Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes

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Ferone, Diego, Rosario Pivonello, P. Martin van Hagen, Virgil A. S. H. Dalm, Elgin G. R. Lichtenauber-Kaligis, Marlijn Waaijers, Peter M. van Koetsveld, Diana M. Mooy, Annamaria Colao, Francesco Minuto, Steven W. J. Lamberts, and Leo J. Hofland. Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes. Am J Physiol Endocrinol Metab 283: E1056–E1066, 2002.—We recently demonstrated the expression of somatostatin (SS) and SS receptor (SSR) subtype 1 (sst1), sst2A, and sst3 in normal human thymic tissue and of sst1 and sst2A on isolated thymic epithelial cells (TEC). We also found an inhibitory effect of SS and octreotide on TEC proliferation. In the present study, we further investigated the presence and function of SSR in freshly purified human thymocytes at various stages of development. Thymocytes represent a heterogeneous population of lymphoid cells displaying different levels of maturation and characterized by specific cell surface markers. In this study, we first demonstrated specific high-affinity 125I-Tyr11-labeled SS-14 binding to thymocyte membrane homogenates. Subsequently, by RT-PCR, sst2A and sst3 mRNA expression was detected in the whole thymocyte population. After separation of thymocytes into subpopulations, we found by quantitative RT-PCR that sst2A and sst3 are differentially expressed in intermediate/mature and immature thymocytes. The expression of sst3 mRNA was higher in the intermediate/mature CD3+ fraction compared with the immature CD2+CD3- one, whereas sst2A mRNA was less abundant in the intermediate/mature CD3+ thymocytes. In 7-day-cultured thymocytes, SSR subtype mRNA expression was lost. SS-14 significantly inhibited [3H]thymidine incorporation in all thymocyte cultures, indicating the presence of functional receptors. Conversely, octreotide significantly inhibited [3H]thymidine incorporation only in the cultures of immature CD2+CD3- thymocytes. Subtype sst3 is expressed mainly on the intermediate/mature thymocyte fraction, and most of these cells generally die by apoptosis. Because SS-14, but not octreotide, induced a significant increase in the percentage of apoptotic thymocytes, it might be that sst3 is involved in this process. Moreover, sst3 has recently been demonstrated on peripheral human T lymphocytes, which derive directly from mature thymocytes, and SS analogs may induce apoptosis in these cells. Interestingly, CD14+ thymic cells, which are cells belonging to the monocyte-macrophage lineage, selectively expressed sst2A mRNA. Finally, SSR expression in human thymocytes seems to follow a developmental pathway. The heterogeneous expression of SSR within the human thymus on specific cell subsets and the endogenous production of SS as well as SS-like peptides emphasize their role in the bidirectional interactions between the main cell components of the thymus involved in intrathymic T cell maturation.

The IMMUNE AND NEUROENDOCRINE SYSTEMS cross talk by using similar ligands and receptors. Neurohormones modulate the function of lymphoid organs and are produced by immune cells as well, thereby exerting a paracrine/autocrine action in immunoregulation (4). Receptors for different neurohormones, such as hypothalamic-pituitary and gastrointestinal hormones, are expressed by immune and lymphoid accessory cells (4, 29). These neuroendocrine circuits seem to exert a pleiotropic control on the physiology of the thymus, the main lymphoid organ (32). Particularly, the intrathymic production of classical neurotransmitters suggests that paracrine and autocrine interactions, mediated by these compounds and their respective receptors, influence both thymic lymphoid and stromal compartments (31, 32). Somatostatin (SS) represents one of the most relevant neuropeptides involved in neuroimmunoendocrine interaction (19, 45). The wide spectrum of actions of SS and the presence of SS receptor (SSR) in lymphoid organs imply a broad functional role of this peptide in the immune system (17, 27, 29, 45).

