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## Age-related decrease of somatostatin receptor number in the normal human thymus

DIEGO FERONE,<sup>1,4</sup> ROSARIO PIVONELLO,<sup>1,4</sup> P. MARTIN VAN HAGEN,<sup>1,2</sup> MARLIJN WAAIJERS,<sup>1</sup> JOKE ZUIJDERWIJK,<sup>1</sup> ANNAMARIA COLAO,<sup>4</sup> GAETANO LOMBARDI,<sup>4</sup> AD J. J. C. BOGERS,<sup>3</sup> STEVEN W. J. LAMBERTS,<sup>1</sup> AND LEO J. HOFLAND<sup>1</sup>  
<sup>1</sup>Department of Internal Medicine, Departments of <sup>2</sup>Immunology and <sup>3</sup>Cardiothoracic Surgery, Erasmus University, 3015 GD Rotterdam, The Netherlands; and <sup>4</sup>Department of Molecular and Clinical Endocrinology and Oncology, Federico II University, 80131 Naples, Italy

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**Ferone, Diego, Rosario Pivonello, P. Martin Van Hagen, Marlijn Waaijers, Joke Zuidervijk, Annamaria Colao, Gaetano Lombardi, Ad J. J. C. Bogers, Steven W. J. Lamberts, and Leo J. Hofland.** Age-related decrease of somatostatin receptor number in the normal human thymus. *Am J Physiol Endocrinol Metab* 279: E791–E798, 2000.—The thymus exhibits a pattern of aging oriented toward a physiological involution. The structural changes start with a steady decrease of thymocytes, whereas no major variations occur in the number of thymic epithelial cells (TEC). The data concerning the role of hormones and neuropeptides in thymic involution are equivocal. We recently demonstrated the presence of somatostatin (SS) and three different SS receptor (SSR) subtypes in the human thymus. TEC selectively expressed SSR subtype 1 ( $sst_1$ ) and  $sst_{2A}$ . In the present study we investigated whether SSR number is age related in the thymus. Binding of the  $sst_2$ -preferring ligand  $^{125}\text{I-Tyr}^3$ -octreotide was evaluated in a large series of normal human thymuses of different age by SSR autoradiography and ligand binding on tissue homogenates. The score at autoradiography and the number of SSR at membrane homogenate binding ( $B_{\text{max}}$ ) were inversely correlated with the thymus age ( $r = -0.84, P < 0.001$ ;  $r = -0.82, P < 0.001$ , respectively). The autoradiographic score was positively correlated with the  $B_{\text{max}}$  values ( $r = 0.74, P < 0.001$ ). Because the TEC number in the age range considered remains unchanged, the decrease of octreotide binding sites might be due to a reduction of  $sst_{2A}$  receptor number on TEC. The age-related expression of a receptor involved mainly in controlling secretive processes is in line with the evidence that the major changes occurring in TEC with aging are related to their capabilities in producing thymic hormones. In conclusion, SS and SSR might play a role in the involution of the human thymus. These findings underline the links between the neuroendocrine and immune systems and support the concept that neuropeptides participate in development of cellular immunity in humans.

octreotide

THE THYMUS, THE PRIMARY LYMPHOID ORGAN responsible for differentiation and maturation of the specific T cell

Address for reprint requests and other correspondence: L. J. Hofland, Dept. of Internal Medicine, Rm. Bd 277, University Hospital Dijkzigt, Dr. Molewaterplein, 40, 3015 GD Rotterdam, The Netherlands.

repertoire, exhibits an aging behavior that is unique because of its irreversible physiological involution (16). This phenomenon is characterized by a progressive structural change of the gland, starting at an early stage of life. Lipomatous atrophy is the most evident age-related change in the thymus, although it represents the final state of the involution (33). The early stages of this process are essentially characterized by a steady decrease in the number of thymocytes, the lymphoid cellular component, and thymic dendritic cells, whereas no major changes are found in the number of thymic epithelial cells (TEC), which represent the most relevant component of the thymic stroma (22). However, the human thymic epithelium is capable of undergoing sequential stages of maturation in the postnatal thymus (18). The factors regulating the involution process of the thymus have not yet been completely clarified. Particularly, contradictory hypotheses have been raised concerning the potential role of hormones and neuropeptides in this process. For instance, thymic involution is considered to be either dependent on or independent of puberty (34, 36). Because several neuropeptides have been localized in lymphoid tissues and because somatostatin (SS) may influence cells of the immune system, we have recently searched for the presence of SS and SS receptors (SSR) in the normal human thymus (7). SS and three different SSR subtypes ( $sst$ ),  $sst_1$ ,  $sst_{2A}$  and  $sst_3$ , were expressed in human thymic tissue, although  $sst_1$  and  $sst_{2A}$  were expressed selectively on cultured TEC (7). Moreover, SS and octreotide administration induced an in vitro inhibition of TEC proliferation, which is presumably mediated by receptors of the  $sst_{2A}$  subtype (7). These data support the concept of a modulatory action of SS on cell functions within the thymus. In addition, a functional role of SS and SSR in the involution process of the thymus can be hypothesized as well. To evaluate whether the SSR pattern shows an age-related change, we studied the binding of the  $sst_2$ -

