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Early Activation of Thyrotropin-Releasing-Hormone and Prolactin Plays a Critical Role during a T Cell-Dependent Immune Response*

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ABSTRACT

Functional interaction between the immune and neuroendocrine systems is mediated by humoral mediators, neurotransmitters, and cytokines, including TRH and PRL. We examined the role of neuroendocrine changes, particularly TRH and PRL, during the T cell-dependent immune response. After immunization of rats with sheep red blood cells (SRBC, a T cell-dependent antigen), an increase of hypothalamic TRH messenger RNA (mRNA) was observed at 4–24 h post immunization, in contrast to the decrease observed after treatment with lipopolysaccharide (LPS). During the above period, with SRBC, there was an increase in pituitary TRH receptor mRNA and plasma PRL levels but no changes in TSH and GH. Also, in contrast to the early corticosterone peak induced by LPS, the activation of the hypothalamic-pituitary-adrenocortical suppressive response appears in a late phase, 5–7 days after SRBC. Intracerebroventricular injection of antisense oligonucleotide complementary to rat TRH mRNA in conscious freely-moving rats immunized with SRBC resulted in a significant inhibition of specific antibody production and a concomitant inability to produce the peak in plasma PRL levels. These studies demonstrate, for the first time, that the T cell-dependent immune response is critically dependent on the early activation of TRH and PRL and that the neuroendocrine changes occurring during it are profoundly different from those occurring during the T cell-independent and inflammatory responses (LPS model). (*Endocrinology* **140:** 690–697, 1999)

THE IMMUNE response is accompanied by homeostatic changes in the neuroendocrine system. Cytokines secreted by immune cells and other cell types act on the central nervous system (CNS), inducing marked changes in neuroendocrine, autonomic, and behavioral processes (1, 2). Neurotransmitters and hormones are the principal messengers by which the CNS influences immune processes (1, 2). Many of the mechanisms involved in these interactions have been characterized by administration of the inflammatory agent lipopolysaccharide (LPS). LPS is a very particular immunogen, insofar as it causes a T cell-independent response through macrophage and B lymphocyte activation. This response, in contrast to T cell antigens [*e.g.* sheep red blood cells (SRBC)], does not induce clonal expansion.

A few studies used T cell-dependent antigens. In contrast to the early hypothalamic-pituitary-adrenal (HPA) axis activation observed with LPS, SRBC led to an increase of both antibody and corticosterone levels within 5–7 days of the injection of antigen (3). A decreased noradrenaline turnover in the hypothalamus of rats was observed at the peak of the immune response to SRBC (4).

There are many reports indicating the influence of PRL and TRH on the immune response. TRH, a tripeptide, is the major hypothalamic-releasing factor for TSH secretion and is the best-known PRL-stimulatory factor. TRH-secreting neurons are located in the medial portions of the paraventricular nuclei of the hypothalamus; their axons terminate in the medial portion of the external layer of the median eminence (5) and stimulate vagal efferent fibers (6). PRL synthesis and secretion from pituitary lactotrophs is inhibited by dopamine (7). PRL is an important immunomodulator. Hypophysectomized rats and mice have been shown to exhibit decreased antibody response, a prolongation of graft survival (8), a decrease in lymphocyte proliferation (9), and a reduction in spleen natural killer cell activity (10). Anterior pituitary transplantation to the kidney capsule (that results in a large increase in PRL) restores the production of IgG and IgM antibodies in SRBC-injected hypophysectomized rats (11). Similarly, *in vivo* administration of the inhibitor of PRL secretion, bromocriptine, inhibits T cell proliferation (12). PRL is necessary, but not sufficient, for lymphocyte proliferation; in interleukin (IL)-2-, IL-4-, or Concanavalin A (Con A)-driven lymphocytes, anti-PRL antisera inhibits *in vitro* proliferation (13, 14), and nuclear translocation of PRL was dem-

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onstrated (15). The PRL receptor is expressed ubiquitously in all immune cells, and PRL may be produced by T-lymphocytes (16). PRL induces up-regulation of IL-2 receptors (17). Human peripheral blood mononuclear cells and rat splenocytes express TRH-receptor (TRH-R) messenger RNA (mRNA) (18). TRH enhances the *in vitro* plaque-forming cell response (19). In healthy human subjects, an increase of IL-2 levels was found 30 min after injection during the standard TRH test (20). TRH injected in rats stimulates thymocyte (21) and splenocyte (18) proliferation.

In the present work, we studied the neuroendocrine changes that take place during the T cell-dependent immune response. Taking into account PRL and TRH actions on T-lymphocytes, we have investigated, in conscious freely moving rats, whether these hormones play a relevant role at the beginning (T cell clonal expansion phase) of the immune response to a T cell-dependent antigen.

Materials and Methods

Animals

Male Wistar rats (180–200 g) were used for all experiments. Animals were individually single-housed and kept under standard lighting (lights on from 0600–1800 h) and temperature (23 ± 2 C) conditions. Food and water were available *ad libitum*.

All experimental protocols were approved by the Ethical Committee on Animal Care and Use, University of Buenos Aires, Argentina.