We (14) have recently demonstrated the expression of SS itself and of three different SSR subtypes (sst)
within the human thymus. Messenger RNAs encoding for sst1, sst2A, and sst3 were found in a series of normal thymic tissues. sst1 and sst2A were selectively expressed on cultured thymic epithelial cells (TEC), and both SS and its analog octreotide inhibited in vitro TEC proliferation. No SSR subtype mRNA was detectable in 7- to 14-day-cultured thymocytes (14), whereas our preliminary data have recently demonstrated a low expression of SSR on cultured thymic epithelial cells (TEC), and SSR are expressed on thymocytes of different animal species (9, 33, 39), and in humans SS is known to modulate different functions of T lymphocytes, which directly derive from thymocytes (45). Moreover, sst3 mRNA has recently been demonstrated to be constitutively expressed in human resting peripheral T lymphocytes (16). Thymocytes are a heterogeneous cell population. In fact, when progenitors enter the thymus from bone marrow, they lack most of the specific T cell markers. The interactions with the thymic microenvironment trigger the expression of T cell-specific surface molecules. First, CD2 is the marker of immature thymocytes when they do not express the TCR-CD3 complex or the co-receptors CD8 and CD4 (42). These cells are called “double-negative” thymocytes and are a highly heterogeneous pool of cells that include several early stages in T cell development (44). Thus thymocytes undergo matura
tion through a series of stages that can be distinguished by the differential expression of the TCR-CD3 complex, CD8, and CD4. CD3-CD4+CD8- represents an intermediate thymocyte subset before the “double-positive” CD4+CD8+ thymocytes stages (20). Finally, the CD3-CD4+CD8+ subset further differentiates into mature CD4+ or CD8+ single-positive thymocytes (15, 44).

The present study was designed to investigate the presence and potential role of SSR in human thymocytes. The receptor expression pattern was evaluated in vitro in freshly isolated thymocytes by SSR-binding studies on membrane homogenate and by RT-PCR to identify and quantify SSR subtypes on different thymocyte subsets. In addition, the in vitro effect of SS and octreotide on cell proliferation was investigated in isolated human thymocytes.

**MATERIALS AND METHODS**

**Samples.** Thymic tissues were removed from 13 patients (age range between 3 mo and 5 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 the patients’ parents. All samples were histopathologically normal and were taken fresh at the operation.

**Protocol of the study.** Thymocytes were freshly isolated from the thymic samples and used for binding studies on membrane homogenates with iodinated SS-14 (Table 1). Thymocytes derived from four samples were separated in subpopulations for RT-PCR studies (sample nos. 5–8, Table 1; see RT-PCR studies). Thymocytes from five samples of the same series (nos. 5–7, 10, and 11, Table 1) were used for the in vitro primary cell cultures. Thymocytes from three samples were used to study the induction of apoptosis (nos. 11–13, Table 1; see Study of apoptosis).

**Cell dispersion, cell separation, and cell culture.** Thymocytes were collected using a filter chamber (NPBl, Emmer Compascuum, The Netherlands) and placed in RPMI 1640 (GIBCO-BRL-Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated FCS, penicillin (100 U/l), and fungizone (0.5 mg/l). The pH of the medium was adjusted to 7.4. Cell viability was determined before each study and was >95%. These thymic cell suspensions generally contain >95% thymocytes, as has been demonstrated by flow cytometry (FACS) and anti-CD2 antibodies, which selectively bind to thymocytes, in a series of normal pediatric thymuses (36). To confirm this, we performed FACS analysis using a FACScan cytometer (Becton-Dickinson, Erembodegem, Belgium) and anti-CD2 antibodies (Becton-Dickinson). Cytometry and additional fluoconjugated antibodies were used to determine the proportion of the intermediate/mature CD3+ thymocyte subset and the CD14+ monocyte-macrophage fraction. Thymic cells (106) were sorted by setting appropriate electronic gates with the dual-laser FACS system (Becton-Dickinson).

