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Altered lymphocyte catecholamine reactivity in mice subjected to chronic mild stress

Valeria Ayelli Edgar[a,1], Dafne Magalí Silberman[a,1], Graciela A. Cremaschi[a], Luis M. Zieher[b], Ana María Genaro[a,*]

[a]*CEFYBO-CONICET, Serrano 669, 3rd Floor, CP (1414), Buenos Aires, Argentina*
[b]*1ra Cátedra de Farmacología, Facultad de Medicina-UBA, Paraguay 2155 CP (1121), Buenos Aires, Argentina*

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Abstract

There is considerable evidence that the sympathetic nervous system influences the immune response via activation and modulation of β_2-adrenergic receptors (β_2R). Furthermore, it has been suggested that stress has effects on the sympathetic nervous system. In the present study, we analyzed the influence of catecholamines on the reactivity of lymphocytes from mice exposed to a chronic mild stress (CMS) model of depression (CMS-animals). The effects of the CMS treatment on catecholamine and corticosterone levels and on β_2R lymphoid expression were also assessed. For this purpose, animals were subjected to CMS for 8 weeks. Results showed that catecholamines (epinephrine and norepinephrine) exert an inhibitory effect on mitogen-induced normal T-cell proliferation and a stimulatory effect on normal B-cell proliferation in response to selective B lymphocyte mitogens. Specific β- and β_2-antagonists abolished these effects. Lymphocytes from mice subjected to CMS had an increased response to catecholamine-mediated inhibition or enhancement of proliferation in T and B cells, respectively. Moreover, a significant increase in β_2R density was observed in animals under CMS compared to normal animals. This was accompanied by an increment in cyclic AMP production after β-adrenergic stimulation. On the other hand, neither catecholamine levels, determined in both urine and spleen samples, nor serum corticosterone levels showed significant variation between normal and CMS-animals. These findings demonstrate that chronic stress is associated with an increased sympathetic influence on the immune response and may suggest a mechanism through which chronic stress alters immunity.

Keywords: Chronic mild stress; Lymphocyte proliferation; β-Adrenergic receptor; cAMP; Catecholamines

1. Introduction

Over the past several years, many studies have suggested an association between chronic stress and immune dysfunction [1,2]. In general, chronic stress has been associated with the suppression of various aspects of the immune response, such as a reduction of lymphocyte proliferation in response to mitogens, reduced natural killer cell activity, decreased antibody response to virus after vaccination, and defects in wound repair [3–6].

Attention has begun to focus on the possible biochemical and physiological mechanisms by which these immune changes may be mediated. The most important mechanism involved has been the activation of both the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system. The sympathetic nervous system and the immune system are linked anatomically by a dense innervation of lymphoid tissues such as the spleen, thymus, and lymph nodes. In these tissues, lymphocytes and sympathetic nerve endings form contacts at a distance that is even shorter than that observed in synapses [7]. Moreover, lymphocytes have been shown to possess receptors for catecholamines, mainly of the β_2 subclass, that are coupled to the cAMP-adenylate cyclase signaling pathway [7,8]. Triggering of these receptors results in modulation of immune reactivity [9].

An extensive literature describes the diverse physiologic effects of chronic stress. Although investigations of the

* Corresponding author. Tel.: +54-11-4855-7194; fax: +54-11-4856-2751.
E-mail address: genaro@cefybo.edu.ar (A.M. Genaro).
[1] Both authors contributed equally to the work.
Abbreviations: cAMP, cyclic AMP; CMS, chronic mild stress; Con A, concanavalin A; [^{125}I]CYP, (−)-[^{125}I]cyanopindolol; LPS, lipopolysaccharide; NE, norepinephrine; S.I., stimulation index; [^{3}H]Tdr, [*methyl*-^{3}H]thymidine.

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effects of chronic stress on the sympathetic nervous system traditionally have focused on neurohormones, recent studies include end-organ receptor function. Animal studies, for example, suggest that there are complex effects of stress on β-adrenergic receptor physiology, with both decreases and increases in β-adrenoceptor density and/or sensitivity, depending on the duration of the stressor and the site of measurement, i.e. brain or lymphocytes [10]. Alterations in neuroimmune communication, in particular those associated with lymphocyte β_2-adrenoceptor expression and function, have been described in other pathologies with immunological abnormalities [11–13].