Immunization and antibody determination

Animals were immunized with SRBC ip. SRBC were washed three times with PBS at pH 7.2, and 5×10^9 cells were injected ip in 0.5 ml PBS, as described (22). The experimental and control groups (injected with endotoxin-free saline) were killed at different times (2, 4, 6, and 24 h; 4 and 7 days) after injection. At each time, pituitary, hypothalamus, and spleen were removed; and blood samples were obtained.

T cell-independent response was induced by LPS (1 mg/kg) (Sigma Chemical Co., St. Louis, MO).

The serum samples were stored at −20 C. Antibody levels were titrated by hemagglutination and whole-cell emzyme-linked immunosorbent assay (ELISA), as described (22). Briefly, for hemagglutination, serial dilutions of sera were performed in wells with fix amounts of SRBC, and antibody titer was expressed as Log 2 of the first concentration in which hemagglutination disappears. For ELISA, serum samples (50 μl/well) were added to the microtiter plates coated with SRBC (fixed with 0.2% glutaraldhehyde for 15 min at room temperature and washed twice with PBS). Next, different antibodies (50 μl/well) were added independently: 1) peroxidase-labeled goat antirat antibody (Dianova, Hamburg, Germany), 1:1,000 in PBS, recognizing total IgG and IgM; 2) peroxidase-labeled goat antirat IgM (Southern Biotechnology Associates, Inc., Birmingham, AL), 1:1,000 in PBS, recognizing total IgM; and 3) monoclonal mouse antirat IgG_{2a} or monoclonal mouse antirat IgG_1 (SERA-LAB, Sussex, United Kingdom), 1:2,000 in PBS, recognizing the iso-type-specific IgG antibodies. These monoclonal antibodies were quantified using peroxidase-conjugated affinity-pure goat antimouse IgG antibody (Dianova), 1:5,000 in PBS, as secondary antibody. Finally, in all cases, 100 μl/well substrate solution (ABTS; 2,2′-azino-bis-[3-ethyl-benzthiazolin-6-sulfonacid] diammoniumsalt) (Sigma Chemical Co.) in buffer (0.1 M citric acid, 0.1 M Na_2HPO_4, pH 4.3; ABTS: 25 mg/50 ml buffer), and 3.4 μl H_2O_2 was added, and optical density was measured at 405 nm in a microtiter plate reader (Bio-Rad Laboratories, Inc., Hercules, CA).

Surgery

Rats were implanted intracerebroventricularly (icv), as described (23). A guide-cannula of fine-bore polyethylene tubing (Portex, Kent, UK) was implanted into the right lateral ventricule (B: +0.5, L: 1.1, and H: −4.0, according to stereotaxic coordinates of Paxinos and Watson) (24) using a stereotactic instrument, under halothane anesthesia (Halocarbon Laboratories, River Edge, NJ), and fixed to the skull with screws and dental acrylic. Placement of the cannula was controlled by neuroanatomical sectioning. After the experiments, 3 μl methylene blue dye was injected icv to check the placement of the cannulae. Only those animals with the injection site confirmed in the lateral ventricle were considered for statistical analysis of the results.

Oligodeoxynucleotides and infusions

On the basis of the sequence of TRH mRNA (Gene Bank Data Base M12138), we used a 29-mer oligo (AACCAAGGTCCCGGCATCCTGGAGGATGC) antisense TRH complementary to the start coding region of rat TRH mRNA (25, 26). The oligonucleotides were phosphorothiolated on the 3′ and 5′ ends for four bases, respectively. The complementary sense TRH (GCATCCTCCAGGATGCCGGGACCTTGGTT), equally phosphorothiolated, and vehicle (saline) were used as controls. The oligonucleotides were purified by QC PAGE chromatography analysis (Pharmacia, Freiburg, Germany). Both the antisense and the sense constructs were compared with the Gene Bank database and found to have little or no homology to mRNAs registered. The oligonucleotides were dissolved in endotoxin-free saline.

Rats were handled during a 6- to 7-day recovery period after implantation. From the onset of the experiment, the conscious freely-moving animals received injections of saline, TRH antisense, or sense oligonucleotides (15 μg/3 μl, each) into the lateral ventricle using a Hamilton syringe connected to a 30-gauge injection needle via polyethylene tubing. The solutions were administered over a 60- to 90-sec period, with the injection needle left in place for an additional 60–90 sec to ensure complete dilution of the probes. Each animal was injected three times at 12-h intervals. No signs of toxicity were observed after injection of the oligonucleotides in ip saline- or SRBC-injected rats. The immunization of animals with an SRBC ip injection was performed at the time of the last icv injection. Dose and injection intervals were chosen, based on results of pilot studies and previous experience (23).