For RT-PCR analysis and for functional studies, thymic cells were first depleted from the monocyte fraction (see below) and subsequently separated into subpopulations by use of magnetic beads coated with specific antibodies (Dynal, Oslo, Norway). The cells were suspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and incubated with the coated beads in plastic tubes kept on ice for 30 min. By continuous rotation of the tubes, the cells and beads were kept in suspension. The tubes were then placed in a magnetic rack to separate the supernatant from the bead-captured cells. The nonselected cells in the supernatant were used for the subsequent rounds of selection with appropriate antibody-coated beads. The bead-captured cells were washed five times with PBS containing 0.5% BSA, counted, and evaluated for specificity by determining the percentage of cells resorted by the beads, which was >98% in all cases. The thymocyte suspension was depleted from the monocyte fraction by using beads coated with CD14 antibodies (CD14+). To isolate intermediate/mature thymocytes...
(CD3+), anti-CD3-coated beads were used. The remaining cells (after a second round of depletion with anti-CD3-coated beads) were further incubated with anti-CD2-coated beads to obtain the immature thymocyte fraction (CD2-CD3+). Additionally, freshly isolated thymocytes (from 5 cases), which did not undergo bead separation, as well as negatively selected CD3+ and CD2-CD3- cell fractions (from 2 cases), were seeded (5 × 10^6 cells/well) in 1 ml of culture medium in 24-well plates (Costar, Cambridge, MA). Then, test substances were added, and the cells were incubated for 24 h for functional experiments. Cell viability was constantly tested during the separation procedure as well as before and after functional studies and was satisfactory.

SSR membrane-binding studies. The method of membrane isolation and the reaction conditions were previously described (14). 125I-Tyr11-labeled SS-14 (Amersham, Houten, The Netherlands) binding to the thymocyte membranes was analyzed. Briefly, membrane preparations (corresponding to 30–50 μg of protein) of freshly dispersed cells were incubated in a total volume of 100 μl at room temperature for 30 min with increasing concentration of 125I-Tyr11-SS-14 with and without excess (1 μM) of unlabeled SS-14 in Hepes buffer (10 mM Hepes, 5 mM MgCl2, and 0.02 g/l bacitracin, pH 7.6) containing 0.2% BSA. After the incubation, 1 ml of ice-cold Hepes buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation for 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 γ-counter (1470 Wizard, Wallac, Turku, Finland). Specific binding was taken to be total binding minus the binding in the presence of 1 μM of unlabeled SS-14 in Hepes buffer at room temperature for 30 min with increasing concentration of 125I-Tyr11-SS-14 with and without excess (1 μM) of unlabeled SS-14 in Hepes buffer at room temperature for 30 min.

Functional studies. In all experiments, SS-14 (Bachem, Hannover, Germany) and octreotide (Novartis Pharma, Basel, Switzerland) dissolved in the culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin, and fungizone), were used at a concentration of 10–12, 10–10, 10–8, and 10–6 M. The culture medium was added to the cells to evaluate the possible effects of the vehicle. After 24 h, proliferation was measured by adding 1 μCi of [methyl-3H]thymidine (91 Ci/mmol; Amersham) for the last 6 h in each well of the 24-well plates. Thereafter, the cell suspension was transferred to 5-ml tubes and precipitated with 10% trichloroacetic acid, and the pellet was washed once again in trichloroacetic acid. After solubilization in 1 M NaOH, the cells were transferred to scintillation-counting vials, and incorporated radioactivity was measured, after neutralization with HCl and the addition of scintillation fluid, in a liquid scintillation counter (Betamatic; Packard, Downers Grove, IL).

RT-PCR studies. RT-PCR was performed as previously described (14). Briefly, poly(A)+ mRNA was isolated using Dynabeads oligo(dT)20 (Dynal) from cell pellets containing 0.5–1 × 10^6 cells per sample. cDNA was synthesized using the poly(A)+ mRNA preparation, the cDNA reactions were performed without reverse transcriptase and amplified with each primer pair (no introns have yet been found in the coding region of these genes, which include the oligonucleotide amplimers used in this study). Amplification of the cDNA samples with the HPRT-specific primers served as a positive control for the quality of the cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of SSR receptor subtypes, 0.001–0.1 μg of human genomic DNA, representing ~300 to 30,000 copies of ss1 template, was amplified in parallel with the cDNA samples. As a positive control for the PCR of HPRT and SS, aliquots of a cDNA sample were amplified, because these primer pairs did enclose introns in the genomic DNA. In the thymocyte preparation, only ss2 and ss3 mRNAs were detectable. To quantify ss2 and ss3 mRNAs, a quantitative RT-PCR was performed by the TaqMan Gold nuclease assay (Perkin-Elmer, Foster City, CA) and the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) for real-time amplification according to the manufacturer’s instructions. The specific primer and probe sequences that were used for the quantitative RT-PCR are reported in Table 3. The amount of ss2 and ss3 mRNA was determined by means of a standard

