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# Effect of cyhalothrin on Ehrlich tumor growth and macrophage activity in mice

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## Abstract

Cyhalothrin, a pyrethroid insecticide, induces stress-like symptoms, increases c-fos immunoreactivity in the paraventricular nucleus of the hypothalamus, and decreases innate immune responses in laboratory animals. Macrophages are key elements in cellular immune responses and operate at the tumor-host interface. This study investigated the relationship among cyhalothrin effects on Ehrlich tumor growth, serum corticosterone levels and peritoneal macrophage activity in mice. Three experiments were done with 10 experimental (single gavage administration of 3.0 mg/kg cyhalothrin daily for 7 days) and 10 control (single gavage administration of 1.0 mL/kg vehicle of cyhalothrin preparation daily for 7 days) isogenic BALB/c mice in each experiment. Cyhalothrin i) increased Ehrlich ascitic tumor growth after *ip* administration of 5.0 x $10^6$ tumor cells, i.e., ascitic fluid volume (control = 1.97 ± 0.39 mL and experimental = 2.71 ± 0.92 mL; $P < 0.05$), concentration of tumor cells/mL in the ascitic fluid (control = 111.95 ± 16.73 x $10^6$ and experimental = 144.60 ± 33.18 x $10^6$; $P < 0.05$), and total number of tumor cells in the ascitic fluid (control = 226.91 ± 43.22 x $10^6$ and experimental = 349.40 ± 106.38 x $10^6$; $P < 0.05$); ii) increased serum corticosterone levels (control = 200.0 ± 48.3 ng/mL and experimental = 420.0 ± 75.5 ng/mL; $P < 0.05$), and iii) decreased the intensity of macrophage phagocytosis (control = 132.3 ± 19.7 and experimental = 116.2 ± 4.6; $P < 0.05$) and oxidative burst (control = 173.7 ± 40.8 and experimental= 99.58 ± 41.7; $P < 0.05$) *in vitro* in the presence of *Staphylococcus aureus*. These data provide evidence that cyhalothrin simultaneously alters host resistance to Ehrlich tumor growth, hypothalamic-pituitary-adrenocortical (HPA) axis function, and peritoneal macrophage activity. The results are discussed in terms of data suggesting a link between stress, HPA axis activation and resistance to tumor growth.

Key words: Cyhalothrin; Corticosterone; Macrophage; Ehrlich tumor; Innate immunity; HPA axis

## Introduction

Experimental and epidemiological evidence shows the relevance of the interactions between stress and changes in immunity. A fixed-effect analysis showed that stress and depression are associated with i) overall leukocytosis, ii) mild reductions in absolute NK-cell counts and relative T-cell proportions, iii) marginal increases in CD4+/CD8+ ratios, and iv) moderate decreases in T-cell and NK-cell function (1). Changes in cell-mediated immune function and susceptibility to cancer were also reported in persons undergoing distressing life experiences (2,3). Accordingly, a variety of stressors have been found to alter immune responses in experimental animals. An inescapable foot shock and a psychological stressor generated with the use of a communication box were shown to concomitantly alter stress levels, macrophage activity, and Ehrlich tumor growth in mice (4); sound stress suppressed the T- and B-cell mediated responses in rats (5); inescapable foot shocks increased total alveolar cell count in OVA-sensitized rats (6); individual housing induced an altered immune-endocrine response and, at the same time, decreased resistance to Ehrlich tumor growth (7); submissive mice displayed more anxiety-like behaviors and decreased macrophage activity, and showed a decrease in resistance to implantation and development of melanoma metastases in their lungs (8); stress induced by cohabitation with sick partners decreased macrophage activity (9) and dendritic cell phenotype (10).