Neuroendocrine experiments

For estimation of early morning basal plasma levels of corticosterone, PRL, TSH, and GH, great care was taken to keep the rats undisturbed the night before the experiment. Sampling was always performed between 0700 and 0900 h in stress-free conditions. Undisturbed animals were anesthetized by introducing the cage into a plastic box containing saturated CO_2 vapor for 30 sec, after which the animals were immediately decapitated. Trunk blood was collected in ice-chilled EDTA-coated (2 ml) tubes containing 140 μg aprotinin (Trasylol, Bayer, Köln, Germany). Plasma samples, for hormone measurement by RIA (ICN Biomedicals, Inc., Costa Mesa, CA), were stored at −20 C. TRH was measured by RIA in hypothalamic homogenates, as previously described (26).

To avoid using stressed animals, subjects whose levels of corticosterone were more than 100 ng/ml at the time of sacrifice were excluded from all experimental groups.

Cell culture

Spleens were removed aseptically and dispersed through a metal mesh into PBS, as described (18). Cells were cultured in RPMI-1640 supplemented with 2 mM L-Glu, antibiotics, 50 μM β-mercaptoethanol, 25 mM HEPES, and 5% FCS (Gibco, Paisley, UK) at a density of 2.5×10^6 cells/ml (200 μl/well). Cell viability was always more than 95%, as determinated by trypan blue exclusion. Cells were stimulated with Con A (2.5 μg/ml; Pharmacia) for 48 h at 37 C, 5% CO_2, and 98% humidity.

IL-2 assay

IL-2 bioactivity in supernatants was assessed by a bioassay based on the proliferation of the IL-2-dependent murine cell line CTLL-2, as described (27). Briefly, CTTL-2 cells were cultured in RPMI-1640 (supplemented as described above) in 96-well microtiter plates (5×10^3/well) with serial dilutions of culture supernatants, compared with a standard curve generated by using recombinant murine IL-2 (Becton Dickinson and Co., Bedford, MA; specific activity 1.1×10^7 U/mg

protein). After 20 h of incubation, [^{3}H] thymidine (0.25 μCi/well) was added to each well. After 4 h of incubation with [^{3}H] thymidine, cells were harvested, and radioactivity on filters was measured by liquid scintillation counting. IL-2 activity in each sample was assessed by comparison with the standard curve. Results were expressed as units per milliliter. One unit of IL-2 activity was defined as the reciprocal of the dilution that yielded 50% of the maximal incorporation of [^{3}H]thymidine by the CTLL-2. The inter- and intraassay coefficients of variance of this bioassay were 30% and 10%, respectively.

Tissue dissection and RNA analysis

Tissues were obtained, and RNA was studied by Northern blot, as described previously (28, 29). The hypothalamic blocks were obtained according to a previously described procedure shown to include the paraventricular nucleus (29). Briefly, hypothalamic blocks were dissected within the following limits: posterior border of the optic chiasm, anterior border of the mamillary bodies, and the lateral hypothalamic sulci. The depth of the segments was approximately 2 mm, chosen to include the paraventricular nucleus (29). For Northern blot, total RNA, isolated by the guanidine isothiocyanate phenol-chloroform extraction method, was denatured with glyoxal, electrophoresed on a 1.2% agarose gel, and transferred to a nylon membrane. Filters were baked for 2 h at 80 C and stained with methylene blue to check for RNA integrity. They were prehybridized for 1 h and hybridized overnight at 42 C with TRH and at 55 C with TRH-R and actin probes in hybridization buffer (50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA). Blots were washed at increasing salt and temperature stringency, with a final wash of 30 min at the hybridization temperature in 0.1 × SSC containing 0.1% SDS. Dried filters were exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY) at −70 C, with intensifying screens, for 2–3 days.

A 1.3-kb rat TRH cDNA fragment (25) and 1-kb *Pst*I fragment of actin cDNA (30) were labeled with a random priming kit using α-^{32}P-dCTP (specific activity, 2–4 × 10^8 cpm/μg). A 1.8-kb mouse TRH-R complementary RNA probe (31) was labeled (specific activity, 2–5 × 10^7 cpm/μg) by *in vitro* transcription using T7 RNA polymerase and α-^{32}P-UTP. The autoradiograms were scanned with an LKB Ultroscan II laser densitometer (LKB, Bromma, Sweden). The blots were reprobed, after eluting the first probe with 5 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.1 × Denhardt's solution at 65 C for 2 h. After the previous signal was removed, confirmed by reexposure of the filter, the blots were prehybridized and hybridized, following the methods described above. The control with the actin fragment cDNA as probe was performed in each blot.

Statistics

Statistics were performed using two-way ANOVA with *post hoc* Scheffé's test. Results are expressed as mean ± SEM. A $P < 0.05$ was accepted as the level of significance.

Results

SRBC immunization

To validate the model of SRBC immunization, we compared the activation of the HPA axis with previous reports (3) and verified T-lymphocyte activation and antibody production. Seven days after SRBC immunization, plasma corticosterone levels were significantly elevated, compared with saline-injected control animals (Table 1A). As shown in Table 1B, a high level of anti-SRBC antibodies production was observed at 7 days post immunization. To verify a correct stimulation of T cells, we measured the concentration of IL-2 in supernatants of splenocytes stimulated with Con A. At early times (6 h post immunization), there was an increase in IL-2 levels, whereas at 7 days post immunization, IL-2 levels in SRBC-injected animals did not differ from control animals (Table 1C).

