Pharmacology Biochemistry and Behavior, Vol. 45, pp. 665–672, 1993
Printed in the U.S.A.

0091-3057/93 $6.00 + .00

Seven-Day Variable-Stress Regime Alters Cortical β-Adrenoceptor Binding and Immunologic Responses: Reversal by Imipramine

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Received 28 July 1992

BASSO, A. M., M. DEPIANTE-DEPAOLI, L. CANCELA AND V. MOLINA. *Seven-day variable-stress regime alters cortical β-adrenoceptor binding and immunologic responses: Reversal by imipramine.* PHARMACOL BIOCHEM BEHAV **45**(3) 665–672, 1993.—Rats were submitted daily to a variable stressor for 1 week with or without concurrent imipramine (IMI) administration. One day after the last injection or stressful event, binding of cortical β-adrenoceptors was determined in all experimental groups. Another group of chronically stressed animals with or without concurrent IMI administration were sacrificed 24 h following the last stress or injection treatment, and several immunologic parameters were evaluated. Chronically stressed rats showed an enhanced number of cortical β-adrenergic sites without changes in their affinity. This effect was not present following concurrent administration with the antidepressant. In addition, a decreased percentage of T lymphocytes and a reduced delayed-type hypersensitivity reaction was also observed in stressed animals. Both responses were no longer evident when stressed rats were previously administered IMI. A possible link between behavioral, neurochemical, and immunologic alterations due to the stress regime is discussed.

Chronic variable-stress regime Imipramine Cortical β-adrenoceptor binding T lymphocytes
B lymphocytes Delayed-type hypersensitivity reaction Hemagglutinin titer

EXPOSURE to uncontrollable aversive experiences leads to a wide range of behavioral disturbances (17,24,34). Some of these changes resemble those reported in depressive patients. Although the role of stress in the etiology of depression is not completely understood, life event stress has been consistently reported to be a precipitating and predisposing factor of depression (6,9). Moreover, if it is of importance it is likely to play a role primarily in individuals with particular vulnerabilities. The fact that these stress-induced behavioral changes as well as the depressive syndrome are both reversed following repeated antidepressant treatment led to the use of these behavioral paradigms as animal models of depression (42). Among these models, the chronic variable-stress (CVS) paradigm is based upon the exposure to several uncontrollable and unpredictable aversive stressors for 2 or more weeks (26,33). Following this experience, stressed animals showed hypoactivity when confronted with a novel aversive stimulus in a novel environment (31), anhedonia (33), increased immobility in the forced swim test (FST) (12), enhanced escape deficit in a foot-shock experience (29), and reduced shock-induced aggression (44). Concomitant with these behavioral changes, an increase in the number of cortical β-adrenoceptors was reported to occur following the CVS experience (26). In addition, most of the behavioral abnormalities seen in chronically stressed rats are also observed in other models of depression (29) and similar neurochemical modifications to those seen after CVS have also been reported in another animal model of depression. Thus, it has been found that previously shocked rats that displayed later escape deficits in a shuttle-box task have an increased number of central β-adrenoceptors (25). Therefore, it seems possible to suggest that at least some of the behavioral disturbances observed in stressed rats could be functionally linked to an upregulation of central β-adrenoceptors.

Strong evidence now indicates that animals exposed to acute uncontrollable aversive experiences show alterations in the functioning of the immune system (15,16,18,20,27). Experience with uncontrollable stressors leads to a reduced plaque-forming cells response and antibody levels to a novel antigen

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(20,28,43), decreased cell proliferation in response to mitogen stimulation (18,21,27), and alterations of natural killer cell activity (15,27,28). Moreover, exposure to inescapable shock (IS), which has been frequently employed to induce experimental depression, increased the vulnerability to tumor growth and decreased the survival following tumor transplantation (36,41). Recent findings from this lab demonstrated that a CVS procedure of 2 weeks facilitated tumor growth (4). Furthermore, this facilitation was clearly reversed when rats were concurrently administered imipramine (IMI) (4). Therefore, it seems possible that depressive-like behaviors, which are induced by uncontrollable stressful experiences, are in fact related to tumor development. This view seems to be supported by clinical findings, which have suggested a possible association between depressive episodes and cancer (22).

