Expression of the Mel_{1a}-melatonin receptor mRNA in T and B subsets of lymphocytes from rat thymus and spleen

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ABSTRACT **In the present work we analyze by reverse transcription, polymerase chain reaction, cDNA cloning, and sequence analysis the expression of membrane melatonin receptors in rat thymus and spleen. Results show, for the first time, that the melatonin receptor mRNA is expressed in both the thymus and spleen. Moreover, the melatonin receptor mRNA was expressed in all the lymphocyte subpopulations ($CD4^+$,$CD8^+$, double positive, double negative, and B cells) studied from the rat thymus. The Southern blot analysis with the melatonin receptor probe and sequence data also showed the identity of the DNA fragments in thymus, spleen, and the lymphocyte subpopulations studied. The melatonin receptor fragments amplified from rat brain, thymus, and spleen share identical nucleotide sequences with the rat Mel_{1a}-melatonin receptor subtype. No signal was obtained with primers used to amplify the rat Mel_{1b}-melatonin receptor subtype in both thymus and spleen. Finally, the melatonin receptor mRNA transcript distribution throughout the rat thymus was examined. Using digoxigenin-labeled cRNA probe to the specific melatonin receptor mRNA, examination of the whole thymus revealed a clear hybridization signal in both cortex and medulla. Melatonin receptor gene expression in the thymus and spleen supports the notion of the immunomodulatory role of melatonin.—Pozo, D., Delgado, M., Fernandez-Santos, J. M., Calvo, J. R., Gomariz, R. P., Martin-Lacave, I., Ortiz, G. G., Guerrero, J. M. Expression of the Mel_{1a}-melatonin receptor mRNA in T and B subsets of lymphocytes from rat thymus and spleen. *FASEB J.* 11, 466–473 (1997)**

Key Words: pineal gland · gene expression · RNA extraction · DNA sequencing

THE PINEAL NEUROHORMONE MELATONIN plays an important role in regulating the immune system (1). Maestroni and co-workers (2) have demonstrated that melatonin enhances antibody production against T-dependent antigens in normal mice. Melatonin also counteracts the immunodepression induced by acute stress or corticosteroids on antibody production, thymus weight, and antiviral resistance (2). Mechanisms involved in the immunostimulatory effect of pineal gland are not well understood, but evidence suggests that the release of opioid peptides and interleukin-2 by T helper cells (3) may participate in this mechanism by activating at least natural killer cell activity (4) and antibody-dependent cellular cytotoxicity (5). Furthermore, melatonin also activates human monocytes, increasing the cytotoxicity, the secretion of IL-1, and the production of reactive oxygen intermediates (6).

The effect of melatonin on the immune system is also supported by the existence of specific binding sites for melatonin in lymphoid cells (7). We have described previously the presence of high-affinity binding sites for melatonin in human blood lymphocytes (8, 9). We have also provided evidence for the presence of low-affinity melatonin binding sites in human granulocytes (10). Melatonin binding sites are present in T lymphocytes, but not in B lymphocytes (11). The affinity of these binding sites (K_d=0.27 nM) suggests that they may recognize the physiological concentrations of melatonin in serum. Moreover, among the lymphocyte subpopulations studied, binding of melatonin was found mostly in $CD4^+$ cells rather than in $CD8^+$ cells, suggesting that $CD4^+$ lymphocytes may be the target of melatonin among the human lymphocytes (11). In other species, melatonin binding sites have been described in spleens of the mouse, guinea pig, duck, chicken, and pigeon,

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with K_d values in the 0.1 nM range (12–14). Similar findings have been shown in spleen and thymus from the rat, where the K_d value of the melatonin binding site is in the nanomolar range (15–17).

Recently, cDNAs encoding membrane melatonin receptor proteins (Mel_{1a} and Mel_{1b}) have been cloned from both the central nervous system (18) and retina (19) of the rat, providing for the first time the possibility to examine mRNA expression of melatonin receptor in rat lymphoid organs. Our results demonstrate the presence of Mel_{1a}-melatonin receptor gene expression in rat thymus and spleen, as well as in T and B cells derived from the thymus.

MATERIAL AND METHODS

Animals and tissue preparations

Male Wistar rats (Iffa Credo) weighting 250–300 g were maintained at constant temperature (23±2°C and 50±5% humidity. Animals received food and water ad libitum and were exposed to an automatically regulated light-dark (LD) cycle of 14:10; the lights were turned off daily from 2000 to 0600 h. Unless otherwise stated, rats were killed between 1200 and 1600 h. The thymus, spleen, brain, and liver were carefully removed and total RNA isolations were performed immediately.

