it corresponds to a single

FIG. 4. Immunoelectron microscopy of a mouse TEC line (a) and a TNC complex (b) labeled with anti-NTR MAb and revealed by colloidal gold conjugate. In the TEC nucleus shown in a, staining is predominantly found in the heterochromatine (*arrowheads*), whereas in b, a lymphocyte nucleus shows positive staining in both euchromatin and heterochromatin regions (*arrowheads*). Some gold particles seen in the extranuclear position (*arrows*), although virtually absent in control preparations, may correspond to difusion of NTR during processing of thymus for electron microscopic analysis. Magnification, ×26,600. *Bar* = 380 nm.

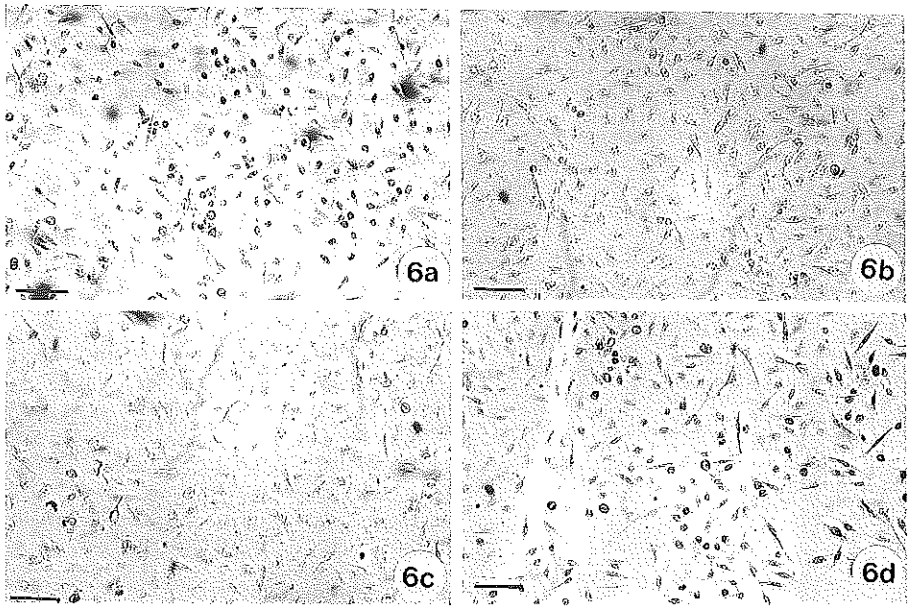

FIG. 6. Modulation of nuclear T_3 receptor expression in the mouse TEC line after T_3 treatment. Epithelial cells were cultured in the absence (a) or presence of the hormone applied in a 10^{-6}-M concentration for 1 (b), 5 (c), or 24 (d) h. Immunoperoxidase technique. Magnification, ×500. *Bar* = 20 μm.

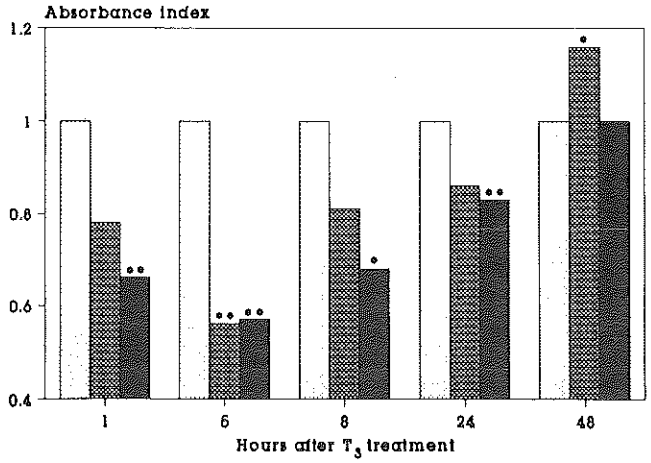

FIG. 7. Semiquantitative evaluation of NTR expression by TEC after modulation with T_3, as determined by ELISA. Data represent the absorbance index of anti-NTR labeling in control TEC cultures (□) or T_3-treated cells in 10^{-8}-M (▨) or 10^{-6}-M (■) hormone concentrations. Three distinct experiments were considered, with SDs not exceeding 15% of the mean values. *, $P < 0.05$; **, $P < 0.01$.

polypeptide chain with a mol wt of 57K. This is similar to the NTR described in brain tissue, but different from what was reported in liver, kidney, and spleen extracts, in which a weaker 45K protein band could be simultaneously detected with the major 57K band, using the same anti-NTR reagent (24). In fact, studies on human and rat cells together with the recent cloning of several thyroid hormone receptor-related cDNAs suggest that different tissues may express more than one form of NTR-related proteins, defined as NTR α and β, which are in the mol wt range of 45–58K, that could account for functional tissue heterogeneity (35–37).