Together, there seems to be a temporal correlation with regard to the impact of a long CVS treatment on the behavioral, neurochemical, and immunologic levels of functioning. However, there is no evidence concerning the consequences of a short CVS regime. It has been recently reported that daily exposure to variable stressors for only 1 week results in an enhanced inactive posture, a marker of depressive-like behavior (30), when stressed animals were confronted to long-duration IS (31). Therefore, and to study if the temporal correlation between behavioral, neurochemical, and immunologic changes could also be present following seven variable stressors, we assessed the effect of a 7-day stress regime on cortical β-adrenoceptor binding and on several immunologic parameters as the percentage of T and B lymphocytes and cellular and humoral immune response to heterologous antigens such as sheep red blood cells (SRBCs). In addition, the influence of IMI on the possible CVS effects on the parameters mentioned above was also evaluated.

METHOD

Animals

Adult, male Wistar rats (3 months old) weighing 250–350 g were used in these experiments. Rats were housed in wire cages (six rats per cage) in the experimental room for at least 7 days before the start of experiments. They were maintained at 22 ± 2°C under a 12 L : 12 D cycle beginning at 0700 h. Animals had continuous access to food and water.

CVS Procedure

Rats were submitted to CVS during 7 days as previously described (29). The stressors' schedule used was: day 1, 60 min of horizontal shake to high speed; day 2, 5-min swim in 4°C icewater; day 3, 1-min tail pinch; day 4, 2 h of restraint; day 5, idem day 1; day 6, idem day 2; and day 7, idem day 3. Stressors were always delivered throughout the lighting cycle from 1100–1400 h.

Immunization

SRBCs were washed three times in phosphate-buffered saline (PBS), pH = 7.2, and made up to an appropriate concentration for injection. Animals were immunized IP on day 1, with 1×10^9 cells in a volume of approximately 1 ml and IV on day 4 with 1×10^9 cells in a volume of 0.2 ml. This dosage was stated in accordance with preliminary studies that revealed that it led to a submaximal antibody titer and a positive delayed-type hypersensitivity reaction.

Determination of the Percentages of T and B Lymphocytes

Animals were bled by cardiac puncture under ether anesthesia and the blood collected into heparinized syringes. Peripheral blood lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation. The blood was diluted 1 : 1 with PBS and 6 ml were layered over 3 ml Ficoll-Hypaque and centrifuged at $400 \times g$ for 30 min at 18–20°C. Interface lymphocytes were removed and washed twice in PBS by centrifugation at $100 \times g$ for 10 min. The third washing was made with PBS and sodium azide (NaN_3) 10% diluted 1 : 100. The measurement of T cells is based upon surface markers known to associate with a different antibody, namely, OX19 monoclonal antibody directed against rat total T lymphocytes (Bioproducts for Science, Inc.) was used. In brief, $1–2 \times 10^6$ cells were incubated with 20 μl of a saturating concentration of monoclonal antibody (dilution 1 : 100) at 4°C for 30 min. After three washes with PBS and NaN_3, cells were incubated for 30 min at 4°C with fluorescein-isothiocyanate-labeled polyvalent rabbit antimouse immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO), washed three times, and the percentage of T lymphocytes assayed with an immunofluorescence microscope.

For the measurement of B cells, lymphocytes previously obtained as described above were incubated for 40 min at 4°C with fluorescein-isothiocyanate-labeled polyvalent rabbit antirat IgM and IgG (Zymed Lab.), washed three times, and the percentage of B cells determined with an immunofluorescence microscope.