The present study was undertaken to analyze the autonomic nervous system–immune interactions underlying the effect of CMS on mice and to investigate the contribution of the autonomic nervous system to the immune alterations associated with chronic stress. For this purpose, we studied the influence of catecholamines on mitogen-induced lymphocyte proliferation and the correlation with β_2 receptors on these cells in normal and CMS-exposed mice. Likewise, we also analyzed catecholamine and corticosterone levels, given that both contribute to the regulation of the β_2-adrenergic receptor and may be altered under chronic stress conditions [14,15].

2. Materials and methods

2.1. Drugs

Con A, LPS, epinephrine, NE, propranolol, butoxamine, and anti-mouse monoclonal antibodies were purchased from the Sigma Chemical Co. [^{3}H]Tdr (20 Ci/mmol) and [^{125}I]CYP (2200 Ci/mmol) were purchased from New England Nuclear (NEN), Life Science Products. Other materials were from standard commercial sources.

2.2. Mice

Inbred female BALB/c mice were purchased from the Instituto Nacional de Tecnología Agropecuaria (INTA). All animals were between 60 and 100 days of age. Animals were cared for in accordance with the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996.

2.3. CMS model

All the animals were housed singly and maintained on a 12-hr light/dark cycle under controlled temperatures between 18 and 22°. Except as described below, food and water were available *ad lib*. First, the animals were trained to consume a 1% sucrose solution. Sucrose consumption was monitored throughout the experiment. After this phase (2 weeks), the animals were distributed into two groups. One group was housed in normal conditions (non-exposed animals). The other group was subjected to CMS throughout the experiment. The stress regime was modified slightly from that previously used in rats by Willner *et al*. [16] and in mice by Monleon *et al*. [17]. It consisted of the following: three 3-hr periods of food and water deprivation, immediately prior to testing for sucrose consumption; one additional 16-hr period of water deprivation; two periods of continuous overnight illumination; two periods (7 and 17 hr) of 45° cage tilt; one 17-hr period in a soiled cage (100 mL water in sawdust bedding); one 8-hr period of food deprivation; one 17-hr period of paired housing (mice were always housed in the same pairs, but the housing location was alternated between the home cages of each member of the pair). The stressors were scheduled through the week, in a similar manner to that previously described [16,17]. Finally, sucrose consumption tests were continued for an additional week following the withdrawal of stress. A depression-like state was induced by the CMS which led to diminished food consumption and a diminished preference for sweet drinks (anhedonia). These parameters reached their maximum values after 6 weeks, and remained high during the subsequent weeks [18].

2.4. Cell suspensions and culture conditions

Lymphoid cell suspensions were obtained from the spleens of control and CMS-exposed BALB/c mice as previously reported [19]. Cells were resuspended in RPMI 1640 medium supplemented with batch-tested non-stimulatory 10% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 50 μM β-mercaptoethanol. T-cell enriched populations were obtained by passage of the cell suspension through a nylon wool column according to the method of Julius *et al*. [20]. B-cell enriched populations were obtained by treatment with anti-Thy-1 antibody plus C as described elsewhere [21]. To remove adherent cells, the lymphocyte suspension was incubated twice in plastic tissue culture dishes in the presence of RPMI 1640 medium supplemented with 5% fetal bovine serum. The B- and T-cell population purity was between 90 and 95% as determined by direct immunofluorescence. T- and B-cell viability was estimated according to the trypan blue exclusion criteria and was higher than 90%.

2.5. Mitogen assay

Lymphocyte proliferation was determined by culturing 2×10^5 cells, in each well of a 96-well plate, in 150 μL of RPMI 1640 medium containing supplements. Aliquots of 50 μL of Con A and LPS were added to the microcultures to yield the following final concentrations: Con A: 0.5, 1, 2, and 4 μg/mL, and LPS: 15, 30, 60, and 75 μg/mL. In control cultures, stimulants were replaced by 50 μL of culture medium. Then cells were cultured at 37° in a 5%

CO_2 atmosphere for different periods. Mitogenic activity was measured by adding 1 μCi [^{3}H]Tdr to each microculture for the final 18 hr of incubation. The thymidine incorporation was measured by scintillation counting after retention over GF/C glass-fiber filters (Whatman) of the acid-insoluble macromolecular fraction. The means of triplicate determinations were calculated for each mitogen concentration. The proliferation kinetics of the mitogen-stimulated cells were as expected, with a peak of proliferation on the third day in culture. To analyze the influence of catecholamines on the proliferative response, cells were co-incubated with either epinephrine or NE, at concentrations ranging from 1×10^{-8} to 1×10^{-4} M. In parallel, a β-adrenergic receptor antagonist (propranolol, 1×10^{-5} M) or a β_2-adrenergic receptor antagonist (butoxamine, 1×10^{-4} M) was added to the cultures in order to determine whether the effects observed were mediated specifically through β_2-adrenergic receptors.