When required, cell suspensions from rat thymus (100×10^6 cells/ml) were prepared by mechanical dissociation. B lymphocytes were purified using immunomagnetic separation. Briefly, cells were labeled with mouse monoclonal antibodies anti-CD45RA (clone OX33, PharMingen). Thereafter, the cells were incubated with anti-mouse IgG magnetic beads and labeled cells were isolated magnetically. For T lymphocyte preparations, cells were double-stained by direct immunofluorescence with FITC-conjugated mouse anti-rat CD4 and PE-conjugated mouse anti-rat CD8 for 1 h at 4°C, then cells (3×10^6 cells/ml) were sorted in a FACStar^{plus} (Becton-Dickinson, Rutherford, N.J.). Thus, the four major thymic subpopulations defined by CD4 and CD8 staining were obtained: $CD4^+$, $CD8^+$, double positive (DP),[2] double negative (DN), and B cells. In all cases, the purity exceeded 98%.

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted by water-saturated phenol/chloroform/isoamyl alcohol (49:49:1) as previously described by Chomczynsky et al. (20). mRNA from thymic subpopulations was purified with oligo(dT) cellulose columns (Invitrogen, San Diego, Calif.) and the amount was determined spectrophotometrically at 260–280 nm. Each 20 µl cDNA synthesis reaction contained 1 µg poly (A)-RNA (for cell preparations) or 5 µg total RNA (for tissues preparations), 1× polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl, 5 mM DTT, 1 mM each of 2′-deoxynucleoside-5′-triphosphates (dATP, dGTP, dCTP, and dTTP; Boehringer-Mannheim, Mannheim, Germany), 66 units placental ribonuclease inhibitor (Promega, Madison, Wis.), 150 units MuLV-reverse transcriptase (Promega), and 2 µg random hexamer oligodeoxynucleotides (Promega). All samples were treated with DNAse; to avoid residual DNA contamination, samples were RNAase treated. The reaction mixture was preincubated 10 min at 23°C before cDNA synthesis. The reverse transcription (RT) reactions were carried out for 50 min at 42°C and were heated to 90°C for 5 min to terminate the RT reaction using a Perkin-Elmer Cetus Thermal Cycler 480.

Polymerase chain reactions (PCR)

Primers used for the amplification of the rat melatonin receptor cDNA fragment were chosen from the RT-PCR-generated fragment of the coding region of rat brain Mel_{1a}-melatonin receptor (18) or from the PCR-generated fragment of the rat Mel_{1b}-melatonin melatonin receptor subtype (19). The positions of the sense and antisense primers were 51–71 and 296–315 for the Mel_{1a}-melatonin receptor subtype, respectively, predicting a 264 bp fragment as a result of mRNA RT-PCR amplification (**Fig. 1**). The Mel_{1b}-melatonin receptor sense (112–135) and antisense (319–342) primers were designed from consensus among all known Mel_{1b}-melatonin receptor members. PCR amplifications were performed in 50 µl reaction volumes. The primers used for Na^+/K^+ ATPase amplification were: sense 5′-GCTTCATGGATTTGATTGTCAAACC-3′, antisense 5′-CGAGGCTGTCATCTTCCTCATTGG-3′. Each reaction contained 5 µl RT reaction product as template DNA, 1× PCR buffer, 200 µM each deoxynucleotide, 2.5 units Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 1.5 mM $MgCl_2$, and 50 pmol sense and antisense primers. The template was initially denatured for 5 min at 95°C, followed by a 35-cycle program with 1 min at 94°C, 1 min annealing at 55°C, 1 min 45 s extension at 72°C, and a final extension cycle at 72°C for 10 min. PCR markers (Promega) were used as size standards.

Plasmid and probes preparation

The plasmid pCRII (Invitrogen) containing the RT-PCR-generated insert of rat brain Mel_{1a}-melatonin receptor was a gen-