Delayed-Type Hypersensitivity Reaction (DTH)

Animals previously submitted to the method of immunization described above were evaluated for this assay. Delayed cutaneous hypersensitivity was elicited 7 days following the first immunization. Rats were challenged with 1×10^8 SRBCs in 0.1 ml PBS under the right hind footpad; the left footpad received PBS alone. Reactions were assessed 24 h later by measuring the increase in dorsoventral thickness of the test over the control footpad using a caliper. All measurements were conducted by the same individual and results expressed as specific increases in footpad thickness.

$$\text{DTH (mm)} = (\Delta\text{SRBC footpad} - \Delta\text{control footpad}) \times 10.$$

Determination of Serum Antibody Titers

Rats were anesthetized with ether and blood was collected by cardiac puncture and allowed to clot. Blood samples were centrifuged at 1,000 rpm for 10 min, the serum was collected, and complement was inactivated at 56°C for 30 min. Hemagglutinin titers to SRBCs were estimated by serial dilutions of inactivated serum in PBS and a 1% SRBC solution in microtiter plates. The highest dilution at which aggregation of SRBCs was evident was considered to be the antibody titer and expressed in $\log_{10}$ units of the reciprocal of the antibody titer.

Binding Assay

β-Adrenergic binding studies were performed on a pool of frontal cortex tissue of two rats belonging to each experimental group. Each binding assay run contained tissue from the four experimental groups. Animals were sacrificed and the brain removed; the frontal cortex was quickly dissected according to Heffner et al. (14). The tissue was preserved at −20°C until the binding assay was performed by a conventional procedure (7). Tissues were homogenized with a Poli-

tron PT 10 (setting 6, 20 s) in 20 vol. ice-cold Tris buffer (0.05 M, pH = 8.0) and centrifuged at 39,000 × *g* for 20 min at 4°C. The resulting pellets were washed in the same volume of Tris buffer and resuspended in 19 vol. buffer. [^{3}H]Dihydroalprenolol (^{3}H-DHA, specific activity 61.6 Ci/mmol, from New England Nuclear Corp., Newton, MA) was used as radioligand for receptor binding assays. Experiments were performed in duplicate with 900 μl membrane suspension (750–950 μg protein) and ^{3}H-DHA (0.5–5 nM) incubated at 23°C for 18 min in a final volume of 930 μl. The incubation was ended by adding cold buffer to each tube and rapidly filtering the contents through Whatman GF/B filters (Whatman, Clifton, NJ) with a cell harvester (Brandel Harvester for Receptor Binding Assays, Biomedical and Development Laboratories, Inc.). The incubation tubes were rapidly washed twice with cold buffer and the filters were dried and transferred to vials to count the radioactivity in a solution containing PPO, Triton X-100, and toluene. Specific binding was defined as the difference in radioactivity in the absence and presence of 2.0 × 10^{-5} M propranolol. The dissociation constant (K_d) and maximum ^{3}H-DHA binding were determined from least square fits to Scatchard plots of the data. Proteins were assayed according to Lowry et al. (23).

Experimental Procedure

Rats were injected with IMI (Lab. Prest, Bs. As., Argentina) 10 mg/kg/day IP or 0.9% saline (SAL) 1 h before each stressful experience through the entire chronic stress regime. Control unstressed rats received SAL or IMI injections for the same period of time as stressed animals. Animals were always stressed between 1100 and 1400 h. Experimental groups consisted of: a) unstressed rats injected with SAL for 7 days; b) unstressed rats injected with IMI for 7 days; c) stressed animals injected with SAL 1 h before each stress; and d) stressed rats injected with IMI 1 h before each stress session.

One day after the last injection or stressful event, animals were sacrificed, the brain removed, and the frontal cortex quickly dissected for binding studies. Another group of rats were bled by cardiac puncture under ether anesthesia 24 h after the last injection or stressful session and the blood was collected into heparinized syringes. The percentages of T and B lymphocytes were determined as previously described. Another group of animals was submitted to the same experimental procedure and immunized with SRBCs on days 1 and 4 of the CVS procedure. One day after the last stressful stimulus or injection, rats were challenged with SRBCs under the footpad to evaluate the DTH reaction. These animals were bled by cardiac puncture without anticoagulant, and serum hemagglutinin titers to SRBCs were estimated on heat-inactivated samples.

Statistical Analysis

The data were analyzed with a two-way analysis of variance (ANOVA), followed by the least significant difference Fisher test set at 0.05. Values corresponding to the percentages of T and B lymphocytes were transformed to the arc sin $\sqrt{\%/100}$ and serum hemagglutinin titers were expressed in $\log_{10}$ units of the reciprocal of the antibody titer for statistical analysis.