A further aspect deserving discussion is related to the *in vitro* modulation of the anti-NTR MAb-defined epitope by T_3. It was previously demonstrated that liver nuclei isolated from T_3-treated rats showed an apparent decrease in total NTR number compared to nuclei obtained from control and thyroidectomized animals (38). It is possible that the fading of NTR immunostaining after T_3 treatment would be determined by conformational changes in the receptor molecule when occupied by its ligand, thus masking the anti-NTR MAb-defined epitope(s). Alternatively, it might be due to an enhancement of NTR breakdown, after its binding to the thyroid hormone. Interestingly, NTR down-regulation upon T_3 treatment was observed in the GH_3 rat pituitary cell line, regarding both NTR α- and β_2-subtypes (39, 40). In contrast, the β_1-receptor was shown to be up-regulated in the same cell line after 24 h of T_3 treatment (40). Taking into account that the anti-NTR MAb used herein recognizes both α- and β-subtypes of T_3 receptors, and that modulation of NTR was noted regarding α-, β_1-, and β_2-subtypes, further studies will be necessary to clearly define the subtype(s) of NTR expressed in thymic cells. In any case, it should be pointed out that these results clearly suggest that the T_3 receptor reported herein is really functional, since it is somewhat altered when in contact with T_3.

Differences in the intensity of NTR labeling were also observed *in vivo*, with a regional strong immunoreactivity in newborn animals. Actually, the finding that neonatal thymic subcapsular and outer cortex concentrate high amounts of NTR is in keeping with previous work showing a significantly greater NTR-binding capacity in thymocytes of newborn than in adult rats (16). As T_3 levels in newborn rats are very low (41), it is conceivable that the higher NTR-binding capacity found in neonatal thymus would ensure T_3 influence during thymus ontogeny. Moreover, the expression of NTR by thymic nurse complexes, including epithelial cells and lymphocytes (the latter mostly bearing the immature phenotype), clearly suggests a role for thyroid hormones in the early stages of intrathymic T-cell differentiation.

The definition of receptors for T_3 in distinct thymic cell types should also be discussed in the context of the extrinsic control of thymus physiology. In this respect, our data pro-

vide further evidence that thyroid hormones play a pleiotropic role in the thymus, directly acting on both thymocyte and microenvironmental compartments. This hypothesis is supported by the following lines of evidence. A direct effect of thyroid hormones on thymocytes was reported by Segal *et al.* (42), who showed that T_3 was able to increase glucose uptake by these cells. In contrast, other effects, such as thymocyte proliferation, may be indirect, being mediated by TEC via thymic hormone production. In fact, the addition of T_3 to adult thymocytes *in vitro* did not influence the [^{3}H] thymidine incorporation rate. However, when supernatants from IT-76M1 cells grown in the presence of T_3 were applied to thymocytes, proliferation occurred to a greater extent than with supernatants from untreated TEC. This thymocyte proliferation was shown to be greatly determined by thymulin, which also modulated the CD4/CD8-defined phenotypes (43). These latter findings are in keeping with data showing that thymulin secretion is modulated *in vitro* by T_3 (21), and that thyroid hormone status *in vivo* influences thymic hormone production in both health and disease (20, 23, 44). Regarding the effects of intracellular events in TEC on thyroid hormone action, recent studies suggest that the T_3-induced enhancement of thymulin secretion by TEC is due to a *de novo* synthesis, since cycloheximide (a potent inhibitor of mRNA turnover) prevents this effect in thymic organ cultures (45).

Lastly, the fact that not only thymic hormone secretion but also TEC growth as well as extracellular matrix production are modulated by T_3 (our manuscript in preparation) clearly indicates that the binding of T_3 to its receptor should trigger a variety of genes in the thymic epithelium.

In conclusion, our data provide further molecular basis for the mechanism by which thyroid hormones modulate thymus physiology. Regarding the lymphoid compartment, we are presently developing flow cytometry studies to determine NTR expression in thymocytes as a function of thymus ontogeny and in terms of distinct thymocyte subsets defined by relevant differentiation molecules, namely CD3, CD4, and CD8. Additionally, the present results together with others previously reported (see reviews in Refs. 8 and 9) extend the concept that the thymic epithelium is a common target for a variety of neuroendocrine influences.

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