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preferring ligand  $^{125}\text{I-Tyr}^3$ -octreotide in a large series of normal human thymuses of different age. SSR density was determined both by SSR autoradiography and by ligand binding studies on tissue homogenates. The results were correlated with the chronological age of the thymuses.

## METHODS

**Samples.** Thymic tissues were removed from 30 patients (15 males and 15 females, age ranging between 15 days and 21 yr) to allow adequate exposure of the heart during cardiovascular surgery. Samples from these thymuses were used in the present study. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation, and informed consent was obtained from patients or their parents. All samples were histopathologically normal and were taken fresh at the operation, quickly frozen on dry ice, and stored at  $-80^\circ\text{C}$  for ligand binding on cryostat sections and membrane homogenates. The 30 thymic tissue samples were divided into five groups on the basis of arbitrary age ranges: *group 1* ( $n = 10$ ), 0–12 mo; *group 2* ( $n = 5$ ), 13–24 mo; *group 3* ( $n = 5$ ), 25–72 mo; *group 4* ( $n = 5$ ), 73–120 mo; *group 5* ( $n = 5$ ), >120 mo.

**SS receptor binding on cryostat sections.** Receptor autoradiography was carried out as described by Visser-Wisseelaar et al. (39). Briefly, 10- $\mu\text{m}$ -thick cryostat (Jung CM3000, Leica, Germany) sections of the tissue samples were mounted onto precleaned gelatine-coated microscope glass slides and stored at  $-80^\circ\text{C}$  for  $\geq 3$  days before the experiment to improve the adhesion of the tissue to the slide. As radioligand, the SS analog  $^{125}\text{I-Tyr}^3$ -octreotide (Novartis Pharma, Basel, Switzerland) was used. Specific activities of the radioligand amounted to  $\sim 2,000$  Ci/mmol. To wash out endogenous SS, the sections were preincubated at room temperature for 10 min in 170 mM Tris·HCl (pH 7.4). Thereafter, the sections were incubated for 60 min at room temperature in binding buffer (170 mM Tris·HCl (pH 7.4), 5 mM  $\text{MgCl}_2$ , 1% BSA, 40  $\mu\text{g/ml}$  bacitracin) with  $^{125}\text{I-Tyr}^3$ -octreotide (final concentration  $\sim 80$ –160 pmol/l). Nonspecific binding was determined in a sequential section in the presence of excess unlabeled  $\text{Tyr}^3$ -octreotide (1  $\mu\text{M}$ ). The incubated sections were washed twice for 5 min in binding buffer containing 0.25% BSA and once in binding buffer without BSA. After a short wash in distilled water to remove salts, the sections were air dried and exposed to Kodak X-OMAT AR or Hyperfilm- $^3\text{H}$  (Amersham) for 3–7 days in X-ray cassettes. Histology was performed on hematoxylin-eosin-stained sequential cryosections. A sample was considered positive for  $^{125}\text{I-Tyr}^3$ -octreotide binding when the signal obtained in a control section was displaced by an excess of unlabeled octreotide by  $>50\%$  (12). The binding signals obtained were analyzed densitometrically by means of a computer-assisted image processing system and were quantified by calculating the ratios between the regions of interest delineated on the total (T) and nonspecific (NS) binding sections. By use of the total-to-nonspecific (T/NS) ratios, the amount of binding in every section was graded as negative (0) for T/NS ranging from 0 to 1.9, positive (1) for T/NS ranging from 2 to 3, and strongly positive (2) for T/NS  $>3$ .