2.6. [^{125}I]CYP binding assay

The iodinated β-adrenergic antagonist (−)-[^{125}I]CYP was used to assay for receptor characteristics. This ligand allows one to obtain high quality binding data from a small number of cells expressing low levels of β-adrenoreceptor binding sites. However, we needed a minimum of 3–4×10^6 cells/tube instead of 1–2×10^6 cells/tube as other authors reported [22] in order to obtain high quality binding data.

For binding assays, cells were incubated with [^{125}I]CYP solutions containing the ligand at concentrations that ranged from 1 to 300 pM, in a final volume of 200 μL of 20 mM HEPES buffer containing 12 mM $MgCl_2$ and made isotonic with 130 mM NaCl. After 30 min of incubation at 30°, the samples were filtered through GF/C filters (Whatman), washed three times with 4 mL of buffer, and assessed for radioactivity using a Packard gamma counter. The amount of non-specific binding [in the presence of 1×10^{-6} M (−)-propranolol] was lower than 25% of the total binding. Specific binding was defined as the difference between binding in the absence and in the presence of (−)-propranolol. The density of specific binding sites for [^{125}I]CYP (B_{max}) and the dissociation constants (K_d) were determined by Scatchard analysis of [^{125}I]CYP binding [23].

2.7. cAMP assay

Cells (1×10^7/mL) in 1 mL of PBS were pretreated with isobutyl methyl xanthine (IBMX) (200 μM) for 20 min and then were incubated alone or in the presence of the indicated concentrations of NE for 10 min. At the end of the incubation time, 2 mL of chilled ethanol was added. Cells were homogenized, supernatants were evaporated at 55° under a nitrogen stream, and the residue was dissolved in 0.5 mL of assay buffer. Nucleotide determination was carried out by a cAMP enzyme immunoassay (Amersham Pharmacia Biotech).

2.8. Catecholamine assay

The concentrations of catecholamines were determined in samples from both 24-hr urines and spleens by the fluorometric assay described by Laverty and Taylor [24]. Briefly, spleens were homogenized in 12.5% sodium sulfite, 10% EDTA in 0.4 N perchloric acid. After 24 hr at 4°, the homogenates were centrifuged at 5000 *g* for 10 min at 4°. The supernatants were brought to pH 8.2 and loaded on a pre-washed alumina column. Urine samples were also brought to pH 8.2 and purified through an alumina column. The eluates were oxidized with iodine in an alkaline medium. Fluorescence was recorded at 375 nm in a spectrofluorometer using an excitation source of 325 nm.

2.9. Corticosterone determination

Blood from animals under different experimental conditions was collected on ice and separated in a refrigerated centrifuge. The plasma was stored at −80° until assayed. Corticosterone levels were determined using highly sensitive double antibody radioimmunoassay kits (ICN Biomedical Inc.).

2.10. Statistical analysis

Student's *t*-test for unpaired values was used to determine the level of significance. When multiple comparisons were necessary after ANOVA, the Student–Newman–Keuls test was applied. Differences between means were consider significant if $P \leq 0.05$.

3. Results

3.1. Effect of adrenergic receptor stimulation on mitogen-induced T- and B-lymphocyte proliferation

Splenocytes from non-exposed mice (control) or mice exposed for 8 weeks to the CMS procedure were used to evaluate the proliferative response sensitivity to β-adrenergic regulation. As can be seen in Fig. 1, T cells from CMS-exposed mice showed a lower response to the mitogen Con A (panel A) than non-exposed T-cells, while the B-cell response to LPS (panel B) was higher compared to lymphocytes from control animals. The addition of NE to the cultures resulted in a nonstatistically significant inhibition of the proliferative response of control T cells to Con A (Fig. 2A), while an increase of LPS-induced B-cell proliferation was observed under these conditions (Fig. 2B). Similar results were obtained in the presence of epinephrine (data not shown). The catecholamine effect was greater on T and B cells from CMS-exposed mice than

