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Cohabitation with a Sick Cage Mate: Effects on Ascitic Form of Ehrlich Tumor Growth and Macrophage Activity

Glaucie Jussilane Alves    Luciana Vismari    João Palermo-Neto
Laboratory of Applied Pharmacology and Toxicology, School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil

Key Words
Psychological stress · Neuroimmunomodulation · Macrophage · Ehrlich tumor · Phagocytosis · Flow cytometry

Abstract
The present study was designed to evaluate the effects of mice cohabitation with a sick conspecific cage mate on peritoneal macrophage activity and on resistance to Ehrlich tumor growth. Female mice housed in pairs were divided into control and experimental groups. One mouse of each control pair was inoculated with NaCl (0.1 ml/10 g) intraperitoneally and the other, called ‘companion of healthy partner’ (CHP), was kept undisturbed. One animal of each experimental pair of mice was inoculated with 5.0 \times 10^6 Ehrlich tumor cells intraperitoneally and the other, the subject of this study, was called ‘companion of sick partner’ (CSP). Peritoneal macrophages were removed from CSP and CHP mice to analyze resident macrophage activity (experiment 1), macrophage activity after Mycobacterium bovis (experiment 2) or Ehrlich tumor cells (experiment 3) in vivo inoculations. The resistance of CSP and CHP mice to Ehrlich tumor growth was also analyzed (experiment 4). Differences between groups were not found on resident macrophage activity. However, Onco-BCG- and Ehrlich tumor-activated macrophages from CSP mice presented a decreased intensity and percentage of phagocytosis and an increased respiratory burst in the presence of Staphylococcus aureus stimulation in vitro. CSP animals at the same time displayed a decreased resistance to Ehrlich tumor growth. These data were discussed in light of a possible psychological stress effect imposed by the housing condition on mice’s peritoneal macrophage activity and, as a consequence, on their resistance to Ehrlich tumor growth.

Introduction
The central premise underlying psychoneuroimmunology is that nervous, endocrine and immune systems are components of an integrated system of defense. The existence of bidirectional pathways between the brain and the immune system reinforces the hypothesis that immune changes constitute an important mechanism through which psychosocial factors influence health and disease [1, 2]. Changes in cell-mediated immune function and susceptibility to cancer are reported in persons undergoing distressing life experiences [2–4].

In both animal and human models, the deleterious effects of stressors on healing have been repeatedly demonstrated. For instance, a variety of stressors have been found to alter immune responses in experimental animals [5, 6]. These effects, however, depend on the type of stressor, its duration and frequency, the temporal rela-
tionship between stress and immunological stimulus and, among many other variables, the subjects' ability to control or escape from stressors [7–9].

Animals, as well as people, need a healthy social relationship and may experience similar responses to chronic psychological stress [10]. Murgulis et al. [6] reported that cohabitation for 11 days with a sick cage mate increased the motor activity of mice within an open field. Recently, we reported in mice that were companions of sick partners (CSP) a decreased level and an increased turnover in hypothalamic noradrenaline (NA) as well as a decreased neutrophil oxidative burst and phagocytosis after in vitro Staphylococcus aureus stimulation [11].

Alterations of immune function are considered to be more relevant when resulting in significant pathological, microbial and/or clinical symptoms. Ehrlich tumor cells were reported to elicit a strong host immune response [12, 13], a fact that, together with other properties, makes this tumor an interesting model for analysis of the effects of drugs and/or external environmental events on tumor growth [7, 14, 15]. It is known that effective immune function against tumor cell invasion requires, among other factors, a strong cooperation among macrophages, T and B lymphocytes. Therefore, suppression of one of these components may compromise immunocompetence, thus changing tumor growth. The present experiment was then designed to analyze the effects of cohabitation with a tumor-bearing mouse on macrophage activity and Ehrlich tumor growth.

**Method**

**Animals**

Naive Swiss female mice (50–60 days old) were used. Female mice were chosen because they are less aggressive than males when grouped [16] and also based on our previous study [6, 11]. The animals were housed under conditions of controlled temperature (22–26°C) and artificial light (12 h light/12 h dark, lights on at 7:00 AM), with free access to rodent chow and water. The experiments were performed in a different room with the same temperature as in the animal colony to which the animals were transferred and maintained in their home cages 10 days before the beginning of the experiments. Animals were housed and used in accordance with the guidelines of the Bioethical Committee of Care and Use of Laboratory Animal Resource of the School of Veterinary Medicine, University of São Paulo, Brazil. These guidelines are similar to those of the National Research Council of the USA.