Table 2. Primers used for RT-PCR studies

<table>
<thead>
<tr>
<th>Sequence (5'-3')</th>
<th>Position†</th>
<th>Size of PCR Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>sst1 (forward)</td>
<td>ATGGTGCGCCCTAGAAGCGCGG</td>
<td>754</td>
</tr>
<tr>
<td>sst1 (reverse)</td>
<td>CCGGGGTGGCGTTAATAGTCAAG</td>
<td>1071</td>
</tr>
<tr>
<td>ss2a (forward)</td>
<td>GCCAAGATGAGAGCCAATCACAG</td>
<td>214</td>
</tr>
<tr>
<td>ss2a (reverse)</td>
<td>GAGTAAACTGCCCTGCTGACCGCC</td>
<td>627</td>
</tr>
<tr>
<td>ss3 (forward)</td>
<td>CCAAGCTGTCACATCCCTACACC</td>
<td>236</td>
</tr>
<tr>
<td>ss3 (reverse)</td>
<td>TCCCGAGAAGAGGCCACAC</td>
<td>549</td>
</tr>
<tr>
<td>ss4 (forward)</td>
<td>ATCTTGGCGAAGACAGAGG</td>
<td>547</td>
</tr>
<tr>
<td>ss4 (reverse)</td>
<td>ATCAAGGCTGCTGAGGAGGA</td>
<td>867</td>
</tr>
<tr>
<td>ss5 (forward)</td>
<td>CGCTTGCTACATCCTACACCG</td>
<td>596</td>
</tr>
<tr>
<td>ss5 (reverse)</td>
<td>GCCTACTGGCACAGCATCAGC</td>
<td>819</td>
</tr>
<tr>
<td>SS (forward)</td>
<td>GATTGGTGCTCCTGCGCTCAG</td>
<td>132</td>
</tr>
<tr>
<td>SS (reverse)</td>
<td>CAAAGTGGGAAAGGTTCTCCA</td>
<td>544</td>
</tr>
</tbody>
</table>

HPRT, hypoxanthine-guanine phosphoribosyltransferase. † The sequences of the primers for SS receptor subtypes sst1, sst4, and sst5 are derived and/or adapted from Kubota et al. (18) and Wulfsen et al. (47). All other primers and probes were designed by use of the Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) and the appropriate GenBank entries. ‡ The position is given of the 5' nucleotide of the primer relative to the first nucleotide of the coding region in the cDNA sequence.

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The amount of sst2 and sst3 mRNA was calculated relative to the amount of HPRT and is given in arbitrary units. Values are expressed as absorbance units (A405 nm — A490 nm).

Statistical analysis. Data are expressed as means ± SE. In functional studies, n = four wells per treatment group. All data were analyzed by ANOVA to determine overall differences between treatment groups. When significant differences were found, a comparison between treatment groups was made using the Newman–Keuls test. SSR-binding data were analyzed by the method of Scatchard. Receptor-binding studies and RT-PCR experiments were performed at least twice.

RESULTS

SSR-binding study. Using membrane homogenate binding, specific $^{125}$I-Tyr$^{11}$-SS-14 binding was demonstrated on freshly isolated thymocytes in all cases. Binding of $^{125}$I-Tyr$^{11}$-SS-14 could be displaced with excess unlabeled SS-14. Scatchard analysis of the binding data revealed a single class of high-affinity binding sites, with an apparent $K_d$ ranging between 0.3 ± 0.1 and 3.1 ± 1.0 nM and a low maximum binding capacity ($B_{max}$) ranging between 11 ± 1.1 and 59 ± 5.1 fmol/mg membrane protein (Table 1). As a control for binding, rat brain cortex membranes were used. An example of saturation binding data with Scatchard analysis is shown in Fig. 1.