**SS receptor binding on membrane homogenates.** The method of membrane isolation and the reaction conditions were the same as those described by Reubi (28). Briefly, membrane preparations (corresponding to 30–50  $\mu\text{g}$  protein) of tissue samples were incubated in a total volume of 100  $\mu\text{l}$

at room temperature for 60 min with increasing concentrations of  $^{125}\text{I-Tyr}^3$ -octreotide without and with excess (1  $\mu\text{M}$ ) unlabeled octreotide in HEPES buffer (10 mM HEPES, 5 mM  $\text{MgCl}_2$  and 0.02 g/l bacitracin, pH 7.6) containing 0.2% BSA. After the incubation, 1 ml ice-cold HEPES buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer, and the final pellet was counted in a  $\gamma$ -counter (1470 Wizard, Wallac, Turku, Finland). Specific binding was taken to be total binding minus binding in the presence of 1  $\mu\text{M}$  unlabeled octreotide.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. All data were analyzed by ANOVA to determine overall differences between groups. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The comparison between categorical data among the groups was analyzed with the Fisher's exact test. The correlation study was performed by use of nonlinear or linear analysis calculating the Spearman or Pearson coefficients, respectively, where appropriate. SSR binding data were analyzed by the method of Scatchard. Receptor binding studies were performed at least twice.

## RESULTS

**SSR binding on cryostat sections.** Figure 1 shows an exemplary case for each age group of the specific binding of the  $\text{sst}_2$  subtype-preferring ligand  $^{125}\text{I-Tyr}^3$ -octreotide on cryostat sections of human thymus. At autoradiography, the binding was not homogeneously distributed but was localized mainly in the medullary region of the thymuses (Fig. 1). With the use of a three-point score, the amount of binding was graded as strongly positive (2) in 8 out of 10 cases (80%) of *group 1* and in 1 out of 5 cases (20%) of *groups 2* and *3*. The binding was graded as positive (1) in 2 out of 10 (20%) of *group 1*, in 4 out of 5 (80%) of *groups 2* and *3*, and in 1 out of 5 (20%) of *group 4*. The binding was graded as faint or negative (0) in 4 out of 5 (80%) of *group 4* and in 5 out of 5 (100%) of *group 5*. The percentage of cases with grades 2 and 1 was significantly higher in *group 1* vs. *groups 4* and *5* ( $P < 0.005$ ) and in *groups 2* and *3* vs. *groups 4* and *5* ( $P < 0.05$ ). The mean values of T/NS ratios displayed a progressive decrease with the increasing age range in the five groups (Fig. 3A). The mean T/NS values were significantly higher in *group 1* than in *groups 4* and *5* ( $P < 0.05$ ). The decrease in the T/NS values with the increasing age of the cases in the five different groups shows an exponential rather than a linear trend (Fig. 3A). Histology was normal in all of the samples, and no major structural differences were found between the different groups.

**SSR binding on membrane homogenates.** With the use of  $^{125}\text{I-Tyr}^3$ -octreotide, specific binding was detectable on membrane preparations of all thymic tissues, except in four cases of *group 4* and three cases of *group 5*. Scatchard analysis of the binding data revealed a single class of high-affinity binding sites with an average apparent dissociation constant ( $K_d$ ) of  $0.6 \pm 0.1$  nm. The maximum binding capacity ( $B_{\text{max}}$ ) was low, with an average of  $18.5 \pm 3.6$  fmol/mg membrane protein, in the cases with detectable  $^{125}\text{I-Tyr}^3$ -