RESULTS

Effect of the CVS Procedure and IMI Administration on Cortical β-Adrenoceptor Binding

As shown in Table 1 and Fig. 1, SAL-treated rats submitted to a series of unpredictable stressors have a significant increase in the number of β-adrenergic binding sites as compared to unstressed SAL-treated animals. Repeated IMI administration to unstressed rats induced a clear downregulation of β-adrenoceptors in the frontal cortex (Table 1). The concentration of β-adrenergic sites after concurrent administration of the CVS regime with IMI was not different from that observed in control rats. In addition, the number of cortical β-adrenoceptors of rats that experienced the associated treatment with CVS and IMI was significantly different from the values observed after CVS alone. No difference in the apparent affinity (K_d) was observed between the different experimental groups.

TABLE 1

EFFECT OF CVS REGIME AND IMI ADMINISTRATION ON CORTICAL β-ADRENOCEPTOR BINDING

Treatment	No. of Assays	B_{max} (fmol/mg protein)	K_d (nM)
Saline	6	135.0 ± 8.24	4.89 ± 0.89
Imipramine	6	96.5 ± 8.90*	3.51 ± 0.39
CVS	6	169.9 ± 10.00*	6.06 ± 1.60
IMI + CVS	6	114.1 ± 16.10$^+$	5.01 ± 0.96

Data are means ± SEM of the number of assays conducted on homogenates from a pool of frontal cortex tissue of two rats for each assay. Each assay run contained tissue from saline-, IMI-, CVS-, and IMI + CVS-treated rats.

*Significantly different from the saline group, $p < 0.05$ (Fisher test).

$^+$Significantly different from the CVS group, $p < 0.05$.

Effect of the CVS Regime and IMI Administration on Immunologic Parameters

Exposure to the CVS regime resulted in an immunosuppressive effect, as evidenced by the reduced percentage of T lymphocytes (Fig. 2) and decreased DTH reaction (Fig. 3). This response was reversed when stressed rats were concurrently administered IMI. The ANOVA for T lymphocytes data showed a significant treatment effect (CVS), $F(1, 21) = 10.6$, $p < 0.05$, a significant IMI effect, $F(1, 21) = 19.2$, $p < 0.001$, and also a significant interaction, $F(1, 21) = 16.3$, $p < 0.001$. Fisher post-hoc analysis showed a clear reduction in the percentage of T lymphocytes in CVS-treated rats as compared to unstressed SAL-treated rats. This effect was no longer evident following concurrent administration of IMI. No difference was observed between unstressed rats treated with either SAL or IMI. The ANOVA conducted on the data from DTH experiments revealed a significant treatment effect (CVS), $F(1, 20) = 7.9$, $p < 0.01$, and a significant (CVS-IMI) interaction, $F(1, 21) = 6.1$, $p < 0.05$. Individual comparisons (Fisher post-hoc analysis) revealed a significant decrease of the DTH reaction in chronically stressed animals as compared to unstressed SAL-treated rats. This effect was not observed after concurrent treatment with IMI and CVS. DTH reactions were not altered in unstressed rats administered either SAL or IMI. Also, exposure to the CVS regime did not modify the percentage of B lymphocytes (Fig. 4) or the hemagglutinin titers against SRBCs (Fig. 5).

DISCUSSION

The present report showed that rats daily exposed to a CVS regime for 7 days have an upregulation of cortical β-adreno-