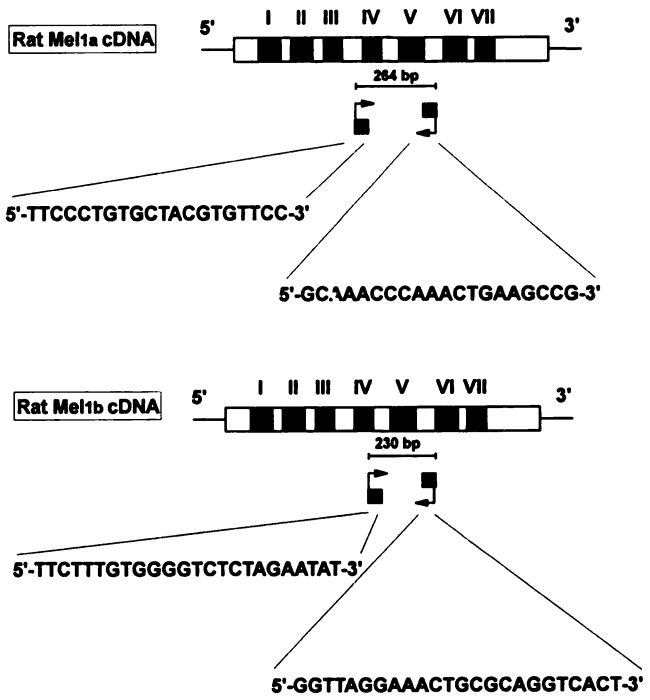

Figure 1. Schematic representation of the RT-PCR strategy used to detect the rat melatonin Mel_{1a} and Mel_{1b} receptor subtypes. The box-arrows indicate the positions of the PCR primers and their extension by Taq polymerase.

[2] Abbreviations: DN, double negative; DP, double positive; PCR, polymerase chain reaction; RT, reverse transcription.

erous gift from Dr. S. M. Reppert (Massachusetts General Hospital, Harvard Medical school, Boston, Mass.). A 468 bp EcoRI fragment containing the sequence of the melatonin receptor gene was isolated from the plasmid and used as a probe to detect melatonin receptor-specific sequences. For DNA probes in Southern hybridization, template DNA fragments reacted with Klenow enzyme, random hexamer oligodeoxynucleotides, dATP, dGTP, dCTP, dTTP, and DIG-dUTP according to the instructions provided with the DIG-DNA labeling kit from Boehringer-Mannheim. For in situ hybridization studies, antisense and sense cRNA probes were generated by in vitro transcription in the presence of DIG-dUTP. The templates for transcription were cDNAs of full-length coding region of rat Mel_{1a}-melatonin receptor subtype. Labeled sense riboprobe or antisense riboprobe was diluted in the hybridization buffer.

Southern blotting

After amplification, 10 µl PCR reaction was electrophoresed in 2% agarose gel in 1× TAE buffer and visualized by staining with ethidium bromide and UV illumination using ϕX174 DNA/Hae III DIG-label (Boehringer, Pearl River, N.Y.) as size marker. The cDNA was transferred to a hy^+-nylon membrane (BioRad, Richmond, Calif.) using a vacuum blotting system (Hoeffer, San Francisco, Calif.), with 10× SSC as transfer solution and cross-linked to the nylon membrane using a calibrated UV light source. Blots were prehybridized at 42°C for 4 h in prehybridization buffer (50% formamide, 5× SSC, 25 blocking reagent, 0.1% N-laurylsarcosyl, 0.02% SDS). The hybridization was performed at 42°C overnight in the same prehybridization buffer plus 10 ng DNA/ml of 468 bp fragment labeled probe. Thereafter, blots were washed twice for 10 min in 2× SSC/0.1% SDS at room temperature and twice for 15 min in 2× SSC/0.1% SDS at 60°C. To detect the hybridization signal, the blots were incubated 30 min in 0.1 M maleic acid/0.15 M NaCl/1% blocking reagent and 30 min with anti-DIG-AP (anti-digoxigenin conjugated to alkaline phosphatase). Finally, they were washed and incubated in Lumigen PPD. Blots were then exposed to Kodak X-OMAT AR film at room temperature.

Cloning and DNA sequencing

PCR products were run on agarose gel and eluted by using a 0.22 µm filter in 100 µl TE pH 8.0. After ethanol precipitation, DNA was ligated in the presence of 50 ng pGEM-T vector (Promega), 0.5 mM ATP, and 5 U of T4 DNA ligase (Pharmacia, Piscataway, N.Y.) overnight at 15°C. DH5α *Escherichia coli* was transformed with the ligation mixture and plated on ampicillin plates containing X-gal (Sigma, St. Louis, Mo.) and IPTG (Sigma). After overnight incubation at 37 and 4°C for 4 h, white colonies were selected and miniprep DNA were characterized by Pst I/Apa I restriction enzyme mapping. Sequencing of the PCR cloned fragments was performed using the T7 DNA Pol Sequencing Kit (Pharmacia), according to the manufacter's instruction, using α-^{35}S-dATP (specific activity 3000 Ci/mmol, Pharmacia). Samples were subjected to electrophoresis in 6% polyacrylamide sequencing gels containing 8 M urea.