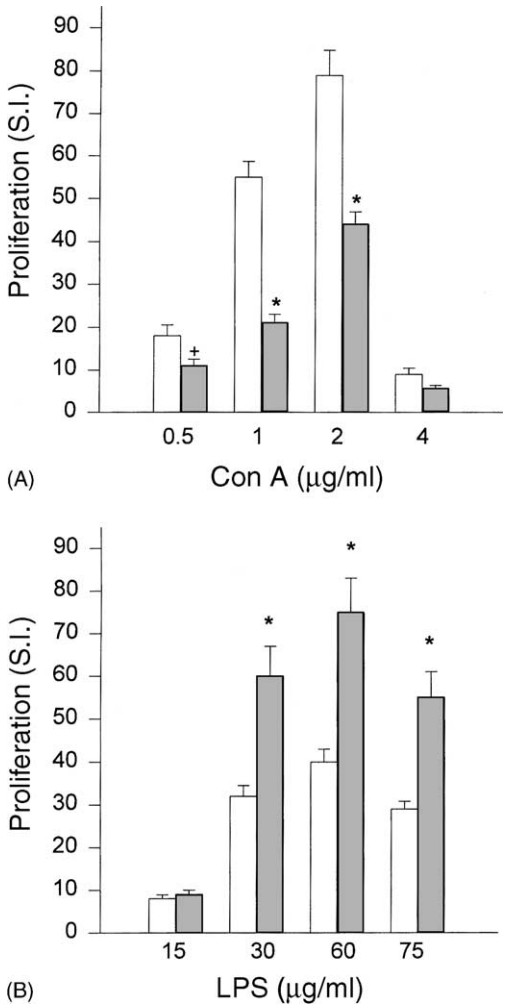

Fig. 1. Mitogen-induced proliferative responses in Con A-stimulated T cells (panel A) and LPS-stimulated B cells (panel B) from non-exposed (open bars) and CMS-exposed (closed bars) mice. Results obtained after 3 days of culture are expressed as the stimulation index (S.I.), calculated as the ratio between dpm values from experimental cultures and those obtained from non-stimulated cells. [^{3}H]Tdr incorporation for negative cultures (non-stimulated cells) was 3401 ± 189 dpm for control and 3034 ± 216 dpm for CMS-exposed animals. Data represent the means $\pm$ SEM from eight independent experiments done on triplicate cultures. Each experiment was performed using one animal from each group. Key: (*) significantly different from the corresponding non-exposed animals ($P < 0.01$); and (+) significantly different from the corresponding non-exposed animals ($P < 0.05$).

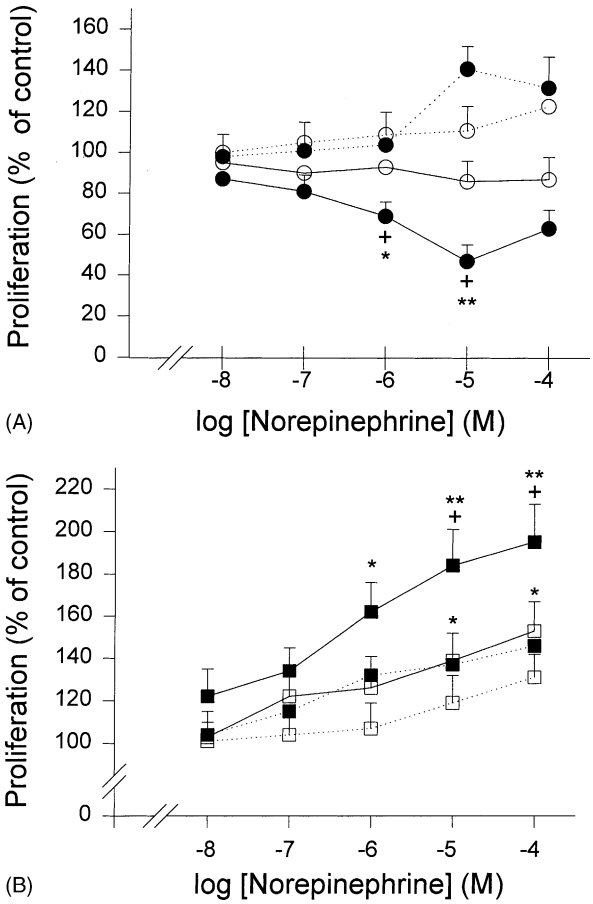

Fig. 2. Effect of norepinephrine (NE) on mitogen-induced proliferative responses. Con A-stimulated T cells (panel A) or LPS-stimulated B cells (panel B) from control (open symbols) and CMS-exposed mice (closed symbols) were co-incubated with increasing concentrations of NE. After 3 days of culture, the S.I. was calculated as described in the legend of Fig. 1. Results are expressed as a percentage of proliferation in the absence of NE (control). NE did not significantly affect the negative cultures (non-stimulated cells). Inhibition of the NE effect by 10^{-5} M propranolol is also shown (dotted lines). Data represent the means $\pm$ SEM from eight independent experiments done on triplicate cultures. Key: (+) significantly different from the corresponding non-exposed mice ($P < 0.05$); (*) significantly different from its corresponding control ($P < 0.05$); and (**) significantly different from its corresponding control ($P < 0.01$).

from non-exposed mice (Fig. 2). The β-antagonist propranolol (Fig. 2) and the β_2-antagonist butoxamine (data not shown) abolished these effects. It is important to note that neither propranolol nor butoxamine alone showed any effect on mitogen-induced proliferation (data not shown).