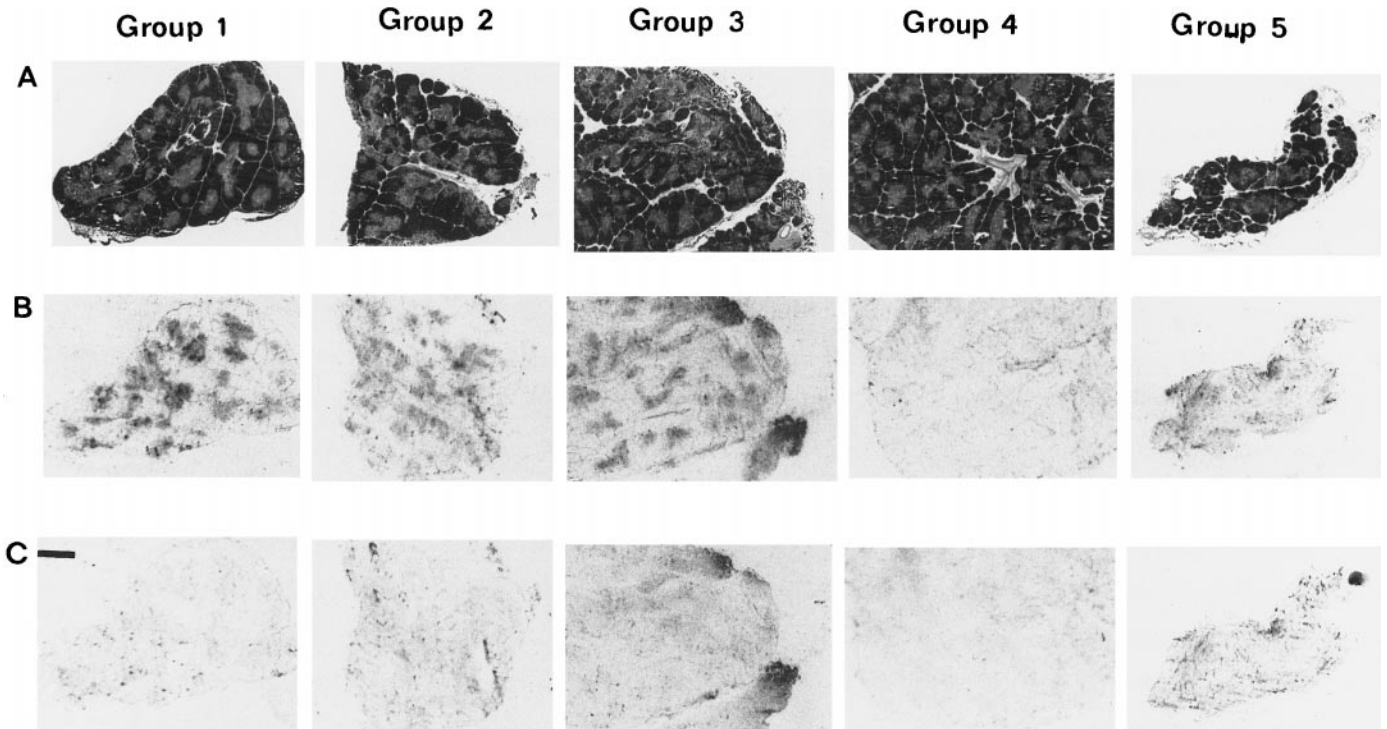


Fig. 1. Somatostatin receptor (SSR) binding in human thymuses of different ages: exemplary cases. Photomicrograph of SSR autoradiography: *group 1* (age range 1–12 mo), *group 2* (13–24 mo), *group 3* (25–72 mo), *group 4* (73–120 mo), *group 5* (>120 mo). A: hematoxylin-eosin-stained section; B: autoradiogram showing total binding of  $^{125}\text{I-Tyr}^3\text{-octreotide}$ ; C: autoradiogram showing nonspecific binding (in the presence of  $1\ \mu\text{M}$  of  $\text{Tyr}^3\text{-octreotide}$ ). Bar, 2 mm.

