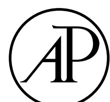
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Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth

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Abstract

The present study analyzed the effects of physical and psychological stressors on behavior, immune function, and serum corticosterone in mice. Adult mice were submitted once daily, for 6 days to one of the following conditions: escapable (ES) or inescapable (IS) footshocks (0.2 mA) signaled by a tone cue or to a psychological stressor (PS) generated through the use of a communication box; in this box, mice received no footshock but were exposed to responses delivered by IS mice. Results showed that IS and PS: (1) decreased locomotor activity observed in an open-field; (2) decreased number of entries into the open arms and decreased time spent in the exploration of the open arms of the plus-maze; (3) decreased macrophage spreading and phagocytosis; (4) increased macrophage H_2O_2 release; and (5) increased growth of the ascitic form of Ehrlich tumor. Behavioral and/or immunological changes were not observed after ES; this absence of effects, however, might not be attributed solely to footshock controllability since mice of groups ES and IS differed with respect to the psychological setting used and the amount of shock they received. An increase of serum corticosterone concentrations was also observed in the stressed mice of all groups; this increment was higher in animals of group IS. These data provide evidence that inescapable footshock and psychological stressors alter, at the same time and in mice, stress levels, macrophage activity, and Ehrlich tumor growth. They also show that ES and PS induced similarly elevated serum corticosterone concentrations, but significantly differ in the immunological and behavioral outcomes they produced in mice. These findings suggest that another factor besides HPA axis activation might be responsible for behavioral and immunological consequences of IS and PS in mice. It is proposed that the final neural link between behavioral and immunological changes observed after physical and psychological stressors might involve catecholaminergic systems within the central nervous system and/or sympathetic autonomic nerve fibers and also opioid peptides.
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Keywords: Inescapable footshock; Escapable footshock; Psychological stress; Macrophage activity; Phagocytosis; Ehrlich tumor; Corticosterone; Neuroimmunomodulation; Open-field; Plus-maze; Stressors

1. Introduction

An increasing body of evidences shows the relevant relationships among stressors and the occurrence of variations in immune function (Ader, Felten, & Cohen, 1991; Besedovsky & Del Rey, 1996; Miller, 1998). The relationship of major depression and naturally occurring stressors to immunological parameters was recently evaluated in humans through the use of a meta-analysis. Thus, a fixed-effect analysis showed that both conditions are associated with (1) an overall leukocytosis, (2) mild reductions in absolute NK-cell counts and relative T-cell

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proportions, (3) marginal increases in $CD4^+/CD8^+$ ratios, and (4) moderate decreases in T-cell and NK-cell function (Zorrilla et al., 2001). Changes in cell-mediated immune function and susceptibility to cancer were also reported in persons undergoing distressing life experiences (Cohen & Herbert, 1996; Irwin et al., 1990). Accordingly, a variety of stressors have been found to alter immune responses in experimental animals. Footshock increased susceptibility to herpes simplex virus in mice (Kusnecov et al., 1992); sound stress suppressed the T- and B-cell mediated response in rats (Sobrian et al., 1997); tail shock decreased the intensity of anaphylactic shock in rats (Maric et al., 1991); inescapable footshock increased total bronchoalveolar cell count in OVA-sensitized rats (Portela, Massoco, Lima, & Palermo-Neto, 2001). The effects of stress, however, depend on the type of stressor, its duration and frequency, the temporal relationship between stress and the immunological stimulus and, among many other variables, the subjects' ability to control or escape from stressors.

Alterations of immune function are considered to be more relevant when resulting in significant pathological, microbiological, and/or clinical symptoms. Ehrlich tumor cells were reported to elicit a strong host immune response (Pessina, Bambilla, & Mocarolli, 1980; Segura, Barbero, & Márquez, 1997) a fact that, together with other properties, makes this tumor an interesting model for the analysis of the effects of drugs and/or external environmental events on tumor growth. In addition, effective immune function against tumors requires cooperation between macrophages, T- and B-lymphocytes. Therefore, suppression of one of these components may compromise immunocompetence, thus changing tumor growth. The purpose of the present study was to analyze the effects of physical and psychological stressors applied to adult mice on macrophage activity and Ehrlich tumor growth. Behavioral measurements indicative of stress levels and serum corticosterone concentrations were also measured in stressed mice. Studies conducted in the field of psychoneuroimmunology are showing that susceptibility to and recovery from infection, allergic reactions, tumor cell inoculation, and autoimmune disorders are strongly influenced by nervous and/or endocrine systems activity (Besedovsky & Del Rey, 1996; Dantzer & Kelly, 1989; Dunn & Berridge, 1990).

2. Material and methods

2.1. Animals

One hundred and twenty genetically similar Swiss mice from our own colony, weighing 25–35 g and about 60 days of age, were used. The animals were housed in controlled temperature ($22 \pm 2\,°C$), humidity (45–65%), and artificially lighted rooms on a 12 h light–12 h dark cycle (lights on at 7:00 a.m.) with free access to rodent chow and water. The experiments were performed in a different room with the same temperature as the animal colony to which the animals were transferred and maintained in their home cages 10 days before the experiments. Animals were housed and used in accordance with the guidelines of the Bioethical Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, which are similar to those of the National Research Council, USA.

2.2. Group formation

Four experiments were conducted in accordance to GLP protocols and quality assurance methods. In each of them, mice were randomly and equally divided into four groups: one control, non-stressed (C), and three experimental stressed groups (ES, IS, and PS). Animals of group ES received escapable footshock (ES), whereas those of group IS were exposed to inescapable footshock (IS). Mice of group PS were submitted to a psychological stressor. In the first two experiments, 5 mice were used within each C, ES, IS, and PS groups whereas 10-mice/group were used in experiments 3 and 4. Data were recorded by investigators that were blinded to the experimental group conditions.