3.2. Expression of β-adrenergic receptors on B and T lymphocytes

Saturation assays with [^{125}I]CYP were performed on B and T cells from non-exposed and CMS-exposed mice. Fig. 3 shows a representative [^{125}I]CYP binding study on B and T cells from normal and CMS-mice using a pool of cells from three animals in order to obtain enough cells to perform a high quality binding assay. As can be seen in Fig. 3, [^{125}I]CYP binding to adrenergic receptors was specific and saturable within a concentration range of 1–300 pM. Scatchard analyses of the binding curves were in agreement with a single-site model for both B and T cells from control and CMS-exposed animals. T lymphocytes from CMS-exposed mice displayed an increase in β-adrenergic receptor number. Similarly, B cells isolated from mice exposed to CMS showed a higher expression of β-adrenergic receptors than B cells from control animals. To assess the variability of the response to CMS exposure between individual mice, we performed experiments on cells from individual animals using a concentration of [^{125}I]CYP that allowed for maximal CYP binding. Fig. 4 shows the results obtained from 10 non-exposed and

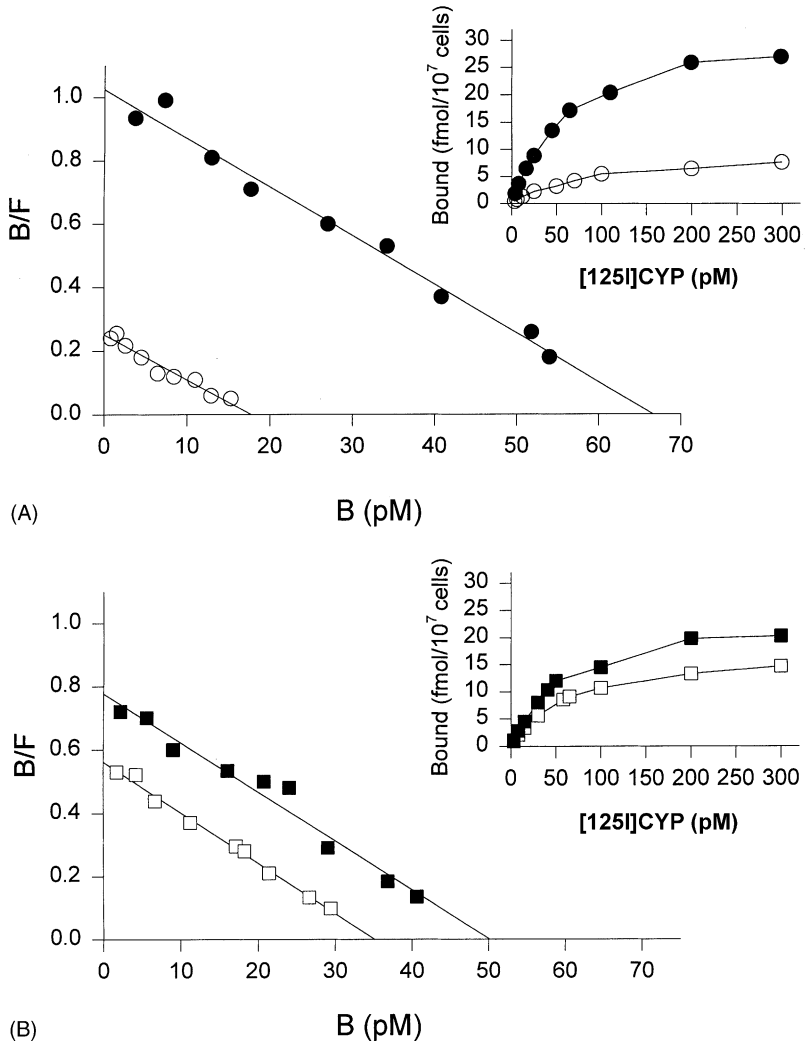

Fig. 3. Binding of [^{125}I]CYP to B and T cells. T (panel A) or B (panel B) cells (4×10^6) from non-exposed (N) (open symbols) or CMS-exposed (CMS) (closed symbols) mice were incubated with increasing concentrations of the radioligand [^{125}I]CYP. Data show one representative experiment from three separate experiments performed in duplicate. Each experiment was performed with a pool of three animals from each group. Scatchard analysis for these experiments showed the following results: B_{max} values for T cells: N: 512 sites/cell, CMS: 1986 sites/cell; B_{max} values for B cells: N: 1060 sites/cell, CMS: 1505 sites/cell; K_d values for T cells: N: 68 pM, CMS: 65 pM; K_d values for B cells: N: 64 pM, CMS: 63 pM.

10 CMS-exposed animals. As can be seen, the binding of [^{125}I]CYP to T cells from CMS-exposed mice (range: 13.3–36.2 fmol/10^7 cells, median: 26.1) was significantly greater than that obtained in control animals (range: 3.5–10.1 fmol/10^7 cells, median: 6.5, $P < 0.001$). Similarly, B cells from mice exposed to CMS bound significantly more CYP than control cells (range: 18.7–27.0 fmol/10^7 cells, median: 14.5; versus range: 9.9–18.6 fmol/10^7 cells, median: 21.9, $P < 0.001$).

3.3. cAMP levels after β-receptor stimulation

To determine if the changes in β-adrenergic expression paralleled the cAMP response, we studied the effect of β-adrenergic stimulation on the intracellular levels of cAMP. As can be seen in Fig. 5, T and B cells from CMS-exposed mice showed a greater cAMP response to the β-agonist, NE, than cells from control animals.

3.4. Catecholamine and corticosterone levels

The results showed in Table 1 indicate that there was not a significant variation in serum corticosterone levels or in urinary or splenic catecholamine levels between control

Table 1
Catecholamine concentrations in urine and spleen samples and corticosterone levels from non-exposed and CMS-exposed animals

Animal conditions	[Norepinephrine]		[Corticosterone] (ng/mL)
	Spleen (ng/mg)	Urine (ng/mL)	
Control	13.9 ± 2.3	4.33 ± 0.67	168 ± 17
CMS-exposed	12.7 ± 1.6 (NS)	4.59 ± 0.58 (NS)	177 ± 19 (NS)

Catecholamine and corticosterone analyses were performed in non-exposed and 8-week-exposed CMS mice. Results represent the means ± SD of six separate experiments. Each experiments was performed using one animal from each group. NS: nonstatistically significant with respect to the control.