Pyrethroid insecticides that are widely used for their

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activity against a broad spectrum of parasites have been reported to induce stress-like symptoms in laboratory animals (11-13). Thus, cyhalothrin (3.0 $mg \cdot kg^{-1} \cdot day^{-1}$ for 7 days) induced no overt signs or symptoms of intoxication in rats, but did produce behavioral effects in the open field and plus-maze apparatuses similar to those induced by anxiogenic-like drugs such as picrotoxin (11). Further analysis of the effects of cyhalothrin showed that this pesticide increased serum corticosterone levels (11), decreased peritoneal macrophage activity in rats (14,15), and increased c-fos immunoreactivity in the paraventricular nucleus of the hypothalamus, but not in the basolateral amygdala of rats (16).

Ehrlich tumor cells have been reported to elicit a strong host immune response (17,18), a fact that, together with other properties, makes this tumor an interesting model for analysis of the effects of drugs or external environmental agents on tumor growth. It has been reported that macrophage activity modulates Ehrlich tumor growth (4,19). Thus, the purpose of the present study was to study the resistance of cyhalothrin-treated mice to Ehrlich tumor growth and to analyze the relationship between this effect and that induced by the pesticide on peritoneal macrophage activity. Serum corticosterone concentrations were also measured in the pesticide-treated mice. Studies in the field of psychoneuroimmunology show that susceptibility to, and recovery from infection, allergic reactions, tumor cell inoculation, and autoimmune disorders are strongly influenced by nervous and endocrine system activity (20,21).

## Material and Methods

### Animals

Sixty male isogenic BALB/c mice from our own colony, weighing 30-40 g and about 90 days of age were used. The animals were housed under conditions of controlled temperature (22 ± 2°C), humidity (45-65%) and artificial light (12-h light/dark cycle, lights on at 7:00 am) with free access to rodent chow (Nuvital Company, Brazil) and water. Sterilized and residue-free wood shavings were used as animal bedding. The experiments were performed in a similar, but separate room, to which the animals were transferred and kept in their home cages 7 days before the experiments. Animals were housed and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo. These guidelines are similar to those of the NIH, USA.

### Pesticide

A commercial formulation of cyhalothrin (Grenade®, Coopers Schering Plough Animal Health, Brazil; 45 g/L, 92.7% purity of the active compound) was used. The formulation consists of four cyhalothrin isomers that comprise two pairs of enantiomers, A and B, in a 60:40 ratio, respectively; within each pair, the enantiomers are present in equal amounts. A 3.0-mg/kg dose of cyhalothrin present in the Grenade formulation was prepared in distilled water (3.0 mg/mL) and administered to the mice by gavage, once daily for 7 days. This formulation dose was chosen based on our previous studies suggesting that it produced no overt clinical signs or symptoms of intoxication but induced stress-like symptoms in rats (11), decreased macrophage and neutrophil activities (14,15) and increased c-fos immunoreactivity in the paraventricular nucleus of the rat hypothalamus (16). The $LD_{50}$ calculated for mice of cyhalothrin present in this formulation was reported to be 37-62 mg/kg (22). The vehicle of cyhalothrin preparation employed in Grenade® (monylphenol ethoxylate, 9.5 EO (Renex®) plus calcium sulfonate and Arol 3700®), diluted in distilled water to the proper volume to be injected (1.0 mL/kg), was used as the control solution and administered by the same route and over the same time. Previous studies from our laboratory have shown no effects of Grenade's vehicle on macrophage activity or behavior (14).

### Clinical observations

The observational grid proposed by Irwin (23) for toxicological screening was used. Control and cyhalothrin-treated mice were observed daily (immediately after each cyhalothrin or control solution treatment) for the following overt signs and symptoms of intoxication: mortality, sedation, excitation, stereotypy, aggressiveness, piloerection, salivation, muscle tone, coarse tremors, convulsions, reactivity to touch, sleep, motor incoordination, gait, respiration, and quality of feces. Each symptom was assessed either by observing the spontaneous behavior of the mice in their home cages or by subjecting the animals to standardized manipulations such as bilateral pressure on their flanks (muscle tone). The animals of both groups were weighed immediately before the treatments on experimental days 1, 3, 5, and 7.