**Groups and Experimental Design**

Four experiments were conducted in accordance with protocols and quality assurance methods, with 16 pairs of mice each. Within each experiment, mice were previously weighed and paired according to their weight, being divided at random and equally into 2 groups: control and experimental. One mouse of each control pair was intraperitoneally injected with 0.9% NaCl (1 ml/kg) and the other animal, referred to as companion of healthy partner (CHP), was kept undisturbed. One animal of each experimental pair of mice was intraperitoneally inoculated with 5.0 × 10⁶ Ehrlich tumor cells, as described elsewhere [6]. The other animal, the subject of this study, was kept undisturbed, being referred to as CSP.

The day on which injections were given was called experimental day 1 (ED1). On ED11, the sick mice were observed in their home cages for Ehrlich tumor signs and symptoms as proposed elsewhere [6]. Briefly, the following scoring system was employed: 0 = predominantly active, no signs and symptoms of disease; 1 = predominantly active with normal feeding and presence of rough hair; 2 = active, normal feeding, rough hair and presence of a small increment in their abdominal volume; 3 = active, normal feeding, rough hair and presence of a mild increment in abdominal volume; 4 = absence of activity, anorexia, dyspnea, rough hair and severe increment in abdominal volume. Mice from the CSP and CHP groups were subsequently removed from their cages and used to analyze the resident macrophage oxidative burst and phagocytosis (experiment 1), Mycobacterium bovis-induced macrophage oxidative burst and phagocytosis (experiment 2), Ehrlich tumor cell-induced macrophage oxidative burst and phagocytosis (experiment 3), and resistance to Ehrlich tumor growth (experiment 4).

In the first experiment, resident macrophage from CSP and CHP mice were collected on ED11 for oxidative burst and phagocytosis evaluation. In the second experiment, an attenuated suspension of M. bovis (Onco-BCG; Butantan Institute, São Paulo, Brazil) was intraperitoneally injected (0.5 ml/animal) on ED4. This procedure was repeated (0.25 ml/animal) on ED9. This Onco-BCG treatment schedule was based on others and reported to activate peritoneal macrophage [5]. On ED11, the peritoneal macrophages of mice of both groups were collected for macrophage activity evaluation. In the third experiment, 5.0 × 10⁶ Ehrlich tumor cells were intraperitoneally inoculated in CHP and CSP mice on ED10. On the following day (ED11), their peritoneal macrophages were collected for activity determination. It was reported that macrophage activation after Ehrlich tumor inoculation only occurred in the first 24 h after tumor injection [14, 18]. Finally, in the fourth and last experiment, CHP and CSP mice were inoculated with 5.0 × 10⁶ Ehrlich tumor cells on ED11 being observed for tumor progressions. After tumor injection, CSP mice were grouped in a plastic cage (41 × 34 × 16 cm), and the same procedure was employed with mice of the CHP group. On ED22, mice were rated for signs and symptoms of the disease as stated above, and were euthanatized under CO₂ for Ehrlich tumor growth evaluation.

**Collection of Peritoneal Macrophages**

Macrophages were obtained by peritoneal lavage using 5.0 ml of phosphate-buffered saline (pH 7.2–7.4). The peritoneal fluid was collected into plastic tubes and kept in an ice bath. Macrophages were subsequently counted using a Neubauer chamber and Trypan blue dye. The number of cells was adjusted to 2.0 × 10⁶ cells/ml. Only cell suspensions with 90% or more of viability were used. Macrophages were identified morphologically, as they are smaller than Ehrlich tumor cells and different from other cells.
Effects of Cohabitation with a Sick Cage Mate

Table 1. Oxidative burst and phagocytosis by resident macrophages from mice that lived (CSP) or not (CHP) with a sick cage mate for 11 days

<table>
<thead>
<tr>
<th>Measures</th>
<th>CSP</th>
<th>CHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal oxidative burst (DCFH)</td>
<td>14.8 ± 4.9</td>
<td>13.3 ± 4.4</td>
</tr>
<tr>
<td>Oxidative burst induced by <em>S. aureus</em></td>
<td>7.4 ± 2.6</td>
<td>7.2 ± 2.8</td>
</tr>
<tr>
<td>Percent of phagocytosis</td>
<td>21.4 ± 6.3</td>
<td>23.8 ± 9.5</td>
</tr>
<tr>
<td>Intensity of phagocytosis</td>
<td>17.4 ± 4.0</td>
<td>18.0 ± 1.5</td>
</tr>
</tbody>
</table>

Data are means ± SD of 8 animals per group. Statistically significant differences were not found. (Student’s t test).