In situ hybridization

All steps were performed under sterile conditions to minimize RNA degradation in unprocessed blocks. Animals were killed and the thymuses were quickly fixed by immersion in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.3, at 4°C for 24 h. After fixation, samples were dehydrated using a routine procedure (21). Briefly, thymuses were extensively washed with sterile isotonic sodium chloride and dehydrated through graded dilutions of ethanol in the same mixture (70–100%). They were finally washed twice in xylol for 15 min at room temperature and imbibed in paraffin wax. Serial 5 µm sections were collected on 3-aminopropyltriethoxysilane coated slides and kept at room temperature until used.

Slides were deparaffined in xylol (twice for 10 min), washed in 100% ethanol for 5 min, and treated with a fresh solution of 10 µg/ml proteinase K, 50 mM Tris-HCl, pH 7.2, 5 mM EDTA, for 30 min at 37°C in a humidity chamber. The slides were finally rinsed with 100% ethanol and covered with 40 µl prehybridization buffer (50% formamide, 10% (w/v) dextran sulphate, 2× SSC, 5× Denhardt's solution, and 1 µg/µl sonicated salmon sperm DNA in 20 mM NaH_2PO_4/Na_2HPO_4, pH 7.2) at 42°C for 1 h. Both DIG-labeled sense (negative control) and antisense (positive control) RNAs trancripts were diluted in the same buffer at a final concentration of 1.6 ng/µl and hybridizations were carried out overnight at 42°C. Finally, slides were washed at room temperature in 2× SSC for 2 h, 1× SSC for 1 h, 0.5× SSC for 30 min at 42°C, and 0.5× SSC for 30 min at room temperature. Digoxigenin was detected using alkaline phosphatase-conjugated sheep Fab anti-digoxigenin at 1:200 for 7 min at room temperature. The alkaline phosphatase was revealed with freshly prepared color solution, 0.1 M Tris-HCl, pH 9.5, 150 mM NaCl containing 3.3 mg nitroblue tetrazolium (NBT), 5.62 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and levamisol for 18 h at room temperature in a dark vat.

RESULTS

Figure 1 shows the location of the PCR primers within the rat melatonin receptor subtype genes. mRNA from rat brain, liver, thymus, and spleen was obtained. Thereafter, an RT and PCR amplification were performed. cDNA obtained from the brain was used as a positive control of the membrane Mel_{1a}-melatonin receptor mRNA. The cDNA obtained from liver, which was treated with and without reverse transcriptase, was used as a negative control. Moreover, a sample using water instead of cDNA was processed using the primers in each PCR run to ensure the absence of exogenous DNA contamination during the preparation of PCR reactions. RT-PCR reactions were processed with Na^+/K^+ ATPase housekeeping gene primers (**Fig. 2*C***).

RT-PCR of mRNA from thymus and spleen resulted in single DNA bands when analyzed by agarose gel electrophoresis (Fig. 2*A*). These RT-PCR products correspond to the predicted size for PCR amplification using the Mel_{1a}-melatonin receptor cDNA as template. RT-PCR products obtained with mRNA from the brain, used as positive controls, also gave an amplification product of the same size. No specific band was obtained with RNA from liver or from reactions in which cDNA was omitted. The Southern blot analysis performed with the 468 bp fragment of the Mel_{1a}-melatonin receptor-specific probe confirmed the identity of DNA fragments (Fig. 2*B*) in the brain, thymus, and spleen. No hybridization signal of melatonin receptor mRNA was observed in liver.