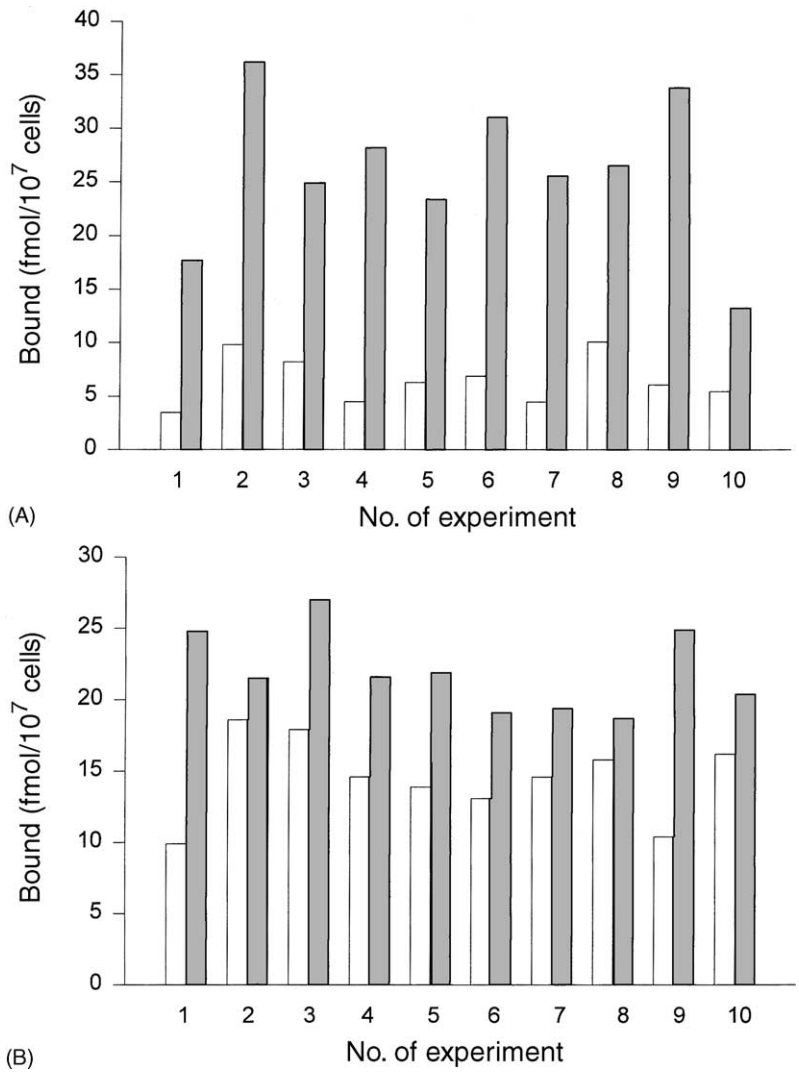

Fig. 4. Maximal binding of [^{125}I]CYP to B and T cells from non-exposed and CMS-exposed mice. T (panel A) or B (panel B) cells (4×10^6) from non-exposed (open bars) or CMS-exposed (closed bars) mice were incubated with saturating concentrations of the radioligand [^{125}I]CYP. The data show maximal binding from 10 independent experiments. Each experiment was carried out in one mouse for each condition and was performed in duplicate.

and CMS-exposed mice. These results were independent of the time the samples were obtained, i.e. during and after stress exposure (data not shown).

4. Discussion

Many studies have suggested that stress has profound effects on immune functions [1,2,25]. It has been known for some time that stress and emotions are associated with neurochemical and hormonal changes that in turn influence the reactivity of cells of the immune system [25–27]. Over the past several years, strong evidence has accumulated indicating the participation of the sympathetic nervous system in the modulation of lymphocyte activity via specific receptors that in turn regulate intracellular signals, such as cyclic nucleotide levels [9]. Moreover, it has been suggested that inadequate communication between the nervous and the immune system could contribute to the pathophysiology of immune disorders [13].