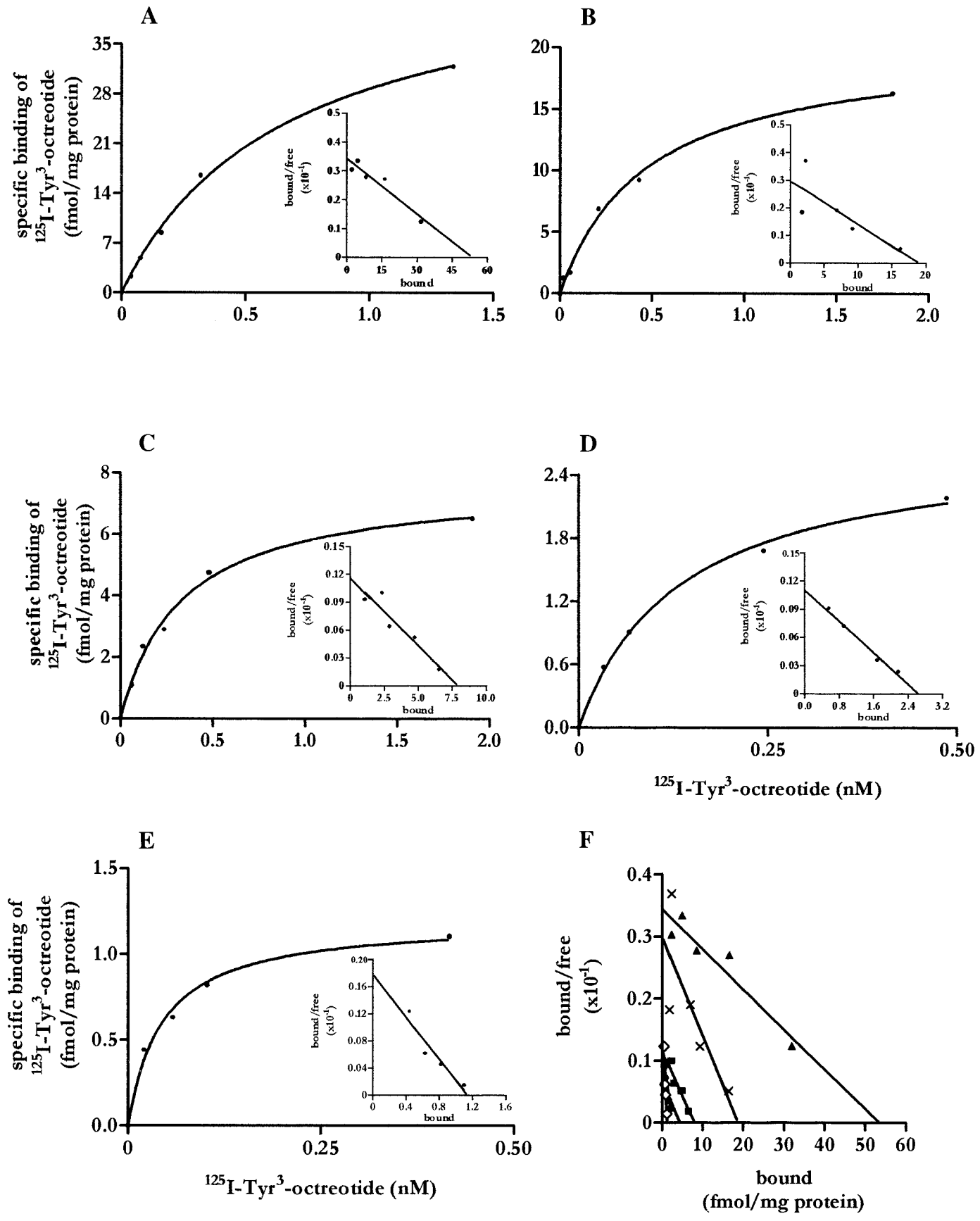
octreotide binding. A sample saturation curve for each group of with Scatchard analysis of the binding data is shown in Fig. 2. The mean values of  $B_{\max}$  displayed a progressive decrease with the increasing age range in the five groups (Fig. 3B). The mean  $B_{\max}$  values were significantly higher in *group 1* than in *groups 4* and *5* ( $P < 0.001$ ). The decrease in the  $B_{\max}$  values with the increasing age of the cases in the five groups shows an exponential rather than a linear trend (Fig. 3B). As a positive control for ligand binding, SSR-positive mouse AtT-20 pituitary tumor cell membranes were used ( $K_d$  of  $0.19 \pm 0.03\ \text{nm}$ ;  $B_{\max}$   $705 \pm 64\ \text{fmol/mg}$  membrane protein). No specific binding was detectable on a proven SSR-negative cell line and tissue (39).

**Correlations.** A significant correlation was found between ligand binding studies on cryostat sections or on membrane homogenates and age of the thymus. In detail, the T/NS ratios at autoradiography ( $r = -0.84$ ,  $P < 0.001$ ) and the  $B_{\max}$  values at membrane homogenate binding study ( $r = \text{minus}0.82$ ,  $P < 0.001$ ) were inversely correlated with the age of the thymus (Fig. 4, A and B). In addition, the T/NS ratios at autoradiography were correlated positively with the  $B_{\max}$  values at membrane homogenate binding study ( $r = 0.74$ ,  $P < 0.001$ ; Fig. 4C). Conversely, no correlation was found between the estimated  $K_d$  values and the age of the thymuses and between gender and both T/NS and  $B_{\max}$  values (data not shown).

## DISCUSSION

The involution of the human thymus with age is a complex phenomenon and remains poorly understood. The human thymus interacts with products of endocrine glands throughout life, and although conflicting results have been reported, thymic involution seems in part dependent on age-related alterations in the interaction between the neuroendocrine activity and the thymus itself (34, 36). Moreover, the intrathymic production of hormones and neuropeptides and the presence of specific receptors represent an autocrine/paracrine pathway, which, in addition to classical endocrine circuits, might modulate the activities of both the lymphoid and stromal components of this organ (32). In fact, TEC produce thymic peptides and other factors known to modulate the main function of the gland, namely the development of the T cell repertoire, and many hormones and neuropeptides may participate to this function by interacting via specific receptors with developing immune cells and with TEC (4). However, the role of neuropeptides and their receptors in thymic involution is still debated.