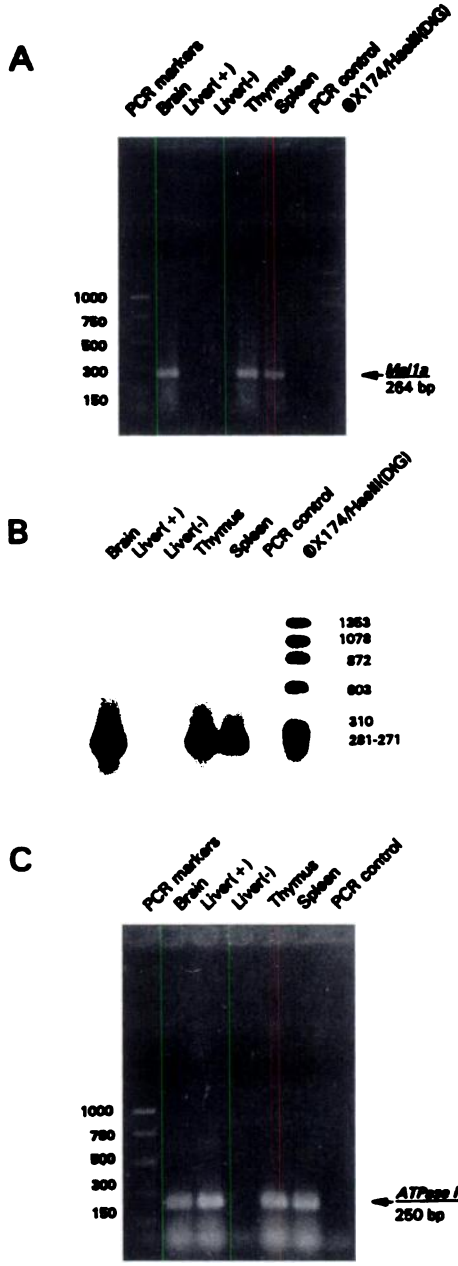

Figure 2. A. RT-PCR analysis of the melatonin receptor mRNA expression in thymus and spleen. The brain was used as positive control, the liver as a negative control. Liver (−) was processed in the absence of MuLV retrotranscriptase. PCR reaction without cDNA substrate (PCR control); molecular size markers (M) were also used. A ϕX174/HaeIII-digoxigenin label was electrophoresed and used for Southern blot analysis as an additional molecular size marker. Arrow indicates the 264 bp amplified PCR product. *B*) Southern blot hybridization of the PCR products shown in panel *A* with the DIG-labeled melatonin receptor-specific probe. *C*) RT-PCR amplification with Na^+/K^+ ATPase primers.

While trying to identify the lymphocyte subpopulation where the membrane melatonin receptor is expressed, RT-PCR was performed using mRNA from purified $CD4^+$, $CD8^+$, DP, DN, or B lymphocytes (**Fig. 3*A***). Single DNA bands were obtained in all cell preparations studied. The products seemed identical in size with that obtained from brain. No message was amplified from the liver or from reactions in which cDNA was omitted. RT-PCR reactions were processed with Na^+/K^+ ATPase housekeeping gene primers (Fig. 3*C*). The Southern blot analysis with the Mel_{1a}-melatonin probe also showed the identity of the DNA fragments in the lymphocyte subpopulations studied. No hybridization signals of melatonin the receptor mRNA were observed in the liver (Fig. 3*B*).