TABLE 1. Immune and endocrine control of SRBC immunization

A.

[a]Time	Treatment	[b]Corticosterone (ng/ml)
2 h	Saline solution	33.6 ± 17.4 (10)
	SRBC	36.7 ± 12.0 (10)
7 Days	Saline solution	9.8 ± 3.1 (10)
	SRBC	80.9 ± 33.9 (10)[c]

B.

[a]Time	Treatment	Log2 antibody titer (Hemagglutination)	[d]ELISA units Ig (M+G)
7 Days	Saline solution	<1 (10)	206 ± 66 (4)
	SRBC	11.4 ± 0.7 (10)	488 ± 45 (9)[e]

C.

[a]Time	Treatment	[f]IL-2 levels (U/ml)
6 h	Saline solution	20.4 ± 2.1 (8)
	SRBC	33.1 ± 3.5 (8)[e]
7 Days	Saline solution	14.5 ± 3.5 (8)
	SRBC	24.1 ± 3.4 (8)

[a] Male rats (180–200 g) were immunized ip with SRBC or saline for 2 h, 6 h, and 7 days. At the time of death, trunk blood was collected, and the spleen was removed. The number of rats in each experimental group is indicated. Data are mean ± SEM. Statistics: ANOVA with Scheffé's test (SRBC *vs.* saline).

[b] Plasma corticosterone levels were measured by RIA.

[c] $P < 0.05$.

[d] We defined 1/10 of antibody titers against SRBC from 7 days after immunization as ELISA arbitrary units.

[e] $P < 0.01$.

[f] IL-2 was measured by bioassay on Con A (2.5 μ/ml) 48 h-stimulated splenocytes.

Hypothalamic TRH mRNA expression

We investigated whether TRH mRNA levels in the hypothalamus are altered after SRBC immunization. TRH mRNA was detected on the Northern blot, by autoradiography, as a single band of 1.7 kb (Fig. 1). An increased level of TRH mRNA was observed in SRBC-injected animals. The densitometric analysis showed the increased level of TRH mRNA at 4, 6, and 24 h post immunization. We have not observed any change of TRH mRNA levels at 2 h or more than 24 h post immunization (Fig. 1). The SRBC-induced effects on TRH mRNA levels are opposite to those induced by LPS, as shown in Fig. 1. As previously reported (32), LPS induces a decrease in hypothalamic TRH mRNA levels that is observed at 24 h and more pronounced at 4 h.

Pituitary TRH-R mRNA levels

TRH-R mRNA expression was examined in the same experiments. Pituitary TRH-R mRNA levels at 6 h post immunization were markedly elevated, in comparison with the control animals (Fig. 2). We observed an increased level of TRH-R mRNA at early times, with a further decrease at 24 h and 4 days, without changes at 7 days post immunization.

Effects on PRL, TSH, and GH plasmatic levels

We next investigated the changes in the plasma PRL levels. As shown in Fig. 3, plasma PRL levels of SRBC-immunized animals significantly increased, with respect to control animals, at 2, 6, and 24 h post immunization. At 4 and 7 days post immunization, there were no differences in the plasma PRL levels. TSH and GH plasma levels showed no significant