In the present study, we investigated the influence of catecholamines on proliferative responses of lymphocytes from animals subjected to a well-recognized model of CMS. We found that mitogen-induced lymphocyte proliferation is altered in CMS-exposed mice. The results specifically showed a decrease in T-cell mitogen responses and an increase in B-cell proliferative responses. Chronic stress has been associated with alterations in immunity. Thus, deficits in cellular immunity as well as some evidence for activation of immunocyte-mediated autoimmunological reactions, such as an increase in the levels of anti-phospholipid, antinuclear, and Epstein-Barr antibodies, have been observed in family caregivers of Alzheimer patients as well as others experiencing severe stress [3–6,28,29].

Concerning the influence of catecholamines on lymphocyte reactivity, we found that lymphocyte proliferation in

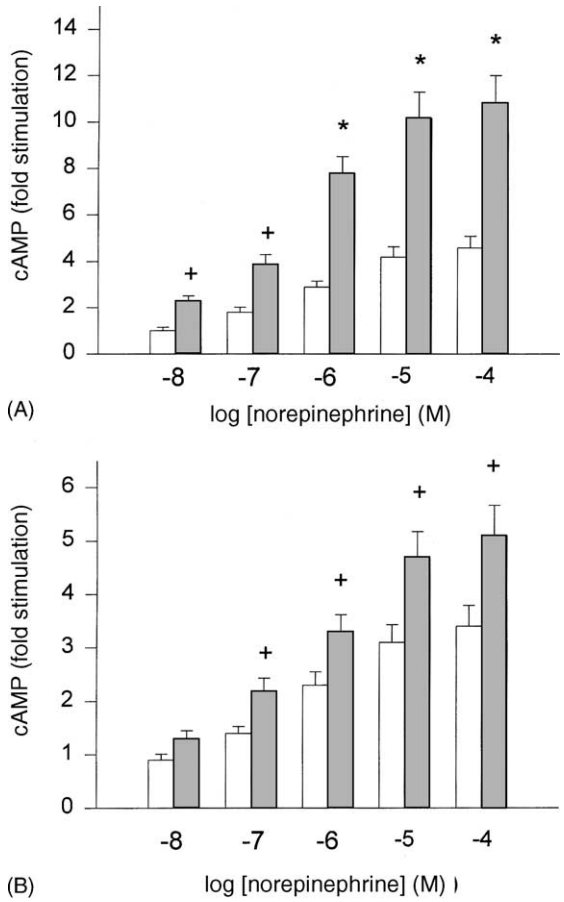

Fig. 5. Stimulated cyclic nucleotide levels in non-exposed and CMS-exposed mice. T cells (10^7) (panel A) and B cells (10^7) (panel B) from non-exposed (open bars) or CMS (closed bars) mice were incubated with increasing concentrations of NE for 10 min. cAMP levels were determined by enzyme immunoassay. The data shown represent the means ± SD of 4 experiments performed in triplicate. Each experiment was performed using a pool of two mice from each group. Key: (*) significantly different from the corresponding normal values ($P < 0.01$); and (+) significantly different from the corresponding normal values ($P < 0.05$).