SS and SSR subtype expression. The bead separation method showed that thymocytes after cell counting were >95% (≥98% of rosetted cells), whereas CD14$^+$ cells were <5% among the filtered thymic cells, in all of the cases examined. This finding was confirmed by FACS analysis (data not shown). Moreover, the data are in agreement with other authors, who performed this evaluation on a larger series of age- and sex-matched pediatric thymuses (41).

The percentage of thymocytes in each subgroup after bead separation ranged from 95.5 to 99.2%. By RT-PCR, sst2A and sst3 mRNA expression was detected in freshly isolated thymocytes from four of four cases tested (nos. 5–8, Table 1), whereas mRNA encoding for SS, sst1, sst4, and sst5 was absent (Fig. 2B). No mRNA encoding for any SSR subtype was detectable in thymocytes after 7–14 days of culture (data not shown), confirming our previous observation (14). In CD14$^+$ cells, the presence of only sst2A mRNA was detected (Fig. 2C). RT-PCR of thymocytes after separation into immature CD2$^+$CD3$^-$ and intermediate/mature CD3$^+$ fractions revealed sst2A and sst3 mRNA expression in both subsets. Table 4 summarizes the results of RT-PCR analysis, and an example is shown in Fig. 2. Quantitative RT-PCR analysis revealed a higher number of sst3 mRNA copies in the intermediate/mature CD3$^+$ thymocyte fraction compared with the immature CD2$^+$CD3$^-$ one (Fig. 3A). Conversely, the number of sst2A mRNA copies was higher in the immature CD2$^+$CD3$^-$ fraction compared with the mature CD3$^+$ thymocytes in three of four cases. The percentage of thymocytes in each subgroup after bead separation ranged from 95.5 to 99.2%. By RT-PCR, sst2A and sst3 mRNA expression was detected in freshly isolated thymocytes from four of four cases tested (nos. 5–8, Table 1), whereas mRNA encoding for SS, sst1, sst4, and sst5 was absent (Fig. 2B). No mRNA encoding for any SSR subtype was detectable in thymocytes after 7–14 days of culture (data not shown), confirming our previous observation (14). In CD14$^+$ cells, the presence of only sst2A mRNA was detected (Fig. 2C). RT-PCR of thymocytes after separation into immature CD2$^+$CD3$^-$ and intermediate/mature CD3$^+$ fractions revealed sst2A and sst3 mRNA expression in both subsets. Table 4 summarizes the results of RT-PCR analysis, and an example is shown in Fig. 2. Quantitative RT-PCR analysis revealed a higher number of sst3 mRNA copies in the intermediate/mature CD3$^+$ thymocyte fraction compared with the immature CD2$^+$CD3$^-$ one (Fig. 3A). Conversely, the number of sst2A mRNA copies was higher in the immature CD2$^+$CD3$^-$ fraction compared with the mature CD3$^+$ thymocytes in three of four
cases (Fig. 3B). The sst₃-to-sst₂A ratio increased with the level of thymocyte maturation (Fig. 3C).

Effect of SS and octreotide on [³H]thymidine incorporation in thymocytes. SS-14 significantly inhibited [³H]thymidine incorporation in all five cultures (nos. 5–7, 10, and 11, Table 1) of freshly isolated thymocytes (whole population) in a dose-dependent manner. The inhibition was statistically significant at a concentration of 10⁻⁶ M (ranging between 45 and 77%) and 10⁻⁸ M (ranging between 27 and 41%) in all five cases at a concentration of 10⁻¹⁰ M in four of five cases (ranging between 21 and 64%) and at a concentration of 10⁻¹² M in 3 of 5 (ranging between 21 and 64%) cases (Fig. 4, A–E). The SS analog octreotide significantly inhibited [³H]thymidine incorporation in only one culture of thymocytes (whole population) at concentrations of 10⁻⁶ (43%) and 10⁻⁸ M (23%) (Fig. 4, F–L). In the CD₂⁺CD₃⁻ thymocyte cultures, both SS-14 and octreotide significantly inhibited [³H]thymidine incorporation in the two cases evaluated (Fig. 5, A–D). The inhibition was dose dependent and statistically significant at all concentrations (except at the concentration of 10⁻¹³ M in one case). Conversely, in the CD₃⁺ thymocyte cultures, only SS-14 significantly inhibited [³H]thymidine incorporation in a dose-dependent manner in both cases evaluated, whereas octreotide was ineffective (Fig. 6, A–D).