2.3. Stress induction

Mice from groups ES and IS were individually placed into a shuttle box (model E 10-16 Mouse ShuttleCage, Coulbourn Instruments, Allentown, PA, USA) housed inside a sound attenuated chamber. In this device, they received, during a 30-min session, 30 ES or IS (0.2 mA scrambled shocks of 5 s) through an electrified grid floor. The following schedule was used for animals of group ES: (1) a discriminative signal (S) (6-dB tone cue of 2 s) preceded the ES; (2) each trial (tone and shock) was repeated 30×, with a random interval between them (from 5 to 50 s); (3) the set of shocks (30 trials) was repeated daily for six consecutive days (experimental day—ED1–ED6); (4) a response during the warning (S) canceled the shock delivery and terminated the S. Every shock was avoided when the responses occurred during both S and grid shocks. The animals of group IS were submitted once daily and for six consecutive days (ED1–ED6) to the same schedule of shock liberation but were unable to avoid the footshock. The mice of the control group (nonshocked, C) were similarly placed in the shuttle box for the same number and period of time (ED1–ED6), but received no aversive stimulation.

During the six footshock sets delivered to animals of group IS, another group of mice (group PS) were individually placed in a communication compartment positioned beside the shuttle cage (5 cm in distance) without

physical contact with those of group IS. In this compartment these mice received no shock but were allowed to observe every day and for six days (ED1–ED6) the emotional reactions of mice of group IS that included: ultrasonic vocalizations, jumping, piloerection, and unusual odors. The PS generated in this situation was reported to induce high levels of stress (Iimori et al., 1982).

Prior to the experiment, all animals were placed in their respective test compartments (shuttle box or communication box) without footshock for 1 h, every day for six days. This was regarded as a habituation phase. During the experiments (ED1–ED6), a 3-min adaptation time was provided in the shuttle box or in the communication box. IS and ES sessions in the operant chamber were controlled by a L2T2 System software (Coulbourn Instruments, Allentown, PA, USA) installed on an IBM-compatible computer placed in an adjacent room.

2.4. Behavioral studies

Locomotor activity in an open-field and the free exploration of a plus-maze apparatus were measured in 10 mice of each of the C, ES, IS, and PS groups. Animals provided from the first two experiments were used, being open-field measures made immediately before plus-maze evaluations on ED7, 24 h after the last experimental session. Previous results from us and others have shown that testing animals in an open-field or in a hole board immediately before the plus-maze test significantly elevates animal activity in this apparatus, i.e., the total number of open and closed arm entries (Palermo-Neto & Guimarães, 2000; Pellow, Chopin, File, & Briley, 1985), with a consequent easier analysis of plus-maze data.

To minimize the influences of possible circadian changes in open-field and plus-maze behaviors, control and experimental animals were alternated for observations that were made at the same time of the day (between 8:30 and 12:00 noon). The experimental devices used were washed with 5% alcohol–water solution before placing the animals.

The open-field employed has been described in detail elsewhere (Palermo-Neto, Massoco, & Fávare, 2001). Briefly, it consists of a round wooden arena (45 cm of diameter, 20 cm high walls) divided into three zones: central, intermediate, and peripheral. For the observations, each mouse was individually placed in the center of the apparatus, being observed for 5 min for total locomotor activity (covered distance in cm) and for locomotor activity performed in each of the three open-field zones using a video camera mounted vertically above the arena. Data were analyzed with an Ethovision System software (Noldus Information Technology, Leesburg, VA, USA) installed on an IBM-compatible computer placed in an adjacent room.

The plus-maze device used was made of wood and had two open arms (23.5 × 8 cm) and two closed arms of the same size with 20-cm high walls. The apparatus used was based on that described by Pellow et al. (1985); it was configured in such a way that the similar arms were placed opposite each other. Each mouse was placed in the central square of the plus-maze ($8\,cm^2$) being observed remotely for 5 min using a video camera mounted vertically above the apparatus for the number of entries into each type of arm (all four paws defining an entry) and the time spent in open arm and closed arm exploration. The measures that reflect anxiety levels in this test are the percentage of entries into open arms versus closed arms and the percentage of time spent in the open arms versus closed arms (Pellow et al., 1985). Data were analyzed as described above for the open-field.

2.5. Immunological determinations

2.5.1. Macrophage activity

Animals provided from experiment number 3 were used for analysis of macrophage spreading, phagocytosis, and H_2O_2 spontaneous and phorbol myristate-acetate (PMA)-induced release. Determinations were made in sequence.

The methods used to study macrophage spreading and phagocytosis were based on that described by Rabinovitch and De Stefano (1973) with modifications introduced by Passeti (1993). Briefly, 2 mg of an attenuated suspension of *Mycobacterium bovis* (Onco-BCG, Butantan Institute) was intraperitoneally injected in mice from all groups 24 h after the last experimental session (ED7). Seven days after inoculation (ED14), all animals were sacrificed under deep anesthesia and the peritoneal macrophages were individually obtained by a lavage with a phosphate-buffered saline ($pH = 7.2$–7.4) solution (PBS). The peritoneal lavage fluid was placed in plastic tubes (10 mL), in an ice bath. The number and viability of the peritoneal cells obtained were evaluated visually by the trypan blue staining exclusion method in a Neubauer Chamber. This procedure yielded 2×10^6 peritoneal cells/mL; more than 90% were macrophages as judged by morphology staining for nonspecific esterase.