### Host resistance and Ehrlich tumor growth

Ehrlich tumor cells were obtained and analyzed as described by Matsuzaki et al. (24). Mice were inoculated intraperitoneally (*ip*) with 5.0 x $10^6$ Ehrlich tumor cells with at least 90% viability. For Ehrlich tumor growth evaluation, experimental and control mice were deeply euthanatized under $CO_2$, the ascitic fluid was collected, its volume was measured, and the number of tumor cells was counted in a Neubauer chamber. Tumor growth analysis was performed 7 days after tumor cell inoculation.

### Collection of peritoneal macrophages

Macrophages were activated by an *ip* inoculation of 5.0 x $10^6$ Ehrlich tumor cells. Peritoneal macrophages were collected for analysis 24 h after tumor cell inoculation, as described elsewhere (25). Control mice were euthanatized under $CO_2$ and the peritoneal macrophages were obtained immediately

after by peritoneal lavage, using 5.0 mL phosphate-buffered saline (PBS), pH 7.2-7.4. The peritoneal fluid was collected into a plastic tube and kept in an ice bath. Macrophages were identified morphologically since they are smaller than Ehrlich tumor cells, being subsequently counted using a Neubauer chamber and Trypan blue dye. The number of cells was adjusted to 2.0 x $10^6$ cells/mL. Only cell suspensions with 90% or more viability were used.

**Measurement of peritoneal macrophage activity**

A flow cytometer (Becton Dickinson Immunocytometry Systems, USA) interfaced with a Macintosh G4 computer was used to analyze peritoneal macrophage activity. Data from 10,000 events were collected in the list mode and analyzed with the Cell Quest Pro® software (Becton Dickinson Immunocytometry Systems). Cell populations were identified based on their properties on forward scatter versus side scatter (FCSP/SCSP) plots, mechanically sorted and evaluated by light microscopy after Giemsa staining. Fluorescence data were collected on a log scale. Green fluorescence from 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, USA) was measured at 530 ± 30 nm (FL1 detector), and red fluorescence from propidium iodide (PI)-labeled *Staphylococcus aureus* (Sigma, USA) was measured at 585 ± 42 nm (FL2). PI and DCFH fluorescence was analyzed after compensation to correct for possible crossing-over between PI and DCFH signals.

Quantification of phagocytosis and oxidative burst was based on mean PI and DCFH fluorescence per cell, respectively. Briefly, 2 x $10^5$ peritoneal cells (100 µL) were mixed with 200 µL 0.3 mM DCFH-DA in PBS and 100 µL of PI-labeled *S. aureus* in polypropylene tubes. Samples were incubated under agitation at 37°C for 30 min. The reactions were interrupted by the addition of 2 mL cold EDTA solution (3 mM) in order to block phagocytosis. After centrifugation at 250 *g* for 10 min, the cell pellets were resuspended in 1 mL cold 3 mM EDTA for flow cytometry measurements. The mean fluorescence of green and red channels was taken as an indicator of oxidative burst and phagocytosis, respectively (26). Percent phagocytosis (percentage of peritoneal macrophages that ingested bacteria) is reported as the number of peritoneal macrophages with red fluorescence divided by the total number of cells, multiplied by 100.

**Corticosterone determination**

Corticosterone was determined with commercial kits (Coat-A-Count, DPC®, USA). This procedure is based on a solid phase radioimmunoassay in which $^{125}$I-labeled corticosterone competes for a fixed time with the corticosterone present in the mouse samples for antibody sites. 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 coefficients of variation were 4.2 and 16.1%, respectively. In order to decrease data variability and the effects of circadian changes, control and experimental animals were intermixed for euthanasia and their blood was taken at the same time of day (between 8:00 and 10:00 am) 24 h after the last cyhalothrin or control solution treatment.