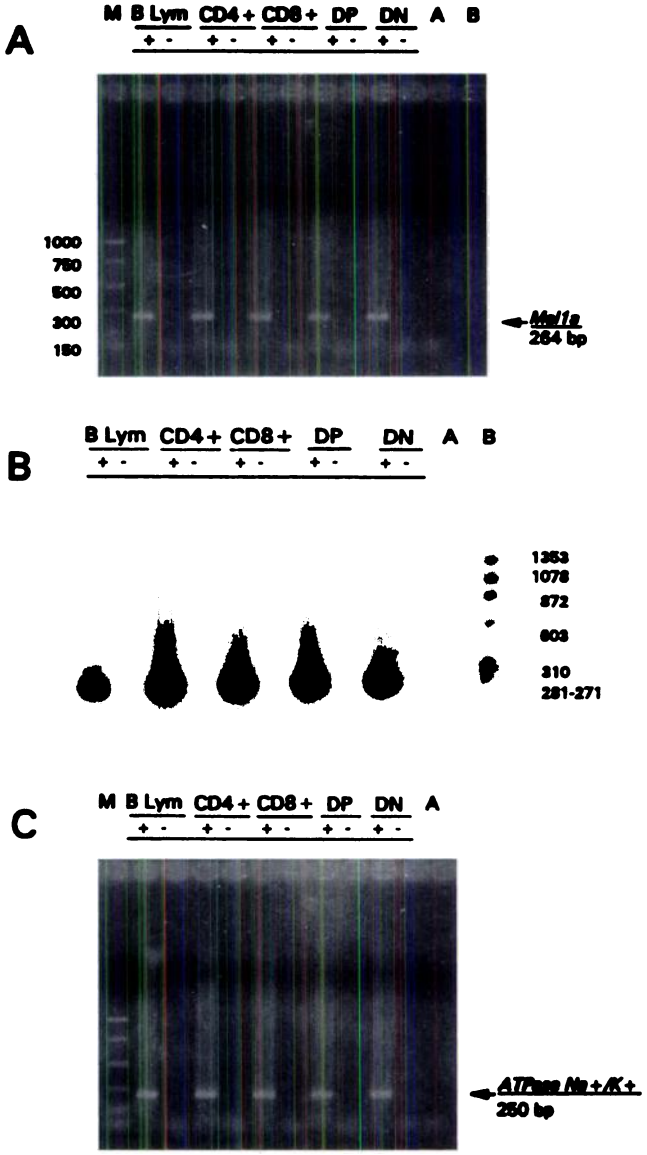

Figure 3. *A*) RT-PCR analysis of the melatonin receptor mRNA expression in $CD4^+$, $CD8^+$, double positive (DP), double negative (DN), and B lymphocytes from thymus. PCR reaction without cDNA (PCR control) and molecular size markers (M) were also used. (—) indicates a RNAase treatment to test DNA contamination. A ϕX174/HaeIII-digoxigenin label was electrophoresed and used for Southern blot analysis as an additional molecular size marker. Arrow indicates the 264 bp amplified PCR product. *B*) Southern blot hybridization of the PCR products shown in panel *A* with the DIG-labeled melatonin receptor-specific probe. *C*) RT-PCR amplification with Na^+/K^+ ATPase primers.

Nucleotide sequences of the melatonin receptors fragments amplified from the brain, thymus, and spleen were studied. Results showed that both thymus and spleen melatonin receptor cDNA fragments exhibited identical nucleotide sequences with the rat brain Mel_{1a}-melatonin receptor subtype. No expression of the Mel_{1b}-melatonin receptor subtype was observed in thymus or spleen when the specific Mel_{1b}-melatonin receptor primers were used. However, the same primers used to amplify mRNA extracted from rat retina, used as a positive control, rendered the expected product corresponding to the Mel_{1b}-melatonin receptor subtype (data not shown).

Finally, we examined the Mel_{1a}-melatonin receptor mRNA transcript distribution throughout the rat thymus. Using antisense cRNA to the specific melatonin receptor mRNA, examination of the whole thymus revealed a clear hybridization signal in both the cortex and medulla (**Fig. 4*A*** and **Fig. 5*A***). When the sense riboprobe was used, however, no hybridization signal was observed (Fig. 4*B* and Fig. 5*B*).

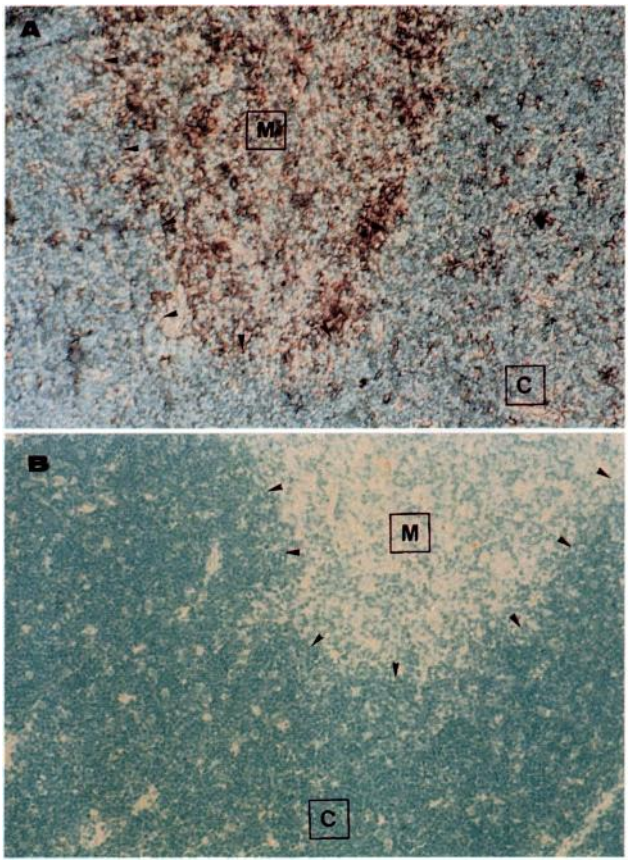

Figure 4. Distribution of the melatonin receptor mRNA expression in the cortex (C) and medulla (M) of the thymus assessed by in situ hybridization. Runoff RNA non-sense transcripts (positive control, *A*) from SP6 phage promoter were made by Eco RV linearization of the plasmid probe. RNA sense (negative control, *B*) transcripts from T7 phage promoter were made by Hind III linearization of the plasmid probe. Arrowheads indicate the border between cortex and medulla. Magnification, ×500.