To study macrophage spreading, 200 μL of cell suspensions derived from each of the 10 mice of all groups were prepared in duplicate in glass slide monolayers (13 × 13 mm) that were kept in multiwell (24 × 13 mm) tissue culture plates (Costar No. 3424, New York, NY, USA) for 20 min. The wells were washed several times with cold (4 °C) PBS; 1.0 mL of RPMI 1640 medium plus 10% fetal calf serum was afterwards added to each well. The plates were then covered and incubated at 37 °C in 5% CO_2–95% air for 60 min. After incubation, the wells were rinsed with cold (4 °C) PBS and the adherent cells obtained were fixed with 0.5% glutaraldehyde for 10 min. These cells were then counted with a phase contrast microscope (Nikon, New York, NY, USA) at 40× magnification. Thus, with the aid of an

ocular grid, 200 macrophages were scored as either round or spread. An index of macrophage spreading (SI) was then calculated for each monolayer in a well as follows: SI, number of spreading macrophages × 100/200 adherent cells, i.e., SI = percentage of spreading macrophages.

Macrophage phagocytosis was performed using the same method described above, except that 1 mg of a zymozan solution was added to each well 1 h before the 60-min incubation period (37 °C). Using the same microscope, objective and methods previously described, a phagocytosis index (PI) was then obtained as follows: PI, number of macrophages with phagocytosis activity × 100/200 adherent cells counted, i.e., PI = percentage of macrophages with phagocytized zymozan particles.

Since two glass slide monolayers were obtained from each mouse within control and experimental groups, the mean SI and PI values were considered to express the results of macrophage activity.

Spontaneous and PMA-induced H_2O_2 releases were measured through the method described by Pike and Mizel (1981) modified by Russo, Teixeira, Marcondes, and Barbuto (1989). The peritoneal macrophages obtained above for animals of all groups were used. Thus, 2.0×10^6 cells/mL derived from each mouse lavage fluid were centrifuged for 10 min and resuspended afterwards in 1 mL of phenol red solution (PRS, containing 140 nM NaCl, 10 nM potassium-phosphate buffer, pH 7.0, 0.5 nM dextrose, 0.28 nM phenol red, and 8.5 U/mL HRPO) for H_2O_2 detection. One hundred microliters of the cell suspension was plated onto each well of the 96-well flat-bottomed tissue culture plate (Costar, New York, NY, USA) and incubated in a humidified chamber at 37 °C in 5% CO_2–95% air for 1 h. Subsequently, the wells containing PRS received 10 μL of 1 N NaOH to stop the reaction. Hydrogen peroxide-dependent phenol red oxidation was measured spectrophotometrically at 620 nm, in a Titertek Multiscan reader (Labsystems, Franklin, MA, USA). The same procedure was employed to determine H_2O_2 release after stimulation with PMA; for that, 10 μL of 10 ng/mL PMA was added in each well before incubation. The concentrations of H_2O_2 were calculated from absorbance measurements, as described by Pike and Mizel (1981). Since spontaneous and PMA-induced H_2O_2 production experiments were obtained four times for each mouse in each group, the mean value of the four measurements was used for estimation of H_2O_2 concentrations.

2.5.2. Ehrlich tumor growth

Ten mice from each control and each experimental groups provided from experiment 4 were used to analyze the ascitic form of Ehrlich tumor growth, through the method proposed by Dagli, Soma, Guerra, and Saldiva (1992). Approximately 3 mL of ascitic fluid was collected from a donor mouse and centrifuged at 200*g* for 3 min. The cells in the pellet were washed three times in PBS solution, resuspended in the same solution, and counted in order to standardize a concentration of 5×10^6 viable tumor cells/mL (using the trypan blue viability test). On ED7, i.e., 24 h after the end of the experimental protocols, mice of all groups were inoculated intraperitoneally with 5.0×10^6 Ehrlich tumor cells, being weighed daily for the next 10 days. On ED16, animals were sacrificed under deep anesthesia, the peritoneal fluid was collected and measured, and the number of tumor cells/mL was counted in a Neubauer Chamber.

2.5.3. Corticosterone determination

The same animals used for behavioral evaluations, i.e., provided from replications 1 and 2, were used for this procedure. For serum corticosterone determination, the mice of all groups were sacrificed immediately after the end of the plus-maze test (ED7). Corticosterone was determined using commercial kits (Coat-A-count). Serum samples were assayed directly without chemical extraction or purification. The limit of corticosterone detection was 16.45 nmol/L and the intra-assay and inter-assay variation coefficients were 4.2% and 16.1%, respectively. In order to decrease data variability mice were handled daily for habituation to the experimental conditions of blood collection that was made at the same time of the day (between 9:00 and 12:00 noon).

2.5.4. Statistical analysis

Serum corticosterone concentration and locomotor activity data obtained on replications 1 and 2, were in the same direction and not statistically different from each other; thus, results of both replications were pooled for statistical purposes and presentation. Bartlett's test (Johnson & Leone, 1974) showed that data concerning locomotor activity, body weight, plus-maze, macrophage spreading, and phagocytosis as well as those of the Ehrlich tumor growth were parametric ($P < .05$). Thus, they were analyzed by one-way analysis of variance followed by the Tukey–Kramer test for comparison of cell means. The body weights were analyzed by two-way ANOVA followed by Student's *t* test. Corticosterone and H_2O_2 data were nonparametric being analyzed by Kruskal–Wallis (KW) Analysis of Variance followed by the Dunnett's test for multiple comparisons. Pearson's correlation coefficients were also employed. The StatPac Statistic Analysis Package was used throughout with the level of significance set at $P < .05$ for all comparisons.