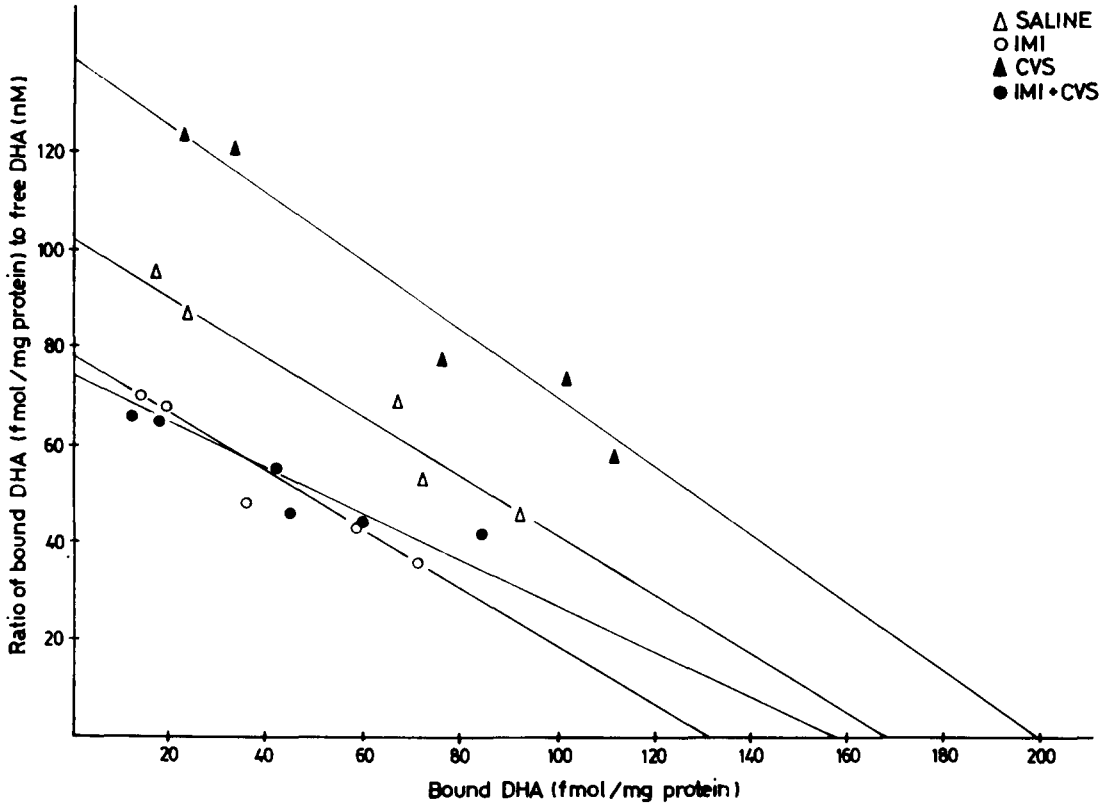

FIG. 1. Scatchard plots of data from representative assays showing β-adrenoceptor binding performed in the frontal cortex. Each assay contained tissue from a pool of frontal cortex tissue of two rats belonging to each experimental group.

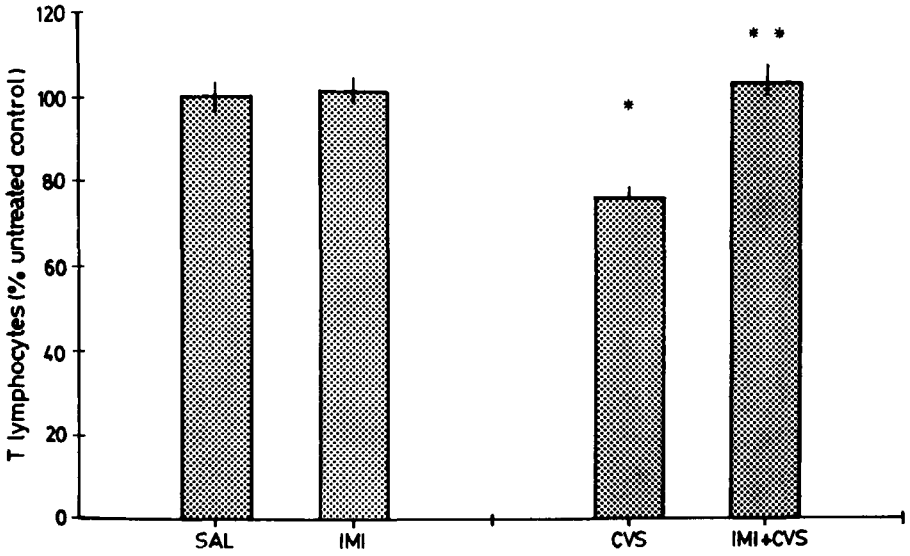

FIG. 2. Effect of chronic variable-stress (CVS) regime and imipramine (IMI) administration on the percentage of peripheral blood T lymphocytes. The percentage of T lymphocytes was determined 24 h after the last injection or stressful session. Values represent means ± SEM (n = 6 for each group). *Significantly different from the saline group, $p < 0.05$ (Fisher test). **Significantly different from the CVS group, $p < 0.05$.