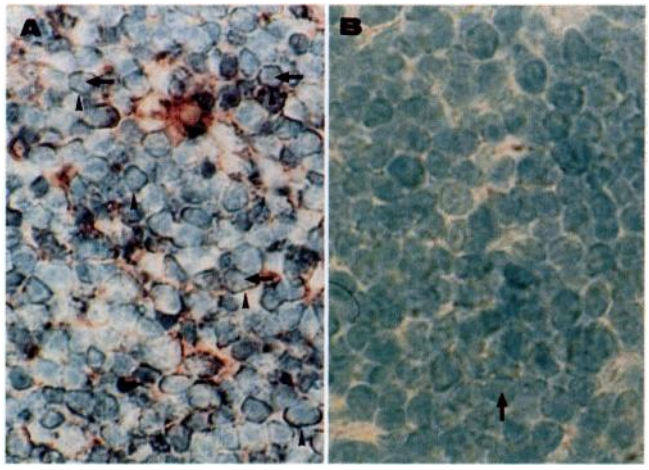

Figure 5. Rat thymus lymphocytes express the membrane melatonin receptor assayed by in situ hybridization. Arrowheads indicate cytosolic RNA duplex hybridization signal (*A*, nonsense riboprobe) and small arrows indicate methyl green nuclear counterstaining. Small arrows in the negative control (*B*, sense riboprobe) show methyl green nuclear staining. ×2500.

DISCUSSION

In the present paper we show, for the first time, the expression of Mel_{1a}-melatonin receptors in both thymus and spleen from the rat. RT-PCR of mRNA from both organs resulted in single DNA bands when analyzed by agarose gel electrophoresis. These RT-PCR products correspond to the predicted size for PCR amplification using the melatonin receptor cDNA as template. The brain was used as positive control since the expression of the melatonin receptors in this structure has been already studied (18, 19, 22). As expected, no specific band was obtained with RNA from liver because, as far as we know, membrane hepatocytes do not bind 2-[^{125}I]iodomelatonin (23). However, it is possible that a specific membrane receptor subtype, different from that in central nervous system, is present in the liver and is not detected with the primers used in this study.

Recently, a G protein-coupled receptor for melatonin has been cloned from *Xenopus* dermal melanophores (24). Using a PCR approach based on the frog sequence, a high-affinity melatonin receptor that was 60% identical at the amino acid level with the frog receptor was subsequently cloned from several mammals including humans (18, 22, 25). These mammalian receptors showed greater than 80% amino acid identity with each other and thus appear to be species homologous of the same receptor, designated the Mel_{1a}-melatonin melatonin receptor. The Mel_{1a}-melatonin receptor gene is expressed in the rodent central nervous system and pars tuberalis. A second melatonin receptor, designated the Mel_{1b}-melatonin receptor, is 60% identical at the amino acid level to the human Mel_{1a}-melatonin receptor and is expressed in the human brain and retina (19).

In this paper, we show that rat Mel_{1a}-melatonin receptor PCR products of the appropriate size (264 bp) were generated from all the lymphoid organs examined. The specificity of the amplification was confirmed by Southern hybridization using the rat Mel_{1a} 468 bp RT-PCR-generated inserts as a probe. We have not found any signal with primers used for the amplification of the rat Mel_{1b}-melatonin receptor subtype in both rat thymus and spleen. Moreover, the nucleotide sequence of the amplified cDNA fragments exhibits the identical nucleotide sequence as that described for the brain Mel_{1a}-melatonin receptor (18). Although we cannot discard the possible existence of different nucleotide sequence in other domains of the receptor, results suggest that the melatonin receptor expressed in the thymus and spleen might be included in the Mel_{1a} receptor subtype.