**Experimental design**

Three independent experiments were carried out. In each two identical replicates were performed according to standard and quality assurance methods. The data obtained were pooled for statistical analysis and interpretation.

In the first experiment, resistance to Ehrlich tumor growth was analyzed. Twenty mice were divided equally at random into an experimental group and a control group. Animals from both groups were treated by gavage with cyhalothrin (3 mg·kg$^{-1}$·day$^{-1}$; in a volume of 1 mL/kg) or with control solution (1.0 mL/kg) for 7 days, respectively. These animals, that were observed clinically for possible signs and symptoms of cyhalothrin intoxication, received *ip* 5.0 x $10^6$ Ehrlich tumor cells (at least 90% viability) on the first day of cyhalothrin treatment; 24 h after the last cyhalothrin treatment, i.e., 8 days after tumor cell inoculation, the animals were euthanatized under $CO_2$ for tumor growth evaluation. Experiments 2 and 3 were designed to analyze the effects of cyhalothrin on macrophage activity after Ehrlich tumor cell inoculation and on serum corticosterone levels, respectively. In each experiment, 20 mice were divided at random into two equal groups, an experimental one and a control one. Animals from the experimental and control groups were treated with cyhalothrin or control solution as described in experiment 1. In experiment 2, control and experimental mice received *ip* 5.0 x $10^6$ Ehrlich tumor cells (at least 90% viability) immediately after the last cyhalothrin or control solution treatment; macrophage activation, collection and analysis were performed as described above, i.e., 24 h after tumor cell inoculation. In experiment 3, trunk blood was harvested from control and experimental mice 24 h after the last cyhalothrin or control solution treatment for the determination of serum corticosterone levels.

**Statistical analysis**

Statistical analysis was performed using the StatPac Statistic Analysis Package. The Bartlett test and Kolmogorov-Smirnov test were initially applied in order to determine whether the data should be analyzed by parametric or nonparametric tests. The Student *t*-test (unpaired, two-tailed) was then used to compare parametric data and the Mann-Whitney U-test was used to compare nonparametric data. The probability of $P < 0.05$ was considered to show significant differences for all comparisons made. Data are reported as means ± SD.

## Results

**Experiment 1. Effects of cyhalothrin on Ehrlich tumor growth**

Administration of cyhalothrin (3.0 mg·kg$^{-1}$·day$^{-1}$, for 7

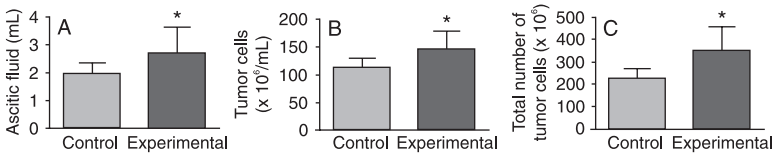


**Figure 1.** Effects of cyhalothrin (3 mg·kg$^{-1}$·day$^{-1}$) for 7 days (black columns) on Ehrlich tumor growth. Control mice (gray columns) were injected with control solution (1.0 mL·kg$^{-1}$·day$^{-1}$ for 7 days). Mice received 5 x 10$^6$ Ehrlich tumor cells *ip* with the 1st treatment with cyhalothrin or control solution. *A*, Ascitic fluid volume; *B*, concentration of tumor cells/mL ascitic fluid; *C*, total number of tumor cells. Data are reported as means ± SD for N = 10 animals/group. *P < 0.05 compared to control (Mann-Whitney U-test).