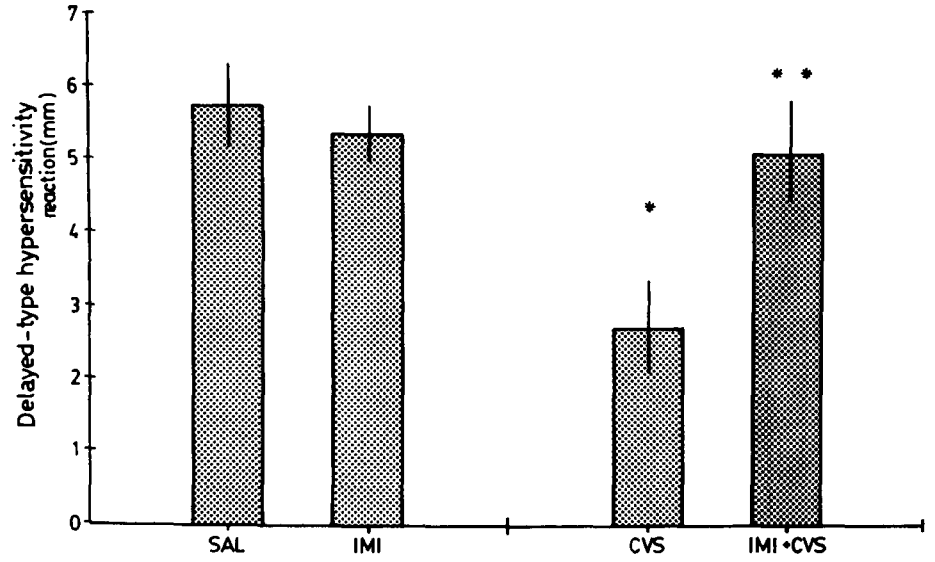

FIG. 3. Effect of chronic variable-stress (CVS) regime and imipramine (IMI) administration on delayed-type hypersensitivity (DTH) reaction. Twenty-four hours after the last stressful stimulus or injection, animals previously immunized with sheep red blood cells (SRBCs) were challenged with 1×10^8 SRBCs in 0.1 ml phosphate-buffered saline under the footpad. To evaluate the DTH reaction, the increase in thickness was measured with a Vernier caliper. Values represent means ± SEM ($n = 6$ for each group). *Significantly different from the saline group, $p < 0.05$ (Fisher test). **Significantly different from the CVS group, $p < 0.05$.

ceptors. A similar change has also been reported following a longer CVS procedure of 2 weeks (26). Moreover, binding studies showed that rats previously exposed to IS that showed later escape deficiency, another animal model of depression, have an enhanced density of central β-adrenergic sites (25). Consistent with the view that stressor uncontrollability is a critical factor to induce escape deficit, it has also been documented in animals previously submitted to the CVS of 2 weeks (29). Therefore, it seems reasonable to propose that this behavioral response to uncontrollable stressors could be, in fact, functionally related to an upregulation of central β-adrenoceptors. On the other hand, several authors observed a decrease