Macrophage Oxidative Burst and Phagocytosis

Resident macrophage oxidative burst and phagocytosis (experiment 1), as well as those obtained after Onco-BCG (experiment 2) and Ehrlich tumor cell (experiment 3) administrations were analyzed. Quantification of phagocytosis and oxidative burst was estimated by PI and DCFH fluorescence, respectively. Briefly, 100 ml of whole peritoneal macrophage lavage fluid (2 × 10^6 cells/100 ml) was mixed with 200 μl of DCFH-DA (0.3 mM) in phosphate-buffered saline and 100 μl PI-labeled *S. aureus* in polypropylene tubes. Samples were incubated under agitation at 37°C for 20 min. Reactions were stopped by adding 2 ml of cold EDTA (3 mM) in order to terminate phagocytosis. After centrifugation (250 g for 10 min), cell pellets were resuspended in 1 ml of cold EDTA (3 mM) for flow cytometry analyses. Direct measurements of mean fluorescence of green and red channels were recorded as oxidative burst and phagocytosis, respectively, as proposed by Hasui et al. [17]. The percentage of phagocytosis (percentage of macrophage which ingested bacteria) was expressed as the number of macrophage with red fluorescence divided by the total number of cells (multiplied by 100).

Flow Cytometry

A flow cytometer (FACS Calibur; Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) interfaced with a Macintosh G4 computer was used to analyze macrophage activity. Data from 10,000 events was collected in list mode and analyzed in Cell Quest Pro (Becton Dickinson Immunocytometry Systems). Discrete cell populations were identified based on their properties on forward scatter/side scatter plots, mechanically sorted (FAC Scan; Becton Dickinson Immunocytometry Systems) and evaluated through light microscopy after staining in Giema. Data from peritoneal macrophages were collected applying gates that sorted out lymphocytes, monocytes and tumor cell clusters. Fluorescence data were collected on log scale. Green fluorescence from DCFH was measured at 530 ± 30 nm (FL1 detector) and red fluorescence from propidium iodide-labeled *S. aureus* was measured at 585 ± 42 nm (FL2). PI and DCFH fluorescence was analyzed after fluorescence compensation to correct for any crossover between the PI and DCFH signals.

Ehrlich Tumor Growth Evaluation

Tumor growth was evaluated on CHP and CSP mice 11 days after Ehrlich tumor cell (5 × 10^6 cells/ml) inoculation (ED22). For that, the ascitic fluid present on CHP and CSP mice was individually collected, measured and the number of tumor cells per milliliter was counted in a Neubauer chamber.

Statistics

Bartlett’s test was performed to evaluate whether data should be handled by parametric or nonparametric procedures. The differences in flow cytometry and ascitic form of Ehrlich tumor growth data were determined by Student’s t test, and presented as means plus standard deviations. The GraphPad Instat® and Sigma Stat 3.0® software packages were used throughout. A probability of p < 0.05 was considered to reflect significant differences for all comparisons performed. Data were presented as means ± SED.

Results

The injection of Ehrlich tumor cells induced behavioral changes in the so-called sick animals. These alterations were progressively settled, being characterized by the presence of lethargy, reduced interest in their surroundings and decreased ability to respond to the other mouse. On ED11, the signs and symptoms induced by Ehrlich tumor cells on sick animals were rated between 3 and 4.

Experiment 1: Resident Macrophage Activity

Table 1 shows the data on resident macrophage activity of CSP and CHP mice. Thus, no differences were found between the animals of the different groups for basal oxidative burst as well as for that observed in vitro in the presence of *S. aureus*. Differences between groups were also not detected for the percentage and intensity of phagocytosis.

Experiment 2: Onco-BCG-Induced Macrophage Activity

As depicted in figure 1, cohabitation with a sick cage mate changed Onco-BCG-induced macrophage activity. Indeed, although no differences were found between CHP and CSP mice for the basal macrophage oxidative burst, differences were recorded for the other macrophage parameters analyzed. Thus, data on macrophage oxidative burst in the presence of *S. aureus* were higher (p < 0.05) and data related to the percentage and the intensity of macrophage phagocytosis were reduced (p < 0.05) in mice of the CSP group.

Experiment 3: Ehrlich Tumor-Induced Macrophage Activity

As before, differences in macrophage activity between CHS and CSP mice were recorded 24 h after Ehrlich tumor cell inoculation. As shown in figure 2, data on macrophage...
oxidative burst in the presence of S. aureus were higher (p < 0.05) and those related to the percentage and intensity of S. aureus phagocytosis were lower (p < 0.05) in CSP mice. Again, no differences were found between mice of the different groups for the basal macrophage activity.

**Experiment 4: Ehrlich Tumor Growth**

No differences were found on ED22 between CHS and CSP mice for signs and symptoms of Ehrlich tumor growth. Indeed, those parameters were rated around 3–4 in all tumor-injected mice (data not shown). However, data on tumor growth evaluation were different between the groups. As seen in figure 3, the number of tumor cells per milliliter of ascitic fluid was higher (p < 0.05) in mice of the CSP group as well as that of the total number of tumor cells per animal. Differences between groups were not found for the volume of the ascitic fluid.
Discussion

The results reported here agree with others for mice paired with a sick conspecific [6, 11] and suggest immunological effects induced by this condition. Indeed, we now show that cohabitation with a sick cage mate (1) decreased the intensity and percentage of phagocytosis and increased the respiratory burst in peritoneal macrophages activated in vivo by Onco-BCG and Ehrlich tumor cells, and (2) decreased the resistance to Ehrlich tumor growth as detected by the increased number of tumor cells per milliliter of ascitic fluid. However, no differences were found on activity of resident peritoneal macrophage in CSP mice.