response to Con A was not affected significantly by NE in control animals; however, NE significantly inhibited mitogen-induced proliferation in lymphocytes obtained from CMS-exposed mice. On the other hand, adrenergic stimulation of control B cells enhanced LPS-induced proliferation. This proliferation-enhancing effect was significantly greater in lymphocytes isolated from CMS-exposed mice than in those from non-exposed control mice. These results suggest that either the number of functional receptors is increased or that the intracellular effector system is enhanced in lymphocytes from CMS-exposed mice. In fact, our results indicate that both B and T cells from mice exposed to CMS showed an increase in β-adrenoceptor expression and a greater cAMP response to β-adrenergic stimulation than control animals.

Various studies suggest rather complex effects of stress on β-adrenergic receptors, with both decreases [30] and increases [10,31,32] in receptor density and/or sensitivity, depending on the duration of the stressor. Changes in the adrenergic receptor response affect lymphocyte activity. It has been reported that stimulation of β-adrenergic receptors can either inhibit or enhance the immune response. In general, an inhibitory effect on T-cell proliferation was described [9,33,34]. On the other hand, β-adrenergic stimulation enhances B-cell maturation and antibody production [22,35,36]. However, down-regulation of β-adrenergic receptors, associated with a lower cAMP response, was described in activated B and T lymphocytes [19,37]. It is probable that changes in the cAMP response after sympathetic stimulation account for the alterations observed in the mitogenic response. Indeed, it has been suggested that altered β-receptor sensitivity, associated with greater life stress, provides a potential mechanism that might explain the decrease in functional immunity described in highly stressed caregivers of dementia victims [10]. Alterations in neuroimmune communication, in particular in lymphocyte β_2-adrenoceptor expression and function, have been described in other pathologies with abnormalities in immunity. A recent study by Baerwald *et al.* [12], showed that patients with rheumatoid arthritis have a significantly reduced density of β-adrenoceptors compared to healthy donors and that this reduction parallels a decreased influence of catecholamines on lymphocyte function.

It is well known that catecholamines and cortisol contribute to β_2-adrenergic receptor regulation, and both may be altered by stress [14,15]. We did not find significant variations in corticosterone levels between control and CMS-exposed animals. Ayensu *et al.* [38] reported high corticosterone levels after 4 weeks of CMS exposure in rats. However, other authors have not found elevated levels of this hormone after prolonged stress situations [39]. Likewise, we did not find any differences in splenic or urinary catecholamine levels between normal and CMS animals. These results are in agreement with those obtained by Azpiroz *et al.* [39], who observed no differences in hypothalamic and hippocampal NE levels in animals under CMS conditions. These data suggest that adrenal (cortical or medullar) activation did not occur in CMS-exposed mice. However, an increase in corticosterone and catecholamine levels was observed in mice exposed to CMS for 1 or 2 weeks (data not shown). The possibility that early increases in these hormone levels may contribute to the induction of long-lasting changes in β_2-adrenergic receptors on lymphocytes is under study.

In summary, to our knowledge, this study presents the first experimental evidence that chronic stress may increase β_2-adrenergic receptor density on lymphocytes and that this increase may, in turn, enhance the impact of catecholamines on mitogen-induced proliferation. This would mean that altered lymphocyte reactivity is due mainly to changes in the number of receptors on target cells rather than from the activation of the symphatetic nervous system. These results suggest that an increased influence of the sympathetic nervous system on lymphocyte function may contribute to the previously reported

alterations in immune function found in chronic stress. However, it is important to note that, although the *in vivo* effects of stressors mimic the *in vitro* effects of NE, further work is needed to establish that the *in vivo* effect is due to β-adrenergic activation. Moreover, since stress signals disturb homeostasis by altering the balance of hormones that have a significant impact on the immune system, the participation of other neuroendocrine mediators will have to be taken into account to better understand the full effect of chronic stress on immune reactivity.

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