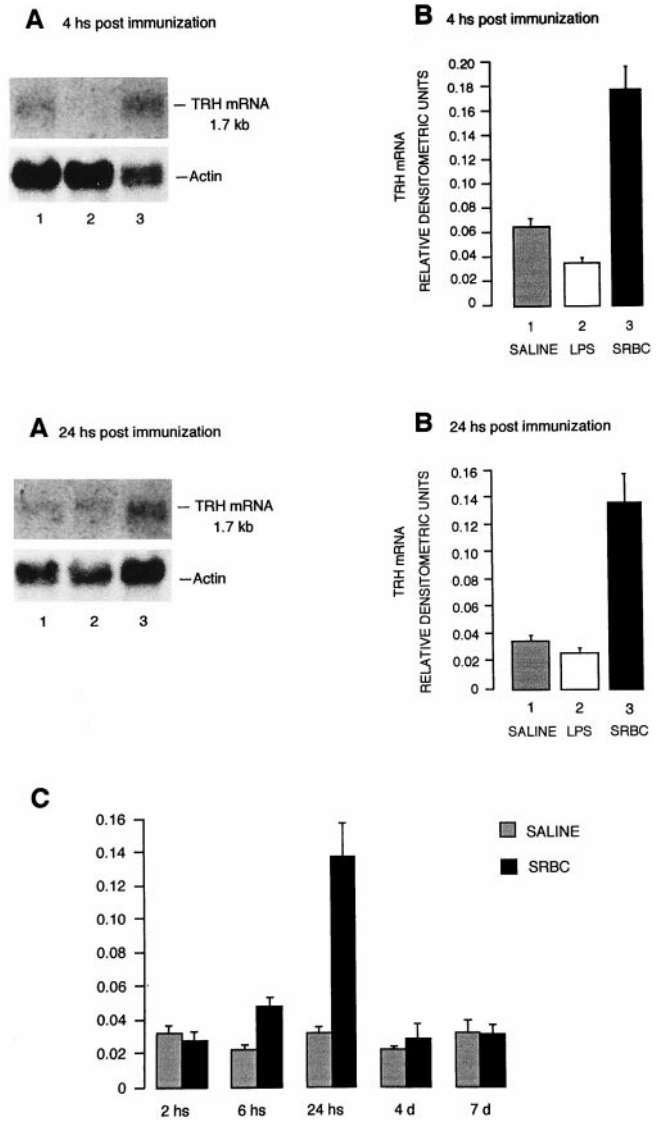

FIG. 1. Hypothalamic TRH mRNA levels. Northern blot of RNA extracted from hypothalamus of ip saline-, ip SRBC-, or ip LPS-treated rats, at the indicated times (similar times for saline, SRBC, or LPS). A, Each lane shows hypothalamic RNA from one representative rat; B and C, densitometric analysis of TRH mRNA content in hypothalamic tissues from ip saline-, ip SRBC-, or ip LPS-treated rats, based on three independent experiments, including three rats for each group for each experiment (n = 9). Data are expressed as ratio of autoradiographic signals of TRH mRNA, relative to β-actin mRNA used as loading control ± SEM.

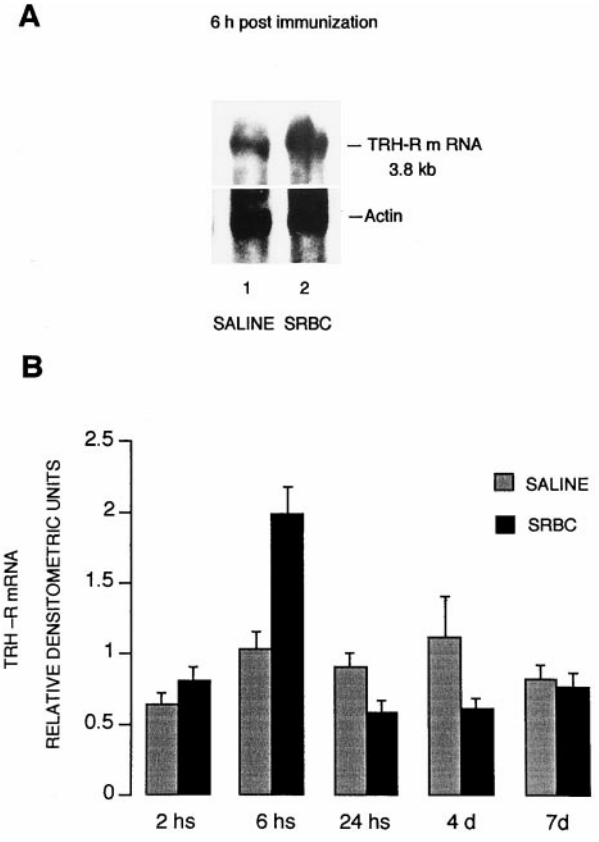

FIG. 2. Pituitary TRH-R mRNA expression. Northern blot of RNA extracted from pituitary of ip saline- and ip SRBC-treated rats, at the indicated times post SRBC or post saline. A, Each lane shows pituitary RNA from one representative rat; B, densitometric analysis of TRH-R mRNA content in anterior pituitary tissues from ip saline- and ip SRBC-treated rats, based on three independent experiments, including three rats for each group for each experiment (n = 9). Data are expressed as ratio of autoradiographic signals of TRH-R mRNA, relative to β-actin mRNA used as a loading control ± SEM.

changes at all times, including 4 and 7 days after SRBC treatment (Fig. 4).

Effects of TRH oligonucleotide icv administration in SRBC-treated animals

We investigated whether the response to SRBC would be suppressed by inhibiting central TRH production with a TRH-antisense treatment. We observed a significant inhibition of antibody production in TRH-antisense-treated conscious freely-moving rats (Table 2A). Primary different anti-SRBC antibody isotypes (IgM and IgG_{2a} and IgG_1) were severely suppressed in TRH-antisense-treated animals (Table 2B). We observed a more pronounced inhibition of IgG_1, in comparison to IgG_{2a}. In contrast, in those animals treated icv with vehicle or TRH-sense, there were no changes in antibody production. Out of the group of 29 rats treated with TRH-antisense, 4 animals did not show any response; however, all the sense-treated rats were similar to vehicle-treated rats. Twelve hours after SRBC immunization, both the hypothalamic TRH mRNA (Fig. 5) and TRH protein (antisense = 338.7 pg/mg tissue *vs.* sense = 587.3 pg/mg tissue; n = 8 each group, $P < 0.05$) were markedly lower in the antisense-treated rats than in the control animals, showing the efficiency of the antisense treatment. Direct measurement of target RNA is validated by the fact that RNase H hydrolyses the RNA strand of a RNA-DNA duplex and is likely to be responsible for the antisense effects of oligonucleotides (33).