A large number of morphological, functional and metabolic changes were reported in activated macrophages in relation to resting cells [18–20]. Activated cells are phagocytic, able to secrete a large variety of biologically active substances, and involved in the processing and presentation of antigens to lymphoid cells. This might be the reason why no differences were found on resting peritoneal macrophages in mice of the CSP group. Thus, it might be suggested that in vivo macrophage activation by Onco-BCG or Ehrlich tumor cells was necessary to display the effects imposed by the housing condition. This suggestion is relevant because it links the reported macrophage data of CSP mice to possible neuroimmune alterations induced by the Mycobacteria or by the tumor cells on the animals’ internal milieu. A similar relationship was already reported to exist after physical and psychological stress application [7].

Within the context of this discussion, stress is defined as suggested elsewhere [21], that is, as a process by which an organism responds to internal or external, environmental or psychological events that pose a challenge or a danger to it. Stressors are known to alter immune function [21–24]. Psychological stressors are reported to increase NA activity within the brain [25, 26]. A positive correlation is reported to exist between the decrease in NA levels and turnover following stressors and the stress responses induced by those stimuli on laboratory animals [27]. Since NA levels and turnover were previously reported to be decreased in mice that lived with a sick cage mate [11], it seems possible to attribute the results reported here on in vivo macrophage activity to the psychological stress imposed to CPS mice by the living condition. In this respect, it was shown that nonshocked mice that observed conspecifics receiving foot shocks developed gastric ulcers [28] and presented decreased NA levels and increased NA turnover similar to the mice that received the foot shocks [29].

An increased respiratory burst, with the consequent generation and release of reactive oxygen intermediates such as H$_2$O$_2$ and superoxide anion (O$_2^-$) is one of the characteristics of activated macrophages [30]. Thus, and at first sight, it might be thought that peritoneal macro-

**Fig. 3.** Ascitic tumor growth in mice that lived (CSP) or not (CHP) with a sick cage mate for 11 days. The figure shows the ascitic fluid volume (a), the number of tumor cells per milliliter (b) and total tumor cells per animal (c). Data are means ± SD of 8 animals per group. * p < 0.05 (Student’s t test compared to respective controls).
phagocytes retrieved from Onco-BCG- or Ehrlich tumor cell-treated CSP mice present an increased in vitro activity compared to those collected from CHP mice. Indeed, data on oxidative burst were higher in the presence of *S. aureus* in CSP mice. However, this fact is in the opposite direction to those reported here for in vitro peritoneal macrophage *S. aureus* phagocytosis. A decrease in both percentage and intensity of in vitro *S. aureus* phagocytosis was found in macrophage collected from CPS mice injected with Onco-BCG and Ehrlich tumor cells. We have no data to further explain this controversy. However, this fact was already reported in other experiments [7, 31–33]. In this respect, the molecular mechanisms related to macrophage activation are not related to identical processes and/or membrane receptor activation [34, 35] and thus, are not necessarily the same.

The decrease in macrophage activity reported here is relevant because it is common knowledge that macrophages play an important role in innate immunity against bacteria and tumor cells [36, 37]. Thus, the decreased resistance of CSP mice to Ehrlich tumor growth might be understood as related to the decreased peritoneal macrophage activity they present. For instance, Ehrlich tumor cells elicit a strong host immune response [12, 13], and an inverse relationship between macrophage activity and Ehrlich tumor growth was reported to exist [7, 15, 38]. Foot shock stress suppresses immune function in rats and decreases their resistance to a tumor challenge [39]. Cold water or immobilization stress suppresses the anti-tumor activity of mononuclear phagocytes [40, 41]. Thus, it seems feasible to suggest that the presently observed decrease in the resistance to Ehrlich tumor might be causally related to the decreased macrophage activity observed after psychological stress imposed by the living situation on CSP mice. Indeed, the psychological stress generated through the use of a communication box (where animals received no foot shock but were exposed to responses delivered by foot shock stress in a conspecific) decreased both peritoneal macrophage activity and animals’ resistance to Ehrlich tumor growth [7]. Thus, the data now being reported provide evidence that the psychological stress imposed in mice by the condition of living with a sick cage mate decreases macrophage activity and, as a consequence, their resistance to Ehrlich tumor growth.

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