3. Results

3.1. Behavioral studies

Figs. 1A–D show that both IS and PS decreased the total locomotor activity (covered distance in cm) of mice

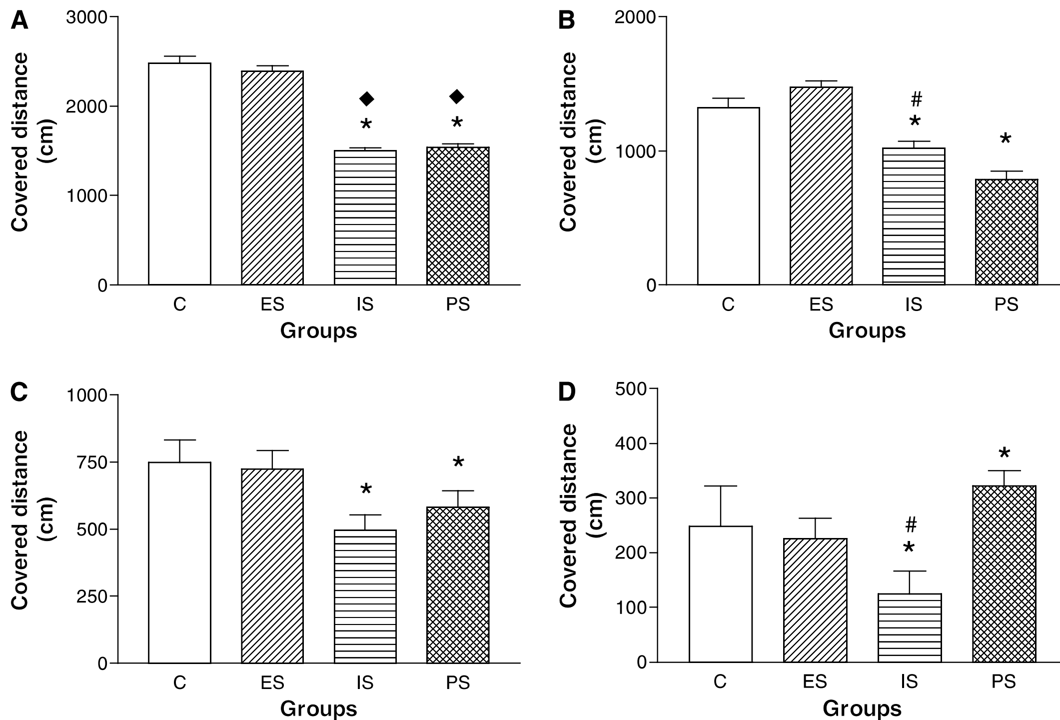

Fig. 1. Effects of escapable (ES) or inescapable (IS) footshocks as well as of a psychological stress (PS) on total locomotor activity (A) and locomotor activities performed in the peripheral (B), intermediary (C), and central zones (D) of an open-field. Data are means ± SEM of 10 mice. ∗, ◆, and # $P < .05$ compared to data of groups C, ES, and PS, respectively. ANOVA followed by Tukey–Kramer's test.

observed in an open-field ($F_{3,36} = 6.84$; $P < .05$) as well as the activities recorded in the peripheral ($F_{3,36} = 6.28$; $P < .05$), intermediary ($F_{3,36} = 8.30$; $P < .01$), and central ($F_{3,36} = 10.3$; $P < .01$) zones of the apparatus, in relation to data of group C. However, as can be seen in Figs. 1A–D, differences were not found between the data of groups ES and C, suggesting that the ES did not change locomotor activity in the open-field. Further analysis showed that data on locomotor activity in mice of the IS and PS groups were significantly lower ($P < .05$) than those recorded in the ES group (Fig. 1A). Finally, locomotion of animals of group IS on open-field central zone was smaller ($P < .05$) than that measured in group PS (Fig. 1D). Opposite data were measured for group IS on open-field peripheral zone, i.e., animals of group IS had higher ($P < .05$) levels of activity in open-field peripheral zone than those of group PS (Fig. 1B).

Plus-maze data are depicted in Figs. 2A–D. As can be seen in Figs. 2A–B, both the percentage of entries in the plus-maze open arms ($F_{3,36} = 6.58$; $P < .05$) and the time spent in open arm exploration ($F_{3,36} = 9.42$; $P < .05$) were lower in mice submitted to IS or to PS in relation to control groups (group C). Conversely, and also in relation to control data (group C), the percentage of entries into the closed arms and the time spent in plus-maze closed arm exploration (Figs. 2C–D) were higher in mice of groups IS and PS ($F_{3,36} = 9.91$; $P < .05$ and $F_{3,30} = 8.00$; $P < .05$, respectively). However, both measures indicative of open arm exploration were lower ($P < .05$) in mice of group IS, in relation to those of group PS. Furthermore, no differences were found between the plus-maze results of groups C and ES, i.e., ES induced no changes in plus-maze arms exploration (Figs. 2A–D).