SS is a well-characterized neuropeptide with a wide spectrum of action, and recent insights have strongly suggested that the thymus might belong to the list of its target organs (7, 29, 30, 38). The five different SSR subtypes recently cloned and characterized (24, 27) show a tissue-specific distribution. The majority of



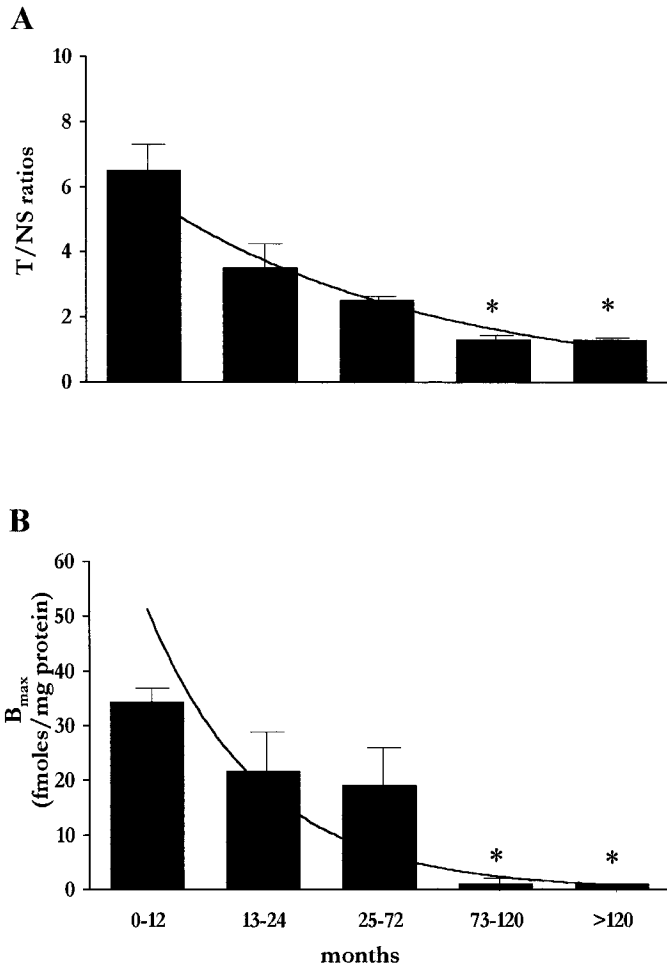


Fig. 3. SSR binding in human thymuses of different ages. *A*: total-to-nonspecific (T/NS) binding ratio values calculated at autoradiographic binding study on cryostat sections; *B*:  $B_{\max}$  values detected at binding studies on membrane homogenates of thymic tissues in groups 1–5. Bars represent the value of T/NS ratios and  $B_{\max}$  (fmol/mg protein) and are expressed as means  $\pm$  SE; \* $P < 0.05$  and  $P < 0.001$  vs. group 1, respectively. Lines represent the exponential trend of the changes in T/NS ratios and  $B_{\max}$  with the increasing age in the 5 different groups of thymuses.

SS-target tissues express multiple SSR, making it difficult to understand the functional role(s) of the individual SSR subtypes. The best known SS analog, the octapeptide octreotide, binds with high affinity to the  $sst_2$  subtype (25). In the endocrine system, where they have been better characterized, SSR subtypes are involved in the control of hormone secretion and cell proliferation, exerting mainly inhibitory effects via distinct mechanisms (15, 17). The  $sst_2$  subtype plays a major role in this system. Whereas in the endocrine system SSR activation leads to inhibitory effects, in the