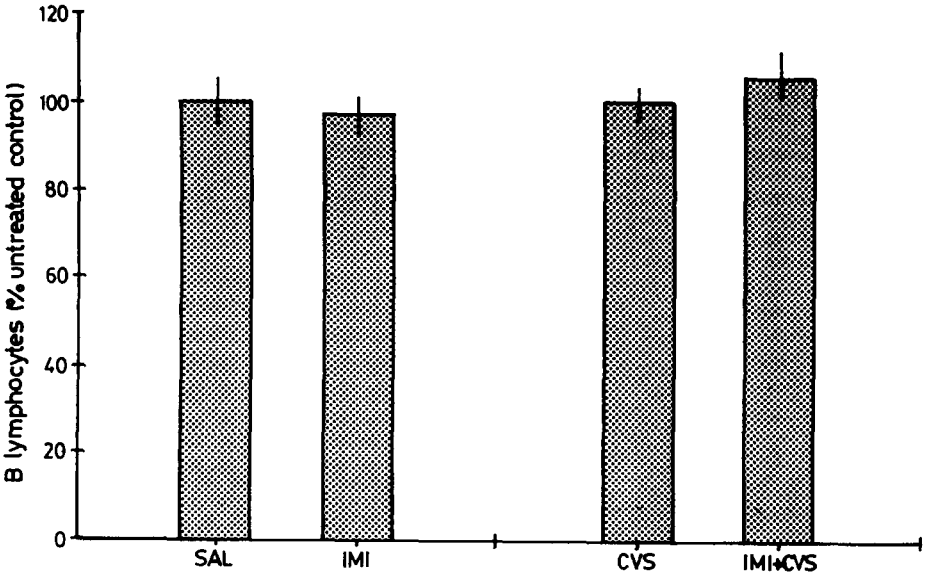

FIG. 4. Effect of chronic variable-stress (CVS) regime and imipramine (IMI) administration on the percentage of peripheral blood B lymphocytes. The % of B lymphocytes was determined 24 h after the last injection or stress session. Values represent means ± SEM ($n = 6$ for each group).

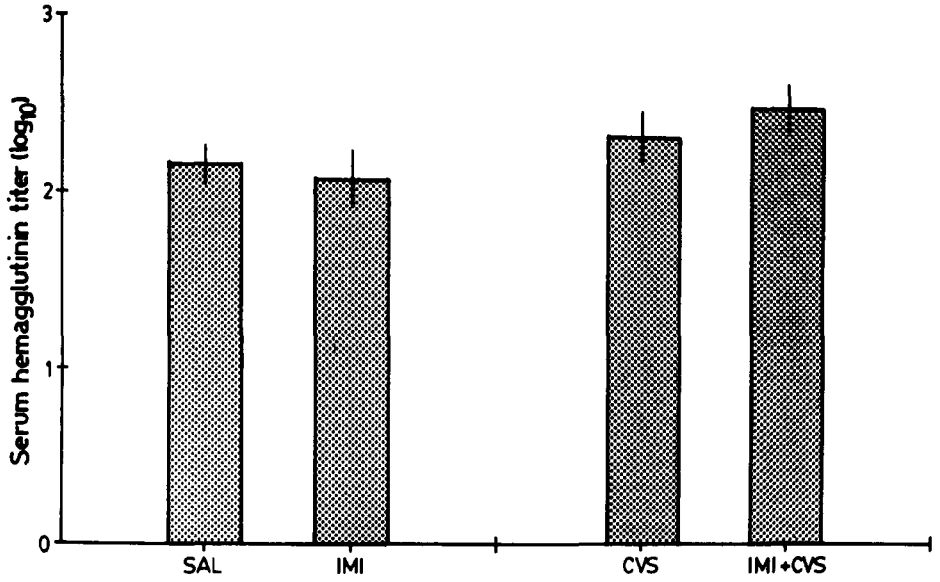

FIG. 5. Effect of chronic variable-stress (CVS) regime and imipramine (IMI) administration on serum hemagglutinin titer. Circulating antibodies against sheep red blood cells (SRBCs) were estimated on heat-inactivated samples, considering the highest dilution at which aggregation of SRBCs was evident. Antibody titer was expressed in $\log_{10}$ units of the reciprocal of the antibody titer. Values represent means $\log_{10} \pm$ SEM ($n = 6$ for each group).

in β-adrenergic receptor density and in norepinephrine-stimulated adenylate cyclase activity in the rat cortex following chronic repeated stress (32,37-39). Although the involvement of a considerable number of neurotransmitter pathways is likely in the physiological and behavioral responses to acute and chronic repeated stress, there is reason to believe that noradrenergic pathways may play one of the more important roles (1,2,8,10,13). In this line, Stone and Platt reported that the reduction in the physiological effects of stress, that is, the formation of gastric ulcers and change in eating behavior, was positively related with a reduction in β-adrenergic receptor density (39). This could represent, as hypothesized by Stone, an adaptation that results in the organism's resistance to stress after exposure to the same stressful situation.