Study of apoptosis. SS-14, but not octreotide, significantly increased the amount of histone-associated DNA fragments, which are measurable after induced cell death (apoptosis), in all of the early cultures of freshly isolated and purified thymocytes (whole population). The number of apoptotic cells was significantly higher at all concentrations in one case (Fig. 7C) and at the concentrations of 10⁻⁶, 10⁻⁷, and 10⁻⁸ M and at the concentrations of 10⁻⁶ and 10⁻⁷ M in the remaining two, respectively (Fig. 7, A and B).

DISCUSSION

The thymus is responsible for promoting the differentiation and maturation of lymphoid precursor cells into mature T lymphocytes. The developing T cells are embedded in an epithelial network known as the thymic stroma. Other cells of hematopoietic origin participate in constituting the complex architecture of this organ; these cells include dendritic cells and thymic macrophages (8, 24). Interactions between thymic stromal cells and thymocytes are mediated by direct contact and via soluble factors and play a crucial role in T cell development (2, 25). Among the soluble factors, neuropeptides have been demonstrated to be involved in the regulation of thymic functions. The intrathymic production of classical hormones suggests that, in ad-

Table 4. Heterogeneity of SS and SS receptor subtype mRNA expression in different fractions of human thymocytes and CD14⁺ thymic cells as determined by RT-PCR

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>sst₁</th>
<th>sst₂A</th>
<th>sst₃</th>
<th>sst₄</th>
<th>sst₅</th>
<th>SS</th>
<th>HPRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated thymocytes</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>CD₃⁻ cells</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>CD₂⁺CD₃⁻ cells</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cultured thymocytes</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>CD14⁺ cells</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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</table>

Each case was evaluated at least two times in independent experiments and yielded identical results.
We (14) have recently described the expression of sst1, sst2A, and sst3 mRNAs in human thymic tissue. Cultured TEC selectively expressed sst1 and sst2A mRNA (14). sst2 mRNA has been detected in murine resting thymocytes (9), in contrast with the expression in the rat where these cells selectively express sst3 and sst4 mRNAs (39). These differences pointed to species-specific expression of SSR subtypes in immune cells. Moreover, another study showed the presence of sst2 mRNA in fresh rat thymocytes and demonstrated that the activation of these cells upregulates the expression of sst1 (33). It should be mentioned that many extrinsic factors and changes in the microenvironmental conditions might regulate the expression of SSR (3, 46). SS itself could be involved in the regulation of receptor expression (5). This might explain why, although we found SS-binding sites on freshly isolated thymocytes (11), in long-term cultured thymocytes SSR mRNA was lost (14).