**Table 1.** Effects of cyhalothrin (3 mg/kg for 7 days) on peritoneal macrophages activated by Ehrlich ascitic tumor cells in mice.

| Parameters | Control | Experimental |
|---|---|---|
| Oxidative burst | | |
| Basal | 151.58 ± 56.39 | 130.41 ± 86.89 |
| *Staphylococcus aureus* | 173.70 ± 40.83 | 99.58 ± 41.72* |
| Phagocytosis | | |
| Intensity | 132.29 ± 19.68 | 116.21 ± 4.60$^+$ |
| Percent (%) | 68.86 ± 6.43 | 67.65 ± 9.47 |

Mice received 5 x 10$^6$ Ehrlich tumor cells *ip* on the 7th day of treatment with cyhalothrin or control solution. Macrophage activity was assessed 24 h later. Data are reported as means ± SD of fluorescence intensity unless otherwise indicated for N = 10 animals/group. Control mice were injected with control solution (1.0 mL·kg$^{-1}$·day$^{-1}$ for 7 days). *P < 0.001 compared to control (Student *t*-test). $^+$P < 0.001 compared to control (Mann-Whitney U-test).

days) did not induce overt clinical signs and symptoms of intoxication in the mice. Indeed, no differences were found between experimental and control mice concerning body weight and the various other parameters included in the observational grid used to analyze toxicity (data not shown).

However, and as shown in Figure 1, cyhalothrin administration (3.0 mg·kg$^{-1}$·day$^{-1}$) for 7 days increased Ehrlich ascitic tumor growth (P < 0.05). In fact, although some overlaps in SD were observed, statistical analysis (Mann-Whitney U-test) showed that treatment with this pyrethroid insecticide increased the volume of ascitic fluid (control = 1.97 ± 0.39 mL; experimental = 2.71 ± 0.92 mL, P < 0.05), the concentration of tumor cells/mL of ascitic fluid (control = 111.95 ± 16.73; experimental = 144.60 ± 33.18, P < 0.05) and the total number of tumor cells found in this fluid (control = 226.91 ± 43.22; experimental = 349.40 ± 106.38, P < 0.05).

### Experiment 2. Effects of cyhalothrin on peritoneal macrophage activity

Table 1 shows that no differences between experimental and control data were detected when the basal oxidative burst was measured in peritoneal macrophages taken 24 h after Ehrlich tumor cell inoculation. However, statistical analysis revealed differences between control and experimental data regarding the intensity of macrophage phagocytosis and macrophage oxidative burst in the presence of *S. aureus in vitro*. Specifically, peritoneal macrophages from cyhalothrin-treated mice presented a smaller intensity of *in vitro S. aureus* phagocytosis compared to control mice (P < 0.001; Mann-Whitney U-test). Accordingly, cyhalothrin treatment decreased macrophage oxidative burst compared to control (P < 0.001; Student *t*-test). No differences were found between control and experimental macrophage data for percent *in vitro S. aureus* phagocytosis (P > 0.05).

### Experiment 3: Cyhalothrin effects on corticosterone serum levels

Biochemical analysis of serum corticosterone levels showed that cyhalothrin (3.0 mg·kg$^{-1}$·day$^{-1}$ for 7 days) increased corticosterone levels compared to control (control = 200.00 ± 48.30; experimental = 420.35 ± 75.50, P < 0.05; Student *t*-test).

## Discussion

Exposure to virtually any stressor activates the hypothalamic-pituitary-adrenocortical (HPA) axis and results in a readily discernible elevation in plasma corticosterone levels. The present results showed an increase in serum corticosterone levels in mice treated with cyhalothrin (3.0 mg/kg once daily for 7 days). Assessment of plasma corticosterone has been used as a reliable measure of stress (27). Thus, the present results agree with data previously reported for a similar cyhalothrin treatment in rats and also with data showing that it increased c-fos immunoreactivity in the paraventricular nucleus of the hypothalamus (16), an area tightly linked to HPA axis activation during stress (28). Thus, the present data agree with and support our previous suggestion of a stressor-like effect for this pyrethroid insecticide. Stress is defined here as a process by which an organism responds to challenging internal or external events (1).