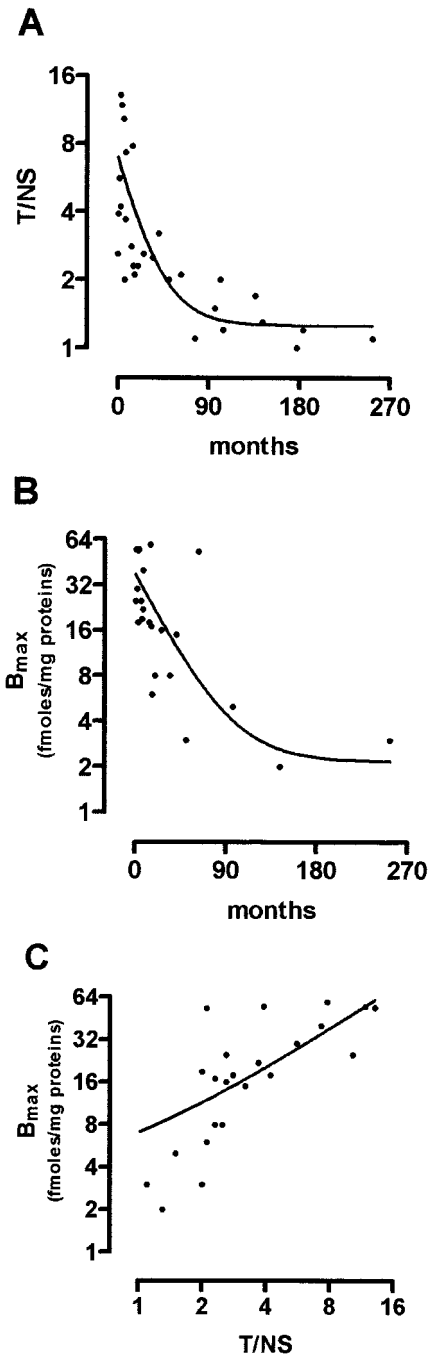


Fig. 4. SSR binding in human thymuses of different ages. *A*: correlation between age and T/NS ratio values. *B*: correlation between age and  $B_{\max}$  values; *C*: correlation between T/NS and  $B_{\max}$  values. T/NS ratios and  $B_{\max}$  (fmol/mg protein) values detected at binding studies on cryostat sections and membrane homogenates of thymic tissues of the 30 cases distributed per age (mo).

Fig. 2. SSR binding in human thymuses of different ages: exemplary cases. Binding of  $^{125}\text{I}$ -Tyr<sup>3</sup>-octreotide to a membrane homogenate preparation of human thymuses: *A*: group 1 [age, 3 mo; maximum binding capacity ( $B_{\max}$ ), 54; dissociation constant ( $K_d$ ), 0.8]; *B*: group 2 (age, 14 mo;  $B_{\max}$ , 18;  $K_d$ , 0.3); *C*: group 3 (age, 35 mo;  $B_{\max}$ , 8;  $K_d$ , 0.3); *D*: group 4 (age, 97 mo;  $B_{\max}$ , 2.7;  $K_d$ , 0.1); *E*: group 5 (age, 144 mo;  $B_{\max}$ , 1.1;  $K_d$ , 0.1). Saturation curves indicate the specific binding (total minus nonspecific binding in presence of 1  $\mu\text{M}$  of Tyr<sup>3</sup>-octreotide). *Insets*: Scatchard analysis of the binding data; *F*: summary of the Scatchard analysis data.  $\blacktriangle$ , Group 1;  $\times$ , group 2;  $\blacksquare$ , group 3;  $\bullet$ , group 4;  $\diamond$ , group 5.

immune system, both stimulatory and inhibitory effects have been reported (6, 38). Moreover, very little is known with respect to the cellular signaling mechanisms coupled to SSR activation in immune cells (13).

In normal human thymus, we recently demonstrated the presence of three different SSR subtypes,  $sst_1$ ,  $sst_{2A}$ , and  $sst_3$  (7). In TEC,  $sst_1$  and  $sst_{2A}$  were selectively expressed, and TEC seemed to produce SS, because SS mRNA was present in these cells (7). All together, these findings pointed toward an important role of the  $sst_{2A}$  receptor in the human thymus, which seems to be confirmed by the heterogeneity of the expression of this subtype among thymic cells (7).

In the present study, we have found additional evidence for a functional role of the  $sst_{2A}$  receptor in the thymus. This SSR subtype may be involved in the processes linked to the thymic age-related changes, because its number undergoes significant changes with increasing age. In fact, using two different techniques, we observed an inverse relationship between the number of binding sites for the  $sst_2$ -preferring ligand  $^{125}\text{I-Tyr}^3$ -octreotide and the age of the thymus. The number of  $^{125}\text{I-Tyr}^3$ -octreotide-binding sites was significantly higher in the younger subjects. This finding is in line with the recently reported evidence of an *in vivo* thymic uptake of  $^{111}\text{In-DTPA-D-Phe}^1$ -octreotide in the three youngest patients (ages 4, 5, and 16 mo) out of 11 who underwent SSR scintigraphy to evaluate abdominal or pelvic neuroblastoma (8). Conversely, no thymic concentration of  $^{111}\text{In-DTPA-D-Phe}^1$ -octreotide was documented in the relatively older children of the same series and in a series of adult patients with thymic hyperplasia (19). In the present study, the ages of the thymus were inversely correlated with both the results of the autoradiographic studies on thymic cryostat sections and the  $B_{\max}$  values measured at the ligand-binding studies on tissue homogenates of the corresponding cases. Moreover, the score of the autoradiography was positively correlated with the values of the  $B_{\max}$ . Although autoradiography is a semiquantitative method for the evaluation of the ligand binding, the  $B_{\max}$  value directly indicates the density of binding sites for a specific ligand expressed on cell membranes. Moreover, it should be pointed out that, at the ligand binding on membrane homogenates, the estimated  $K_d$  values were not correlated with thymus age. This suggests that the change in the number of SSR binding sites is not related to changes in receptor affinity. Considering that  $sst_{2A}$  is expressed mainly on TEC in the medullary compartment of the human thymus (7, 29, 30), it might appear that the reduction of the number of binding sites is related to the decrease in the number of  $sst_{2A}$ -expressing cells. This seems unlikely, however, because the number of TEC in the age range considered in our study remains almost unchanged (22, 33). In fact, little or no decrease in the number of keratin-positive cells has been demonstrated, whereas thymocytes especially, and to a much lesser extent dendritic cells, decrease dramatically in number during the aging process (22). During postnatal development, the thymic epithelium displays a