3.2. Immunological data

3.2.1. Macrophage activity

No differences were found among control and experimental groups concerning the total number of leucocytes found in mouse peritoneal lavage fluid (data not shown). Nevertheless, differences were found in macrophage activity after Onco-BCG activation (Table 1). Thus, IS and PS decreased both macrophage spreading capacity ($F_{3,36} = 10.25$; $P < .05$), and phagocytosis ($F_{3,36} = 8.07$; $P < .05$) compared to control mice (group C). Again, no differences were found between the data of groups C and ES and between those of groups IS and PS. Thus, the ES did not change macrophage spreading and phagocytosis, whereas the IS and PS employed induced similar effects

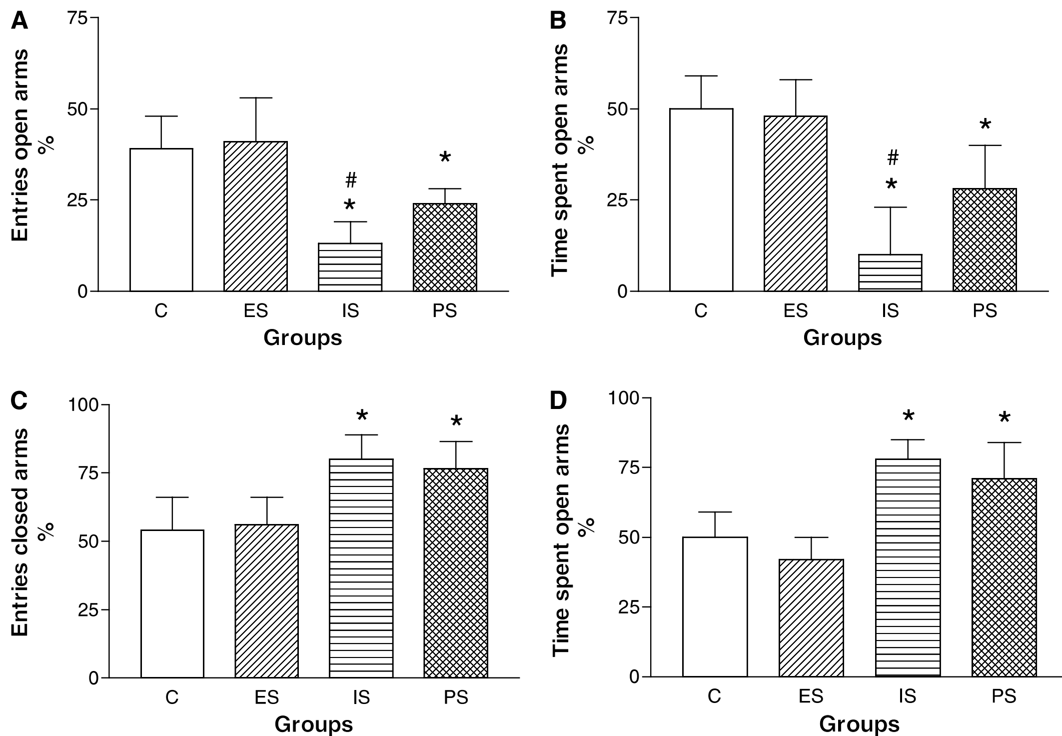

Fig. 2. Effects of escapable (ES) or inescapable (IS) footshocks as well as of a psychological stress (PS) on percentage of entries into plus-maze open arms (A), percentage of time spent in open arm exploration (B), percentage of entries into closed arms (C) and percentage of time spent in closed arm exploration (D). Data are means ± SEM of 10 mice. * and # $P < .05$ compared to data of groups C and PS, respectively; ANOVA followed by Tukey–Kramer's test.

Table 1
Effects of escapable (ES) or inescapable (IS) footshock as well as of a psychological stress (PS) on macrophage activity of mice

Groups[a, b]	Macrophage (%)		Macrophage H_2O_2 (nmol)	
	Spreading	Phagocytosis	Spontaneously	PMA-induced
C	59 ± 10	78 ± 12	5.75 (4.4–7.1)	11.65 (6.5–13.7)
ES	56 ± 13	77 ± 14	4.88 (2.5–8.0)	10.82 (7.8–8.9)
IS	42 ± 8*	58 ± 10*	13.02 (7.4–17.4)**	18.80 (6.4–25.4)**
OS	43 ± 9*	62 ± 12*	13.56 (4.2–15.4)**	17.46 (9.7–4.7)**

Macrophage activation was induced by an intraperitoneal injection of 2.0 mg of an attenuated suspension of *Mycobacterium bovis* (Onco-BCG).

[a] Data on macrophage spreading and phagocytosis are means ± SEM; those of H_2O_2 are median (range).

[b] 10 mice were used per group.

* $P < .05$ (ANOVA and Tukey–Kramer's test).

** $P < .05$ (Kruskal–Wallis and Dunnett's test).

on macrophage activity. As can be seen in Table 1, IS and PS increased H_2O_2 spontaneously ($KW_{3,36} = 12.67$) and PMA-induced ($KW_{3,36} = 15.37$) release in relation to control data (group C). Further analysis showed that no differences were found between the data of groups ES and C nor between the data of groups IS and PS on H_2O_2 liberation.

3.2.2. *Ehrlich tumor growth*

Fig. 3 depicts the data obtained for Ehrlich tumor growth. Thus, IS and PS increased the ascitic form of the Ehrlich tumor growth in relation to control (group C) data, as evidenced not only by the increased number of tumor cells found in mice of those groups ($F_{3,36} = 8.55$; $P < .05$) but also by the higher volumes of ascitic fluid