The TRH-antisense treatment blocked the plasma PRL increase induced by SRBC immunization. The PRL levels of the TRH-antisense-treated animals, after SRBC immunization, do not peak as do the SRBC-treated, sense, or vehicle rats (Table 2C).

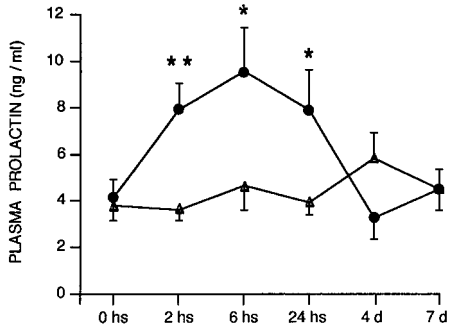

FIG. 3. Effects of SRBC treatment on plasma PRL levels. After injection of SRBC (●) or pyrogen-free saline (△), in four independent experiments (mean ± SEM, n = 10), plasma PRL was measured by RIA at the indicated times. 0 h, Initial PRL levels previous to SRBC or saline treatment. Statistical analysis of the results was performed by two-way ANOVA with *post hoc* Scheffé's test. Effect of time: F (4, 4) = 1.774, $P = 0.1442$. Effect of treatment: (SRBC *vs.* saline): F (1, 4) = 9.105, $P = 0.0036$. Analyzing the effect of treatment with the effect of time post immunization, the statistic (SRBC × time, F (1, 4) = 4.298, $P = 0.0037$) indicates that the observed differences between different times are caused by the antigen treatment. **, $P < 0.01$; *, $P < 0.05$, *post hoc* Scheffé's test.

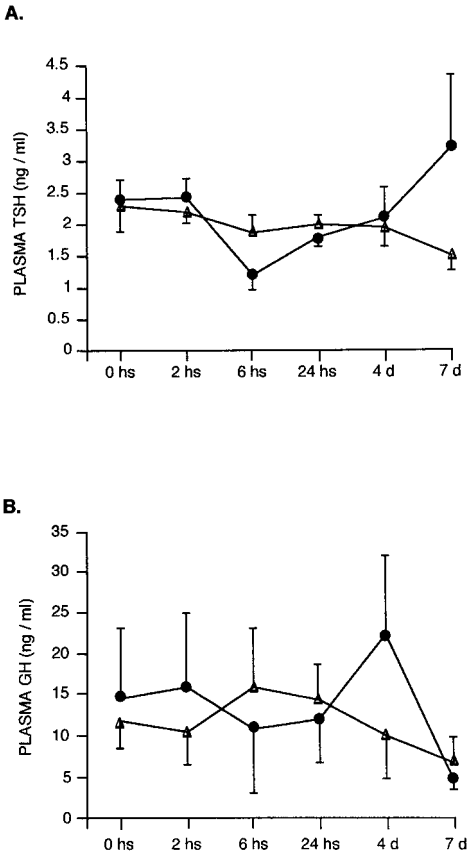

FIG. 4. Effects of SRBC treatment on plasma TSH and GH levels. After injection of SRBC (●) or pyrogen-free saline (△), in four independent experiments (mean ± SEM, n = 10), plasma TSH and GH levels were measured by RIA at the indicated times. 0 h = TSH and GH levels previous to SRBC or saline treatment. Statistical analysis of the results was performed by two-way ANOVA with *post hoc* Scheffé's test. A, Effect of time: F (4, 4) = 1.17, $P = 0.3317$. Effect of treatment (SRBC *vs.* saline): F (1, 4) = 0.819, $P = 0.3695$. B, Effect of time: F (4, 4) = 0.88, $P = 0.4807$. Effect of treatment (SRBC *vs.* saline): F (1, 4) = 0.201, $P = 0.6554$. SRBC-treated animals showed no significant changes, compared with control animals.

Discussion

In the present work, we demonstrate, for the first time, that the neuroendocrine changes occurring during the course of a T cell-dependent immune response (*i.e.* SRBC model) are fundamentally different from those occurring during the T cell-independent and inflammatory responses (LPS model). Thus, in the first 24 h, the T cell-dependent response leads to a peak in hypothalamic TRH mRNA and plasma PRL, whereas this circuit is inhibited during the LPS-IL-1-dependent response. This peak is critical for mounting an adequate response, which is inhibited by its blockade, as we can see in TRH-antisense-treated animals. In coordination with this, the T cell antigen induces the HPA-corticosterone suppressive response only during a late phase (5–7 days), in contrast to the early response induced by the T cell independent antigen.