Also, it is widely accepted that one of the most frequently adaptive changes on monoamine sites observed following continuous antidepressant treatment is a subsensitivity of adenylate cyclase to noradrenaline and isoprenaline and a downregulation of brain β-adrenoceptors (3,5,40). In accordance with these observations, repeated administration with IMI during 7 days clearly reduced the number of cortical β-adrenergic sites. The fact that the CVS procedure failed to induce both behavioral and neurochemical modifications following repeated IMI administration supports the notion of a functional association between the behavioral response to stress and central β-adrenergic sites. Thus, an increased behavioral inhibition could be facilitated when an enhanced density of these catecholaminergic receptors are induced. In support of this assumption, we recently described that exposure to seven daily variable stressors, similar to the CVS procedure employed in the present study, caused a higher frequency of inactive behaviors in response to long-duration shocks (31) and an increase in shuttle-box escape deficits (29).

Besides behavioral and neurochemical changes, exposure to uncontrollable stressors has been described to disrupt the normal functioning of the immune system. In fact, a great deal of evidence indicates a reduced immunologic response after exposure to different types of physical and psychological stressors (15,16,18,20,21,27). In addition, uncontrollable stressful experiences seem to facilitate tumor growth and reduce the chance of survival in implanted stressed animals (36,41). Our findings agree with these reports because administration of CVS induced a clear suppression of cellular immunity with respect to the percentage of T lymphocytes and their ability to respond in the DTH reaction. Interestingly, this immunosuppression was reversed when stressed animals were concurrently administered IMI. It should be stated that this reversing effect occurred when the behavioral changes observed in stressed rats were also normalized to control values after IMI treatment. On the contrary, the CVS regime has no effect on the humoral response, as evidenced by no change in either the percentage of B lymphocytes or the hemagglutinin titer against SRBCs. These data coincide with other reports that demonstrate no effect of IS (another experimental model of depression) on the primary humoral immune response (16). Meanwhile, other authors indicate the suppression of specific antibody production by IS (20). Given that contradictory results are obtained on humoral immune response, further studies are required before a final conclusion can be formed. Our data also show that CVS-induced immunosuppression was no longer present when chronically stressed rats were concurrently administered IMI. In addition, a comparable reversal effect on the facilitation of tumor growth produced by CVS procedure was observed following concurrent IMI administration (4). Thus, there seems to be a temporal coincidence between the alterations on the neurochemical, behavioral, and immunologic systems following different CVS procedures. In addition, previous data reported that similar modifications to those observed in chronically stressed rats are also present in "helpless" rats, another animal model of depression. Hence, it

may be possible to speculate that these findings as well as those previously documented suggest a probable link between the disturbances at different systems produced by uncontrollable stressors. Further, the fact that administration of an antidepressant drug such as IMI can normalize CVS-induced changes other than behavioral ones may lead to the notion that these psychopharmacological agents could possess therapeutic potential in their ability to reverse alterations observed in the functioning of the immune system in depressive illness. As there have been other neurotransmitter pathways found to be altered by different stress paradigms and IMI treatment (11,19), besides the noradrenergic (3,40), the failure of CVS to induce behavioral and immunologic effects after IMI could be a complex phenomenon involving several mechanisms. Further studies are necessary to understand the changes that undergo with CVS and/or IMI and the importance of these changes for the pathophysiology of depression.

ACKNOWLEDGEMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and from CONICOR, Córdoba, Argentina. The authors thank Dr. S. Gruttadauria for kindly providing SRBCs. Also, the technical assistance of Servicio Nacional de Chagas is gratefully acknowledged. A.M.B. is the recipient of a Fellowship from CONICOR, Córdoba, Argentina.

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