In the thymus, approximately 80% of lymphocytes are $CD4^+CD8^+$ (DP). Double-negative cells are few (1–2% of thymic lymphocytes) and are concentrated in the subcapsular area of the outer cortex. The remaining thymic lymphocytes express either CD4 or CD8 and are found predominantly in the medulla (26). B lymphocytes can also be identified in the thymus, and are present in the medulla and within the perivascular space (27). Therefore, the RT-PCR approach was also used with mRNA from purified $CD4^+$, $CD8^+$, DP, DN, or B lymphocytes. In all cell preparations studied, single DNA bands were obtained, indicating that the melatonin receptor is expressed in all B and T subsets of lymphocytes. In previous experiments performed in our own laboratory, however, we had found that 2-[^{125}I]iodomelatonin interacts with human circulating T lymphocytes rather than with B lymphocytes (11). Moreover, specific binding of the ligand by $CD4^+$ cells was around four- to eightfold higher than that found in the other T cell preparations studied (11). These results suggested that $CD4^+$ lymphocytes, in addition to other possible subpopulations, were the target of melatonin among the immunocompetent cells. However, RT-PCR, as normally used, is a qualitative procedure specially designed to detect low abundance transcripts (28). Therefore, further experiments using quantitative PCR are required in order to know whether melatonin receptor expression correlates with the binding of melatonin to lymphocyte subpopulations.

Microscopically, the thymus has a lobular configuration, with each lobule surrounded by fibrous tissue. The lobules are separated into the darkly staining cortex and lightly staining medulla (29). The cortex and medulla are composed predominantly of lymphocytes ("thymocytes") and epithelial cells. As shown in Fig. 4, the lymphocytes are more closely packed in the cortex than in the medulla. Also, the cortical lymphocytes are less mature than those in the medulla. Prothymocytes enter the thymus from the bone marrow and migrate to the outer cortex and possibly the corticomedullary junction. With maturation, the thymic lymphocytes are thought to move from the outer cortex toward the medulla (30). We have studied the distribution of mRNA encoding the melatonin receptor in the rat thymus. In situ hybridization studies revealed hybridization signals in both cortex and medulla, suggesting that the melatonin receptor is probably present during the maturation of T cells.

In the immune system, the functional significance of melatonin mRNA expression by lymphocytes must be elucidated. Thus, immunomodulatory actions of melatonin might be mediated via binding of the hormone to specific receptors on the target cell with activation of intracellular signals. However, other mechanisms of action for melatonin should be also considered. Other studies have shown nuclear localization of melatonin in different mammalian tissues, suggesting that melatonin may have a function at the nuclear level in these tissues (23). At the nuclear level, recent studies have shown that nuclear melatonin binding sites may be a family of orphan receptors called RZRβ and RZRα (31, 32). Binding of melatonin to the RZRα nuclear receptor represses 5-lipoxygenase gene expression in human B lymphocytes (33). Moreover, other effects of melatonin appear to be independent of its ability to bind to a receptor. Thus, it was recently shown that melatonin per se is a potent hydroxyl radical scavenger (33, 34). Moreover, melatonin also appears to modulate the activity of calmodulin-dependent enzymes via binding to calmodulin (35, 36).

The physiological role of melatonin binding sites in thymus remains unclear. However, several investigators have shown a physiologic link between pineal gland and thymus (37). The major thrust of early hypotheses was keyed to the concept that the thymus and pineal gland acted jointly to regulate cell, tissue, and organism growth (38). Several decades later it was shown that administering pineal extracts in long-term experiments resulted in an increase of thymus weight accompanied by lymphoid cell hyperplasia in both the medullary and the cortical zones of the gland (39). Moreover, neonatal pinealectomy leads to thymic atrophy (40) and an impairment of immune potential expressed as diminution of both antibody production (41) and Arthus reactivity to bovine serum albumin (42). Melatonin is also recognized as an immuno-enhancing factor in a mode counteracting the immunosuppressive influence of corticosterone (43, 44). Immuno-enhancing activity of melatonin is observed only in mice injected with T-dependent antigens, suggesting that the hormone exerts its effects on the thymus gland. Recent studies have also shown that melatonin causes a marked in-

crease in the affinity and a decrease in the density of thymic receptors for adrenal steroids, suggesting that hormone steroid receptors in the thymus may be a target site for the interaction between melatonin and adrenal steroids in modulation of the immune response (45, 46). Adrenal steroids are known to inhibit the immune response, and involution of the thymus is generally accepted as a reliable sign of the immunosuppressive action of steroid hormones (47). Therefore, melatonin receptors in thymocytes might be considered, in addition to other possibilities, a mechanism that could explain the functional interactions between the pineal hormone melatonin and the thymus.

In conclusion, our results show that the melatonin receptor mRNA is expressed in both rat thymus and spleen. Moreover, the melatonin receptor mRNA was expressed in all the lymphocyte subpopulations studied from the rat thymus. Finally, a clear in situ hybridization signal was found in both cortex and medulla. From this report, approaches like cloning possible new types of melatonin receptors on immune system have now been opened for more investigation. These and previous results support the notion of the immunomodulatory role of melatonin. FJ

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