Lymphocyte proliferation stimulated by activated human macrophages treated with Canova

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Introduction: Canova (CA) is a homeopathic medication with immunomodulatory properties, recommended for patients with a depressed immune system. CA has been reported to increase in leukocyte numbers, cellular differentiation and reduction in tumor size.

Aim and method: Since CA may stimulate lymphocyte differentiation, proliferation, and/or survival, the aim of the present study was to compare the mitotic index (MI) of phytohemagglutinin-stimulated human lymphocytes cultured in a medium supplemented with human macrophages activated by CA, with lymphocytes cultured in a medium without CA-treated macrophages.

Results: In this study, the MI of lymphocyte cultured received the medium containing CA-stimulated macrophages showed a higher proliferation index (p < 0.01) than the lymphocytes cultured in a medium without CA-treated macrophages. Our results suggest that CA treatment, in addition to activating macrophages, indirectly induces lymphocyte proliferation and has potential as a new adjuvant therapeutic approach.

Keywords: Canova; Activated macrophages; Lymphocyte proliferation; Mitotic index; Homeopathy

Introduction

Canova (CA) is a complex homeopathic medicine containing Aconitum napellus, Thuya occidentalis, Bryonia alba, Lachesis muta and Arsenicum album. CA is indicated for patients whose immune system is depressed. CA treatment seems to enhance an individual’s ability to trigger a specific immunologic response against several pathologic conditions.1

CA activates macrophages both in vivo and in vitro. In vitro production of tumor necrosis factor-alpha by macrophages is significantly diminished by CA.2 NADPH oxidase activity was increased as was that of inducible nitric oxide synthase (iNOS), producing reactive oxygen species (ROS) and nitric oxide (NO), respectively.3 Cesar et al.1 reported the effect of in vitro administration of the medication on the mononuclear differentiation of the bone marrow cells. Our research group has previously demonstrated that CA treatment does not induce cytotoxic or genotoxic effects at the chromosomal level.4

Since CA may lead to lymphocyte differentiation, proliferation, and/or increased survival, the aim of the present study was to evaluate the mitotic index (MI) of human lymphocytes stimulated by the mitogenic action of phytohemagglutinin (PHA)5,6 cultured in a medium supplemented with human macrophages activated by CA compared with the MI of lymphocytes cultured in a medium without macrophages treated with CA. The MI represents the proportion of cells in the M-phase of the cell cycle.7 Thus, an increased MI reflects a stimulation of cell-cycle progression and/or gain of proliferative capacity.
Material and methods

Canova

‘Canova do Brasil’, a Brazilian company, holds the international patent of this medicine (www.canovadobrasil.com.br). CA is produced in drops, inhalant and intravenous forms, and sold only in authorized pharmacies and laboratories. CA is standardized and authorized by competent agencies for medicinal application. It is currently registered as a magistral formula, according to Law No 5991/73. The preparation of this commercial medicament follows Hahnemannian homeopathic techniques. Mother tinctures are purchased from suppliers authorized by the Brazilian Health Ministry. The final product contains A. napellus (Ranunculaceae) 11dH, B. alba (Cucurbitaceae) 18dH, Thuja occidentalis (Cupres-saceae) 19dH, A. album (arsenious trioxide) 19dH and L. muta (Viperidae) 19dH and less than 1% ethanol in 90% of adherent cells were macrophages2 and the prepara-
tion contains streptomycin (USB, Cleveland, OH, USA). More than 0.0016% was added 2 h prior to harvest. Cells were harvested by centrifugation and treated with 0.075 M KCl at 37°C for 20 min, the hypotonic solution caused cells to swell and increased metaphase spreading. Cells were then centrifuged and fixed in 1:3 (v/v) acetic acid:methanol. Finally, air-dried slides were prepared and stained with 3% Giemsa solution (pH 6.8) for 8 min.

Slides were analyzed with an optical microscope and MI (number of metaphases per 2000 lymphoblasts per culture) was determined in CA-treated cultures and controls. All MIs were counted twice by two different researchers (Figure 1).

Statistic analysis

F test (ANOVA) and χ² test were used to assess differences between MI values and between activation index of CA-treated and non-treated macrophages (activation ratios), respectively. In both analyses, the confidence interval was of 99% and p values less than 0.01 were considered significant.