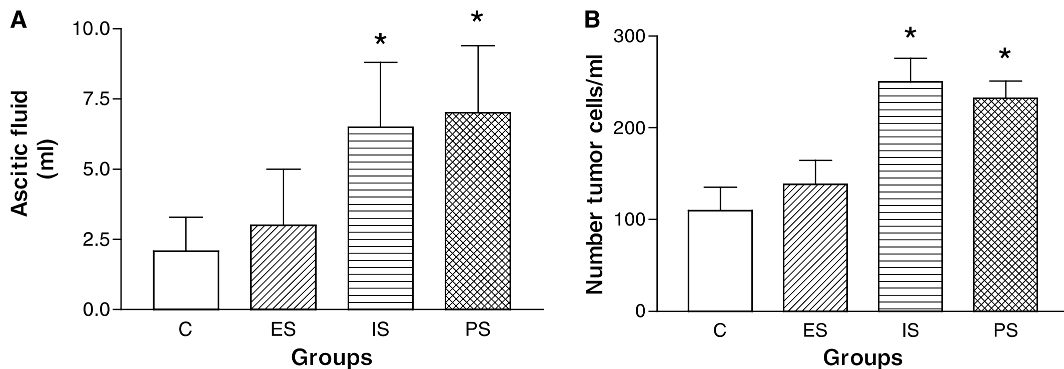

Fig. 3. Effects of escapable (ES) or inescapable (IS) footshocks as well as of a psychological stress (PS) on Ehrlich tumor growth. Data are means ± SEM of the volumes of ascitic fluid (A) and of the number of Ehrlich tumor cells/mL of ascitic fluid (B). Data are means ± SEM of 10 mice. * $P < .05$ compared to control data (C); ANOVA followed by Tukey–Kramer's test.

detected in those animals ($F_{3,36} = 6.01$; $P < .05$). No differences were found between the data of groups C and ES and between those of groups IS and PS on Ehrlich tumor growth. Other data showed that mice of groups IS and PS were heavier ($F_{3,9,37} = 15.16$; $P < .05$) than those of groups ES and C from ED15 to ED17 (data not shown).

3.2.3. Corticosterone serum data

As shown in Fig. 4 the physical (IS and ES) and psychological (PS) stressors used increased ($KW_{3,36} = 7.83$; $P < .05$) the serum concentrations of corticosterone in relation to animals of the control group (group C). Post hoc tests showed that the IS produced an effect significantly higher ($P < .05$) than those induced by ES and PS. Finally, serum corticosterone concentrations were not different between mice submitted to ES and PS.

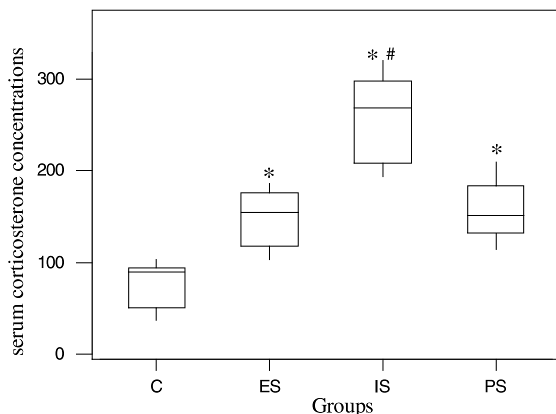

Fig. 4. Effects of escapable (ES) or inescapable (IS) footshocks as well as of a psychological stress (PS) on serum corticosterone concentrations. Data are median with ranges of 10 mice. * and # $P < .05$ compared to data of groups C and PS, respectively; ANOVA followed by Tukey–Kramer's test.

3.2.4. Behavioral, immunological, and endocrinological correlations

Table 2 shows the correlation coefficients (Pearson's correlation = r) obtained for behavioral, biochemical, and immunological alterations induced by IS and PS in the present experiment. Thus, significant direct relationships ($P < .05$) were found between (1) the decrements induced by both stressors in total locomotion in the open-field and macrophage spreading and phagocytosis indices; (2) the decrements observed in plus-maze open arms entries and the index of macrophage phagocytosis (PI); and (3) the decrements in the percentage time spent in open arm exploration and macrophage spreading and phagocytosis. Significant inverse relationships ($P < .05$) were found between the effects of both stressors on: (1) serum corticosterone concentrations and macrophage spreading (SI) and phagocytosis indexes (PI); and, (2) number of Ehrlich tumor cells/mL of ascitic fluid and macrophage phagocytosis (PI).

4. Discussion

The present results demonstrate behavioral and immunological effects for inescapable shock in mice. They also show that a psychological stress generated through the use of a communication box induces stress responses that are similar in magnitude to those generated by an inescapable footshock. These conclusions were based on the following data observed in mice of groups IS and PS: (1) decreased locomotor activity observed in an open-field; (2) decreased number of entries into the open arms and decreased time spent in the exploration of the open arms of the plus-maze; (3) decreased macrophage spreading and phagocytosis; (4) increased macrophage H_2O_2 release; and (5) increased growth of the ascitic form of Ehrlich tumor. Behavioral and immunological changes were not observed in animals of group ES. An

Table 2
Correlation coefficients (r) calculated for the behavioral and immunological alterations induced by inescapable footshock and psychological stressor in mice

Parameters[a]	Inescapable footshock[b]		Psychological stressors[b]	
	SI	PI	SI	PI
Total locomotion	0.72*	0.85*	0.76*	0.70*
Open arm entries	0.58	0.92*	0.68	0.91*
Open arm time	0.77*	0.79*	0.79	0.81*
Corticosterone	−0.85*	−0.96*	−0.82	−0.90*
Ehrlich cells/mL	−0.58	−0.81*	−0.47	−0.75*

[a] Total locomotion in the open-field, number of plus-maze open arm entries, percentage time in plus-maze open arm exploration, serum corticosterone concentration, numbers of Ehrlich tumor cells/mL of ascitic fluid (Ehrlich cells/mL).
[b] Macrophage spreading (SI) and phagocytosis (PI) indexes.
* $P < .05$ (Pearson's correlation coefficients).

increase of serum corticosterone concentration was also observed in mice of groups ES, IS, and PS, this increment being higher in animals submitted to IS. These findings are in accordance with results reported previously in this laboratory in offspring of maternally stressed mice (Palermo-Neto et al., 2001). Some of them also agree with data described elsewhere and in different contexts for stressors applied to adult animals (Fleshner, Bellgrau, Watkins, Laudenslager, & Maier, 1995). Finally, they also match data that reported immunological changes in humans after acute and chronic stressors (Zorrilla et al., 2001).