In the present study, using freshly isolated thymocytes, we first demonstrated specific 125I-Tyr11-SS-14 binding on thymocyte membrane homogenates. The number of SS-binding sites was very low on these thymic cells, which are a heterogeneous population mainly formed by intermediate/mature thymocytes (41, 44). Subsequently, we characterized the SSR subtype expression by RT-PCR. In the whole population of freshly isolated thymocytes, sst2A and sst3 mRNA expression was detected, whereas in thymocytes after 7–14 days of culture, no mRNA encoding for SSR subtypes was detectable, confirming our previous findings (14). Because in freshly isolated thymocytes cells are present at different levels of maturation, we investigated whether sst2A and sst3 mRNA could be differentially expressed in the diverse stages of maturation. We separated the whole thymocyte population into intermediate/mature CD3⁺ and immature CD2⁺CD3⁻ fractions, and by RT-PCR we detected sst2A and sst3 mRNA in both thymocyte subpopulations. However, by quantitative RT-PCR analysis, we demonstrated the predominant expression of sst3 mRNA in CD3⁺ thymocytes. These cells represent the subset of thymocytes that have reached a higher level of maturation during the complex cascade of events occurring in the thymic network (15). Interestingly, sst3 mRNA has been found constitutively expressed in peripheral T lymphocytes, which directly derive from mature thymocytes (16). Conversely, a predominant expression of sst2A mRNA was found in the CD2⁺CD3⁻ thymocytes, which are the immature fraction. The CD2⁺CD3⁻ thymocytes form in the developed thymus a very small but highly heterogeneous pool of cells, whereas the CD3⁺ intermediate/mature cells represent the major proportion of thymocytes (1). Most of these cells are destined to die as a consequence of failing selection (26). Cell death in the thymus occurs by a process known as programmed cell death, or apoptosis, which is a common feature in many developmental pathways (26). The sst3 expressed on these cells might be involved in SS-mediated apoptosis (34). In fact, we have found that SS-14, but not octreotide, which has a lower affinity for sst3,

In addition to the endocrine circuits, paracrine/autocrine interactions may exist in the thymus, influencing both the lymphoid and the stromal compartments of the organ (32).

SS is a neuropeptide with a wide spectrum of actions (19). The biological effects of SS are mediated via five specific, high-affinity, G protein-coupled receptors (20). The presence of the neuropeptide and its receptors has been demonstrated in the human thymus (14, 29, 30).
may increase the amount of apoptotic cells when incubated in human thymocyte cultures as a whole population. Moreover, preliminary data indicated that the number of apoptotic cells is significantly higher in the intermediate/mature CD3\(^+\)/H11001 fraction, compared with the CD2\(^+\)/H11001 CD3\(^+\)/H11002 one, when tested separately (Ferone D, van Hagan PM, Lamberts SWJ, and Hofland LJ, unpublished observations). It is intriguing that another synthetic SS analog has been found to induce apoptosis in cultured human lymphocytes as well (42). Moreover, it has recently been shown that octreotide has a modulatory effect on anti-CD3 and dexamethasone-induced apoptosis of murine thymocytes (43). However, because the immature CD2\(^+\) CD3\(^-\) thymocytes are intensively proliferating cells undergoing a rearrangement process, the predominant presence of the sst2A on this very small subset suggests the involvement of this SSR subtype in the early phase of thymocyte development within the thy-

Fig. 4. Effects of somatostatin (A-E) and octreotide (F-J) on \([^{3}H]\)thymidine incorporation in thymocyte cultures from 5 different thymuses (whole population). Thymocytes were incubated in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin, and fungizone for 24 h in quadruplicate without or with the drugs indicated at the concentrations of \(10^{-13}, 10^{-12}, 10^{-10}, 10^{-8}\), and \(10^{-6}\) M. Values are expressed as counts/min and are means ± SE \((n = 4\) wells/treatment group). *P < 0.01 vs. control; **P < 0.05 vs. control.
mus. Furthermore, the data presented in this study demonstrate that both SSR subtypes on human thymocyte subsets may be activated upon binding with their own ligands. In fact, after the administration of SS-14, we found an inhibition of [³H]thymidine incorporation in all of the early cultures of either whole population or isolated CD2⁺CD3⁻ and CD3⁺ thymocyte fractions. Conversely, the inhibition of [³H]thymidine incorporation by the SS analog octreotide occurred in only one of the five cultures of whole thymocyte population and only at high concentrations. On the contrary, octreotide administered in the CD2⁺CD3⁻ isolated thymocytes induced a significant inhibition of [³H]thymidine incorporation in a dose-dependent manner, whereas this SS analog was ineffective in cultures of CD3⁺ isolated thymocyte fractions. This evidence is in line with the higher affinity of this SS analog for sst²A compared with that for sst³ (20). The possibility cannot be fully ruled out that, at high concentration, octreotide may act via the sst³. On the other hand, emerging data pointed to the role of either sst²A or sst³ in promoting apoptosis by p53-dependent and -independent mechanisms, respectively (37). However, the intracellular pathways mediating the SS-dependent activities regulating cell growth, proliferation, and death might still be partially inactive in the immature thymocyte fraction.