pathway of differentiation similar to that observed in other epithelial organs throughout the human body rather than a real change in the number of its cellular elements (18). This observation supports the concept that the thymic epithelium is capable of undergoing sequential stages of maturation during fetal and postnatal thymic development. Moreover, even considering that, at a certain point, the number of TEC declines, contributing to the significant reduction of the octreotide-binding sites, we have observed a progressive decrease of the number of octreotide-binding sites starting after the first years of life when the number of TEC is still unchanged. Because two different techniques gave a comparable result, it is suggested that the decrease in the number of  $sst_{2A}$  occurs on TEC. Although in cultured thymocytes no mRNA encoding for SSR subtypes was detectable (Fig. 7), we have recently demonstrated SSR binding on freshly isolated thymocytes (5). Preliminary RT-PCR data have shown the expression of  $sst_{2A}$  and  $sst_3$  mRNAs in resting thymocytes. However,  $sst_3$  seems to be the SSR subtype predominantly expressed in the heterogeneous pool of T lymphoid cell precursors, whereas  $sst_{2A}$  mRNA expression is limited to a very small subset of immature cortical thymocytes (unpublished observations). The recent evidence of a selective expression of  $sst_3$  mRNA in peripheral human T lymphocytes (10) is in line with our observation in thymocytes, which are the natural precursors of circulating T cells. Although the immature thymocytes are localized in the cortical region of the human thymus, a contribution of their loss to the decline of octreotide-binding sites cannot be fully ruled out. However, it should play a minor role in this phenomenon, because, according to the autoradiographic pattern, the decline of octreotide-binding sites occurs mainly in the medullary region of the thymus, where TEC are the predominant cell type displaying  $sst_{2A}$ -binding.

$sst_{2A}$  is the SSR subtype involved in controlling secretory processes in SS target cells. Thus the reduction of this receptor on TEC might be in line with the evidence that the major changes occurring in these cells during aging are related more to their functional capabilities in producing thymic hormones rather than to modification in their number (22). The decrease in  $sst_{2A}$  receptor numbers might be related to the production of substances modulating the thymus involution, as well as the maturation of T cells. Which is the factor(s) involved in regulating receptor expression needs to be further investigated. However, in light of studies in which stimulation of neuropeptide receptors by their own ligand was shown to result in receptor internalization (20, 21), it is possible that a downregulation of  $sst_{2A}$  receptors might occur as a consequence of ligand-induced internalization. In fact, the  $sst_{2A}$  receptor has been demonstrated to efficiently internalize bound ligand in many cell systems (11, 14, 23). Furthermore, it has been demonstrated in rat brain that endogenous SS regulates cell surface  $sst_{2A}$  receptors (3). The presence of endogenous SS within the human thymus (1, 7, 9, 26, 31, 35) might account for a regu-





lation of sst<sub>2A</sub> receptor expression on TEC by this mechanism. Conformational changes and/or chemical alterations of the internalized receptor might explain why the exogenous ligand does not recognize its specific receptor (23). However, the influence of additional factors, like cytokines or other neuropeptides, cannot be ruled out either in such a complex organ. It is known that hormones and neuropeptides can modulate TEC physiology by exerting a pleiotropic action on thymic stroma. In fact, glucocorticoid, thyroid, and pituitary hormones can modulate extracellular matrix ligands and receptors (32). Moreover, the expression of receptors for neuropeptides, such as vasoactive intestinal polypeptide, appears to be developmentally regulated in several systems, including the thymus (2, 37).

In conclusion, the number of sst<sub>2A</sub> is inversely correlated with the age of the human thymus. The receptor itself and obviously SS might play a role in the involution of the thymus, consequently affecting the main function of this organ. Although further studies are required to clarify this complex but fascinating network between the neuroendocrine and the immune systems within the human thymus, our findings raise the possibility that neuropeptides may participate in the intrathymic maturation and differentiation of T cell repertoire, which leads to the development of cellular immunity in humans.

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