When mice are introduced into an open-field, they are inclined to explore mainly the peripheral zone of the apparatus, i.e., they present a so-called positive thigmotaxis (Simon, Dupuits, & Constentin, 1994). According to Kelley (1993), stressors and/or anxiogenic drugs increase the amount of time spent by rodents in the peripheral zone of the open-field, decreasing at the same time their permanence in the central zones. Also, increments in the number of entries or in the time spent by mice in open arm exploration in the plus-maze are commonly taken as indicative of the presence of decreased levels of anxiety in these animals (File, 1984, 1991). Thus, reductions in total locomotion and/or in the locomotor activity in open-field central zones (Smith et al., 1988) and in plus-maze open arm exploration (File, 1984, 1991) are good indices of stress response. The open-field and plus-maze results obtained in this experiment showed high levels of stress in mice submitted to both IS and PS. Indeed, the present study showed a decrease in total locomotor activity in the open-field and in the plus-maze open arm exploration in mice of groups IS and PS. The opposite occurred for animals of groups IS and PS in closed arm exploration. Additionally, the present data also showed that the total amount of time spent by mice subjected to IS in the open-field central zone was smaller than that spent by animals exposed to PS, the contrary being found for the amount of time those mice spent in the peripheral zone of the apparatus. Altogether, these open-field and plus maze data suggest an increased level of stress in mice of group IS. The data obtained on serum corticosterone concentrations were in the same direction. They not only indicate increased stress in mice of groups IS and PS, in relation to those of groups C, but also that mice submitted to IS were more stressed than those placed in the communication chamber. However, as it will be discussed ahead serum corticosterone concentration was increased in animals of group ES and they presented no behavioral changes.

Activated macrophages present a large number of morphologic, functional, and metabolic differences from resting cells. They are bigger and display pronounced ruffling of the plasma membrane, an increased capacity for adherence and spreading on surfaces, increased formation of pseudopods, and increased number of picnotic vesicles, as well as functional differences (North, 1978). Activated macrophages were found to exhibit a high antitumor cytotoxic response (Ruco & Meltzer, 1978). The IS and PS treatments used in the present experiment decreased macrophage spreading and phagocytosis. These effects agree with those reported elsewhere for stressor effects on T- and B-cell mediated responses, NK-cell cytotoxicity, lymphocyte proliferation, cytokine, and antibody productions (Dhabhar & McEwen, 1997; Fleshner et al., 1995). According to Okimura, Ogawa, and Yamauchi (1986) restraint stress suppress the uptake of zymozan particles as well as the clearance of carbon particles by macrophages studied in vivo. Although the role of spreading in phagocytosis may be controversial, several recent papers from this and other laboratories have shown differences in macrophage spreading after several different manipulations (Massoco & Palermo-Neto, 1999; Schmidt, Caron, & Hall, 2001). The present data also showed that ES induced no significant changes in macrophage spreading and phagocytosis indices.

Both IS and PS increased Ehrlich tumor growth in mice. This finding is in keeping with previous reports

from this and other laboratories showing that susceptibility to and recovery from infection, tumor cell inoculation, and autoimmune disorders are strongly influenced by central nervous system (CNS) activation states (Dunn & Berridge, 1990; Palermo-Neto & Guimarães, 2000; Portela et al., 2001). Accordingly, cold water or immobilization stress have been shown to suppress the antitumor activity of mononuclear phagocytes (Aarstad, Kolset, & Seljelid, 1991; Pavlidis & Chirigos, 1980). Furthermore, footshock stress was shown to suppress immune functions in rats and decreased their resistance to a tumor challenge (Visintainer, Volpicelli, & Seligman, 1982). Thus, it seems feasible to suggest that the presently observed decrease in the resistance to Ehrlich tumor might be causally related both to the increment in CNS activation states and decrements found in macrophage spreading and phagocytosis after stress. In fact, significant direct relationships were found in this experiment between macrophage PI and stress response measured in open-field and plus-maze apparatuses. Furthermore, inverse relationships were found between macrophage PI and the number of Ehrlich tumor cells/mL of ascitic fluid.

An increased respiratory burst, with the consequent generation and release of reactive oxygen intermediates such as H_2O_2 also characterizes macrophage activation (Somers, Weiel, Hamilton, & Adams, 1986). In the present experiment, IS and PS increased macrophage H_2O_2 release, a phenomenon not observed after ES. Accordingly separation stress in monkeys (Coe, Rosemberg, & Levine, 1988) and conflict stress in mice (Lyte, Nelson, & Baissa, 1990) were shown to stimulate the respiratory burst activity of immune cells. Thus, the present data on H_2O_2 production appear to contradict the results on macrophage spreading and phagocytosis reported above for mice of groups IS and PS. However, a number of relevant differences reported to exist for the type of cytokine released by activated macrophages and/or lymphocytes under the influence of stress as well as for cytokine action on macrophage activity preclude any simplistic interpretation. In fact, it was shown that stress, through glucocorticoid hormone activation, could modulate the switch between TH_1/TH_2 responses (Elenkov & Chrousos, 1999; Elenkov, Papanicolau, Wilder, & Chrousos, 1996) and thus, the expression, production,and release of pro-inflammatory or anti-inflammatory cytokines (Elenkov & Chrousos, 1999; Webster, Torpy, Elenkov, & Chrousos, 1988).