Cyhalothrin treatment (3.0 mg/kg once daily for 7 days) decreased mouse resistance to Ehrlich tumor growth as inferred by the increased volume of ascitic fluid, the total number of Ehrlich tumor cells and the concentration of Ehrlich tumor cells/mL of ascitic fluid found in the experimentally treated mice after being injected with 10$^5$ tumor cells. Physical and psychological stressors have been reported to decrease resistance to Ehrlich tumor (4,7,9). A decreased resistance to mammary tumor growth and its response to

chemotherapy have also been reported in stressed mice (29). Submissive and socially stressed mice displayed more anxiety-like behaviors and showed a decreased resistance to the implantation and development of melanoma metastases in their lungs (8). The present findings on Ehrlich tumor growth are thus consistent 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 states of central nervous system activation (2,10,30).

The development of the ascitic form of the Ehrlich tumor is tightly linked to the activity of the immune system (19) and particularly to the activation of resident macrophages (31). Indeed, macrophages are the prevalent mononuclear cell population that migrates to Ehrlich tumor focus where they operate at the tumor-host interface and modulate Ehrlich tumor growth (32). As a matter of fact, the macrophages analyzed here were collected from mice 24 h after Ehrlich tumor cells inoculation and it is known that stimulation of peritoneal macrophages by Ehrlich tumor cells occurs on the first 3 days after injection of tumor cells (19,23,32).

Activated macrophages, which present a large number of morphologic, functional, and metabolic differences from resting cells (33), exhibited a high antitumor cytotoxic response (34). The cyhalothrin treatment used in this experiment (3.0 mg/kg once daily for 7 days) decreased peritoneal macrophage activity as inferred from data on both intensity of *S. aureus* phagocytosis (number of bacteria phagocytized) and *S. aureus*-induced oxidative burst. Increased phagocytosis and respiratory burst rates with the consequent generation and release of reactive oxygen intermediates such as $H_2O_2$, characterize macrophage activation (35). In this respect, the present results on Ehrlich tumor-activated macrophage phagocytosis in mice agree with and support those previously reported by our laboratory for onco BCG-activated peritoneal macrophages in rats (15). Contrary to our previous data, no differences were found here in the effects of cyhalothrin on percent macrophage phagocytosis (number of macrophages presenting phagocytosis). Differences in *in vivo* macrophage activation (onco BCG x tumor cells) and in the animals studied (rats and mice) might account for the differences observed.

An inverse correlation between peritoneal macrophage activity and resistance to Ehrlich tumor growth has already been reported in stressed mice (4,7). Other stressors suppressed the antitumor activity of mononuclear phagocytes, leading to a decrease in host resistance to tumor challenges (4,8). Thus, it seems reasonable to suggest that the changes induced by cyhalothrin treatment (3.0 mg/kg once daily for 7 days) in macrophage activity may account for the presently described decrease in resistance to Ehrlich tumor growth. On this basis, it would be reasonable to hypothesize also that the present data regarding both peritoneal macrophage activity and/or host resistance to Ehrlich tumor growth depend on the effects of the pesticide on HPA axis activity, particularly on blood corticosterone levels. An altered endocrine response to stress is well known to be linked to immune disorders/disease susceptibility (7,14,36). A decrease in cell-mediated immune function with a concomitant increase in susceptibility to cancer was reported in persons undergoing distressing life experiences (2-4) and also in stressed animals (4,7-9).

Therefore, we suggest here that the effects of cyhalothrin in mice would be in overall agreement with data from other studies that have examined the link between HPA axis activation and host animal resistance to tumor growth (2,7-9,36). Although the present data do not permit additional comments, it seems relevant to point out that glucocorticoid hormones such as corticosterone modulate the profiles of $Th_1$ and $Th_2$ cytokines (37) and activate Toll-like receptors and NF-κB gene expression (38). Cytokines play a relevant role in innate immunity (39) and NF-κB is a relevant transcription factor for macrophage activation (40). It should not be forgotten, however, that recent research in the field of psychoneuroimmunology suggests that there are several pathways beside HPA axis activation by which stress may affect immune function (1-3).

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