Fig. 5. Effects of somatostatin (A and B) and octreotide (C and D) on [³H]thymidine incorporation in isolated CD2⁺CD3⁻ thymocyte cultures from 2 different thymuses. For culture conditions and legends see Fig. 4. Values are means ± SE (n = 4 wells/treatment group; nos. 10 and 11, Table 1). *P < 0.01 vs. control; **P < 0.05 vs. control.

Fig. 6. Effects of somatostatin (A and B) and octreotide (C and D) on [³H]thymidine incorporation in isolated CD3⁺ thymocyte cultures from 2 different thymuses. For culture conditions and legends see Fig. 4. Values are means ± SE (n = 4 wells/treatment group; nos. 10 and 11, Table 1). *P < 0.01 vs. control; **P < 0.05 vs. control.
Finally, it is also noteworthy to mention that, in CD14\(^+\) cells, which are cells belonging to the monocyte-macrophage lineage, the presence of only sst\(_{2A}\) mRNA was detected. This finding is in agreement with our previous reports on the selective expression of this SSR subtype on human macrophages and monocytes (21, 38, 40). SSR are widely distributed within the human thymus on the different cell subsets forming this organ. The stromal compartment preferentially expresses sst\(_1\) and sst\(_{2A}\) mRNA, whereas lymphoid cells express mainly sst\(_3\) and, to a lesser extent, sst\(_{2A}\) mRNA. Preliminary observations seem to confirm this evidence at the protein level. In fact, using polyclonal antibodies, we have recently studied SSR subtype expression by immunohistochemistry in the normal as well as in neoplastic thymic tissues, where this pattern of receptor distribution is basically maintained (12). However, in thymic tumor, sst\(_{2A}\) immunoreactivity has been found on the endothelium of intratumoral vessel as well, whereas sst\(_3\) immunoreactivity has been clearly observed on normal reactive thymocytes but also on some tumor cells (10, 13). These data support the evidence of strong compartmentalization of neuroendocrine peptide receptors in lymphoid tissue (29), as it has also been shown for the expression of vasoactive intestinal peptide (VIP) receptors on murine and rat thymocytes (7). In fact, the two VIP receptors display a distinct distribution in different thymocyte subsets, suggesting that the expression of neuropeptide receptors could be developmentally regulated and vice versa (7).

We have previously demonstrated (14) that SS mRNA is present in the human thymus in TEC, whereas, as is shown in the present study, SS mRNA was undetectable in thymocytes. However, preliminary data from our group have shown that the SS-like peptide cortistatin-17 is highly expressed in human lymphoid cells, including thymocytes (6). This evidence suggests that SS produced by a subset of TEC, but perhaps endogenous cortistatin produced by thymocytes as well, may affect thymic cell populations in a paracrine and/or autocrine manner. Therefore, SS and SS-like peptides may participate in regulating T cell differentiation and selection in the thymus.

In conclusion, the heterogeneous expression of SSR in different cell subsets within the human thymus,
together with the endogenous production of SS, SS-like peptides, and other neurohormones, emphasizes once more the pivotal role played by neuropeptide hormones in this organ. The maturation and selection of the T cell repertoire are two of the most intriguing processes and involve a number of factors. SS, likely produced by TEC (14), seems to affect both the lymphoid and the microenviromental compartments of the thymus. TEC are known to drive the most important phases of T cell maturation and differentiation; however, thymocytes might affect TEC functions as well (23). Thus a bidirectional interaction pathway exists between the two main cell components of the thymus, and SS might be part of this complex circuit. Moreover, SS is known to affect the production of immunoglobulins and interleukins, which are well recognized factors participating in the sophisticated and elegant process leading to the maturation of cellular immunity (28, 36). In this light, SS represents an important molecule involved in the chain of events that results in the generation of the T cell repertoire.

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