In recent years significant insights into the progressive states of tumor growth, particularly tumor invasion and metastatic spread, have arisen from basic research dealing with the molecular biology, biochemistry, cell biology, and immunology. Because macrophages operate at the tumor–host interface they may critically influence tumor growth. Indeed, macrophages are important components of natural immunity involved in inhibition of tumor growth and destruction of tumor cells, being activated for tumoricidal activity by cytokines and bacterial products (Ribeiro-Dias et al., 1998; Ruco & Meltzer, 1978). Thus, changes in cytokine profile induced in this experiment by IS and PS might have changed tumor–host interface thus decreasing animals resistance to Ehrlich tumor growth. Particularly relevant in the context of the present discussion are the possible effects of stressors on cytokines such as TNF-α and IL-1 (Bonta & Shlomo, 1993) and also on nitric oxide (DiNapoli, Calderon, & Lopes, 1997; Yim, Bastian, Smith, Hibbs, & Samlowski, 1993). These mediators are produced by macrophages and were reported as being relevant effector mechanisms against tumor cells. In this respect, the release and gene regulation of TNF-α and IL-1 are under the control of other inhibitors of inflammation among which prostaglandin E_2 (Kunkel et al., 1988; Renz, Gong, Schmidt, Nain, & Gemsa, 1988). Exogenous PGE_2 was shown to regulate macrophage-mediated tumor cell-killing and modulation of biosynthesis of PGE_2 was already related to induction of antitumor activity of macrophages (Taffet, Eurell, & Russell, 1982). Finally, and importantly, it was observed in this experiment an increment in H_2O_2 liberation in IS and PS groups; it was already suggested that inhibition of tumor growth occurred concomitantly with increments in peritoneal macrophage H_2O_2 release (Fecchio, Russo, Sirois, Braquet, & Jancar, 1990).

No changes were observed in this experiment between mice from groups ES and C in the open-field and plus-maze behavioral parameters, in macrophage spreading, phagocytosis, and H_2O_2 release and also in Ehrlich tumor growth. Thus, it could be thought that the psychological dimension of stressor controllability was an important modulator of footshock stress effects in this study. However, mice of IS and ES groups differ markedly not only with respect to the psychological setting used but also and mainly in the amount of electric shock they received. Thus, the lack of behavioral and immunological changes reported in this study for mice of group ES might not be attributed solely to controllability but also to the amount of shocks they received.

Animals from groups ES and PS showed similarly elevated serum corticosterone concentrations and they significantly differ in macrophage function and behavioral outcomes. Accordingly, mice lacking the gene for corticotropin-releasing factor (CRF) also showed a clear dichotomy on stress-induced effects, since the stress-related HPA activation was absent in these mice whereas the stress-related behavioral responses thought to be mediated by CRF were unaffected (Dunn & Swiergiel, 1999); this finding led the authors to suggest that stress-induced HPA axis activation although critical might not be essential for its effects on behavior. Although using a different approach, the present results provide further evidence to support their hypothesis. Altogether, our results along with those of Dunn and Swiergiel (1999)

run counter a large number of studies suggesting that HPA axis activation plays a major role in the behavioral and immunological responses normally observed during stress. In this respect, the ability to escape a footshock was reported as being unable to abolish stress effects on ACTH levels (Maier, Ryan, Barksdale, & Kalin, 1986). Thus, other mechanisms triggered by the stressors applied in this work, besides corticosterone release, might be involved with the present data. Indeed, reliance on steroid elevation as a sole mechanism of stress-induced suppression of immune responses is overly simplistic.

Recent research in the field of psychoneuroimmunology suggests that there are several pathways beside HPA axis activation, by which stress may affect immune function. Interactions between the nervous system and immune system are extensive and include other hormones, catecholamines, other amines, neuropeptides, and opioids. Thus, for instance, stress induced in a communication box similar to that presently used was reported to activate catecholaminergic neurotransmission within the CNS (Iimori et al., 1982). Ether stress (Vellucci, 1977), cold stress (Ritter & Pelzer, 1978), and immobilization stress (Nakagawa, Tanaka, Kohno, Noda, & Nagasaki, 1981) were shown to increase noradrenaline release and turnover in the hypothalamus. A pituitary-independent suppression of peripheral blood lymphocyte proliferation has been related to activity of central and peripheral catecholaminergic systems (Keller et al., 1988). Macrophages show receptors for catecholamines and it has been reported that catecholamines and adrenergic agonists modify macrophage function (Madden & Livnat, 1991; Ortega, Garcia, Saez, & De Le Fuente, 2000). Thus, the final neural link between the behavioral and immunological changes now observed after IS and PS might have involved also catecholaminergic systems within the CNS and/or the sympathetic autonomic nerve fibers that terminate in the parenchyma of lymphoid organs in close association with both T-lymphocytes and macrophages (Felten & Felten, 1991). However, it should not be forgotten that opioid peptides released by a form of footshock stress in rats was already related to a decrease in animals resistance to a tumor challenge (Shavit, 1991). Therefore, future studies involving measures of cellular and humoral immune parameters together with catecholaminergic activities in autonomic and CNS and also of opioid peptide involvement on Ehrlich tumor growth will be useful to determine the precise mechanisms underlying the stressor effects reported in the present study.

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