The instrumental role of the early activation of the TRH-PRL axis is demonstrated by the inhibition of the antibody response induced by the injection of the TRH mRNA antisense oligonucleotides in conscious freely-moving rats. Both the functional effectiveness of the antisense injection and the role of this axis in the response are confirmed by the absolute lack of inhibition in the sense-treated animals, the histological confirmation of the site of injection, the dampening in the plasma PRL peak, and the hypothalamic TRH mRNA and protein after this injection. A small proportion of animals did not respond at all to the antisense treatment, probably because of a lack of penetration of the antisense in the target cells. In support of our finding, it has been demonstrated that: treatment of rats with bromocriptine, which inhibits PRL secretion, decreases the contact sensitivity skin reaction and antibody formation to SRBC (12); and that, after transplantation of chemically-induced tumors in syngenic rats, an early increase in PRL is induced (34). The TRH and PRL response to LPS (an antigen that does not induce T cell clonal expansion) is the opposite, because it lacks the early-phase peak observed during the T cell-dependent antigen response. In fact, it has also been demonstrated that IL-1 (induced by LPS) inhibits both TRH mRNA (32) and PRL (35) levels. The absence of a TRH and PRL peak can be attributed to the fact that the inflammatory response does not require TRH/PRL for its activation, as does the IL-2-T cell-dependent response (8–15, 18–21), strongly supporting both the specificity and the role of this peak during the T cell-dependent immune response.

Although, in the primary immune response to SRBC, IgM is the main antibody involved, there is also a low level of IgG production. Thus, we investigated also the possible influence of the antisense treatment on T-helper (Th)1 and Th2 responses, taking into account that the IgG1 and IgG2a antibodies are predominant during the Th2 and Th1 response, respectively. We observed a more pronounced inhibition of IgG1, in comparison with IgG2a. This may reflect a greater influence of antisense treatment on the Th2 response.

Several mechanisms could account for the neuroendocrine response to SRBC. First, cytokines, among them IL-2 (induced systemically during the T cell clonal expansion or

TABLE 2. Effect of icv administration of TRH-antisense in SRBC-treated rats

A.

[a]Post immunization time	Treatment	[b]Log 2 antibody titer
7 Days	Vehicle	9.9 ± 2.0 (10)
	TRH-sense	9.8 ± 0.6 (15)
	TRH-antisense	5.8 ± 0.5 (25)[c]

B.

[a]Treatment	Antibody type ([d]ELISA Units)			
	Ig(M+G)	IgM	IgG_{2a}	IgG_1
Vehicle	477 ± 42 (10)	306 ± 31 (4)	58 ± 24 (4)	38 ± 16 (4)
TRH-sense	384 ± 27 (11)	277 ± 13 (5)	38 ± 5 (4)	46 ± 14 (4)
TRH-antisense	193 ± 22 (11)[e]	169 ± 29 (5)[c]	16 ± 5 (6)[f]	8 ± 2 (6)[c]

C.

[a]Post immunization time	Treatment	[g]PRL (ng/ml)
12 h	Vehicle	7.9 ± 1.7 (6)
	TRH-sense	8.3 ± 1.4 (6)
	TRH-antisense	2.2 ± 0.5 (6)[c]

[a] Male rats (180–200 g) were icv-infused with oligonucleotide or vehicle and killed at the indicated times after SRBC treatment. The number of animals in each experimental group is indicated. Data are mean ± SEM. Statistics: ANOVA with Scheffé's test, TRH antisense *vs.* corresponding sense and saline.

[b] Anti-SRBC antibodies were determined by hemagglutination.

[c] $P < 0.01$.

[d] Levels of anti-SRBC antibodies at 7 days were determined using isotype-specific ELISA. We defined 1/10 of antibody titers against SRBC from 7 days after immunization as ELISA arbitrary units.

[e] $P < 0.001$.

[f] $P < 0.05$.

[g] Plasma PRL was measured by RIA. PRL levels without SRBC injection were similar to the TRH-antisense treated rats.

locally in the CNS) may be inducing the TRH and/or PRL expression. The lacto/somatotrophic cells in the pituitary express receptors for IL-2 (36); and *in vitro*, IL-2 induces PRL synthesis (37), which is inhibited by dopamine (38). A combination of dopamine inhibition and TRH stimulation could be acting to stimulate PRL release. The PRL elevation observed 2 h after immunization seems to be too early to be attributed to a rise in cytokine synthesis. This early peak, as well as the action on the hypothalamic TRH gene, is most probably caused by nervous system signals. SRBC is mainly processed at the spleen, which is richly innervated by the autonomous nervous system, through afferent and efferent fibers, particularly sympathetic and peptidergic (39). Furthermore, fibers from the paraventricular nucleus of the hypothalamus project to the periphery, through neurons in the spinal cord (40). In fact, changes in hypothalamic norepinephrine turnover (4), as well as in serotonin metabolism (41) after SRBC immunization, have been described. Through this pathway, CNS-induced cytokines (42) and other factors could be responsible for TRH gene induction. In addition, specific T cell-activated lymphocytes, known to cross the blood-brain barrier, could also act at the CNS (43).

At the pituitary level, TRH-R mRNA shows an early increase followed by a decrease, which is very likely caused by feedback signaling, either of PRL or TRH. In contrast, GH and TSH do not show any change during the entire timeframe studied, after antigen inoculation. PRL and TSH secretion patterns, after TRH stimulation, may vary according to the dose and administration pathway of TRH and to the previous status of the pituitary and hypothalamic glands (44). For example, a repeated administration of TRH, every 8 h for 7 days, leads to decreased TSH plasma levels, and repeated administration of TRH in monkeys has been shown to decrease TSH and increase PRL plasma levels (45). In addition, it has been shown that TSH increases under pulsatile TRH administration, but this increase is not maintained after continuous administration, thyrotrope desensitization being the most likely explanation for the attenuated stimulation after continuous TRH application (46). The feedback action of elevated T_3 and/or T_4, induced by a putative direct action of the antigen or cytokines at the thyroid level (47), or the inhibition of serotonin by SRBC (41), known to stimulate TSH (48), could also contribute to counteract the putative elevation of TSH. IL-2 (which stimulates PRL) inhibits GH by a direct action at the pituitary level (37). The action of various cytokines on the thyrotroph is also different from that on the lactotroph. The lack of changes in the kinetic response of TSH and GH may therefore reflect the compensatory integration of multiple regulatory pathways. Interestingly, regardless of the exact nature of the mechanisms involved, the specific kinetic of PRL, compared with GH and TSH, seems to be related to its instrumental role for the T cell response.

A second major difference between the LPS and SRBC models is the activation of the HPA axis. Whereas, after LPS stimulation, the axis is activated immediately, in the early phase, after SRBC inoculation, the peak of corticosterone appears in the late phase, 4–7 days later. It has been shown that for another T cell-dependent antigen (phosphocholine-keyhole limpet hemocyanin), the response of the HPA axis depends on the dose: at low doses it does not induce any change, whereas at high doses, it induces an activation of the HPA axis at day 5, which correlates with the peak of the antiphosphocholine-keyhole limpet hemocyanin antibody response (49). Thus, in contrast to the acute effect of the LPS-IL-1-inflammatory response, the immunosuppressive effect of glucocorticoids seems, in the T cell-dependent re-

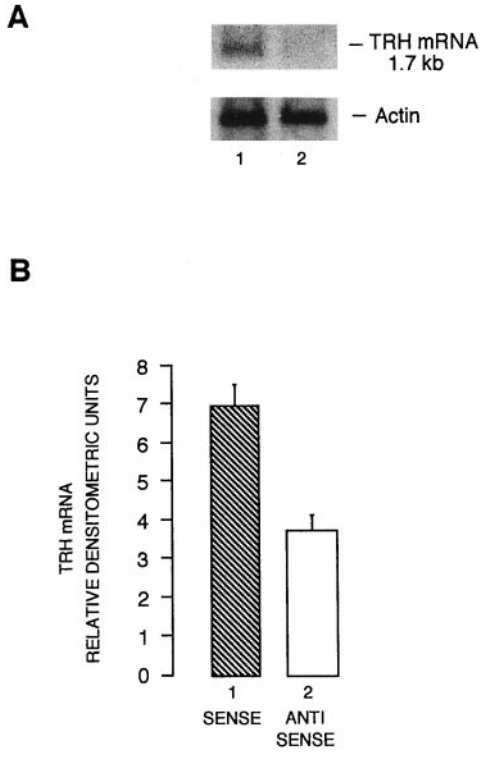

FIG. 5. Hypothalamic TRH mRNA levels in TRH oligonucleotide-treated animals. Northern blot of RNA, extracted from hypothalamus of TRH-antisense and TRH-sense-treated rats, 12 h after SRBC immunization. A, Each lane shows hypothalamic RNA from one representative rat; B, densitometric analysis of TRH mRNA in hypothalamus from control (TRH-sense) and icv TRH-antisense rats after SRBC treatment, based on three independent experiments, including three rats for each group for each experiment (n = 9). Data are expressed as ratio of autoradiographic signals of TRH mRNA, relative to β-actin mRNA used as a loading control ± SEM.

sponse, concomitant with the peak of antibody production, to suppress the late activation of nonspecific clones.

It has been demonstrated recently that prepro TRH gene contains a sequence (178–199 bp) encoding a 22-amino acid peptide, named corticotrophin release inhibiting factor, that inhibits ACTH secretion and may provide the clue for the coordinated regulation of pituitary-adrenal and pituitary-thyroid functions (50, 51). In fact, hypothyroidism results in elevated levels of hypothalamic TRH mRNA and reduced anterior pituitary POMC mRNA and plasma ACTH and corticosterone levels (52). This mechanism may explain the different response of the HPA axis to the inflammatory/T cell-independent and the T cell-dependent stimulation. TRH gene expression, during the response to the T cell-dependent antigen, may thus not only be instrumental in the activation of the immune response but may also be responsible for coordinating the inhibition of the HPA axis during this early activation phase and its elevation in the late phase, when the TRH expression declines, controlling the expansion of putative nonspecific clones. In the LPS inflammatory/T cell-independent response, not only is the TRH/PRL activation not necessary for T cell clonal expansion, but also the glucocorticoid inhibitory action on the inflammatory mediators (*i.e.* IL-1) is immediately necessary.

Our results provide a new insight into the role of the TRH/PRL and HPA axis during the course of the T cell-dependent response, showing that the clonal expansion of T-lymphocytes is critically dependent on this coordinated response.

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