Results

Human macrophage culture

Cells in the control groups were mainly resting macrophages, however activated macrophages were also present.

Human lymphocytes proliferation in supplied medium

Short-term lymphocyte cultures were initiated according to a standard protocol.6 Human lymphocytes were cultured in the supplemented medium (SM) used for macrophage activation by CA treatment, with the addition of 2% PHA. This culture medium was adjusted to 78% HAM-F10 and 20% heat-inactivated fetal calf serum. Lymphocytes were cultured for 72 h (37°C, humidified atmosphere with 5% CO2).

Lymphocyte cultures with the medium used for macrophage activation control groups 1, 2 and 3 (control medium – CM) were also performed. In a 4th control group, lymphocytes were cultured as for standard lymphocyte culture (new medium – NM).5 Metaphase preparations were obtained as described by Moorhead et al.8 In order to obtain a sufficient number of metaphases, colchicine at a final concentration of 0.0016% was added 2 h prior to harvest. Cells were harvested by centrifugation and treated with 0.075 M KCl at 37°C for 20 min, the hypotonic solution caused cells to swell and increased metaphase spreading. Cells were then centrifuged and fixed in 1:3 (v/v) acetic acid:methanol. Finally, air-dried slides were prepared and stained with 3% Giemsa solution (pH 6.8) for 8 min.

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Results

Human macrophage culture

Cells in the control groups were mainly resting macrophages, however activated macrophages were also present.
The index of macrophage activation in control group 1 (no treatment) was 41.5%, in control group 2 ((10% PBS), 46.9% and in control group 3 (10% 0.1% ethanol), 45.2%. Almost all cells (88%) from the CA-treated group were activated, as defined by morphological alterations. There were no statistical differences among the control groups. The CA-treated group was significantly different from the control groups ($p < 0.01$) (Table 1).

### Human lymphocytes proliferation in supplied medium

The MI of lymphocytes that were cultured in supplemented medium was significantly higher than the MI of groups cultured in control medium ($p < 0.01$) and the NM group ($p < 0.01$). No significant difference was observed between CM groups 2 and 3. However, the MI of CM group 1 was significantly higher than the MI of CM groups 2 and 3. The MI of NM group was also significantly higher than the CM groups ($p < 0.01$) (Table 2).

### Discussion

Macrophages play an essential role in host defence against infection and tumoral cells. A large body of data indicates that macrophages must be activated in order to be effective. In this study, almost all cells from the treated group were activated (88%). This activation ratio was similar to the ratio observed in CA-treated mice macrophages.

Cells from our control groups were mainly resting macrophages, but activated macrophages were also present. The presence of activated macrophages in these samples may be due to the low grade of inflammation generally observed in ascites patients. The index of macrophages activation in control groups was about 41.5%. The macrophage activation index in the treated group was significantly different from control groups. Previous studies demonstrated that CA activates macrophages both in vivo and in vitro. Improvement in immune response of CA-treated mice was demonstrated in studies with Sarcoma cell line 180. A reduction in sarcoma size was observed and a significant infiltration of lymphoid cells, granulation tissue and fibrosis occurred, surrounding the tumor. All animals from the treated group survived, and in 30% of them a total regression of the tumor was observed. Treatment with CA increased total numbers of leukocytes, specifically T, CD4, B and NK cells increased. These results suggested a direct or indirect action of the CA on hematopoiesis. So the bone marrow cells were treated and analyzed by light, transmission and scanning electron, and bifocal microscopy, and flow cytometry. All microscopy techniques showed that monocytic lineage (CD11b) and stromal cells (adherent cells) were activated by treatment. CA also increased cell clusters over adherent cells, suggesting areas of proliferation and differentiation.

Activated macrophages stimulate T-cells and lead to an increased cytotoxic effect in response to tumoral growth or infections. Clinical observations include decreased in infection and concomitant reduction of inflammation in patients treated with CA.

Since CA stimulates macrophages, and indirectly stimulates and accelerates T-cell action, we evaluated the index of lymphocyte proliferation in a culture supplemented with macrophages activated by CA. Our results suggest cytokines and growth factors secreted by macrophages activated with CA treatment lead to an increased T-lymphocyte proliferation stimulated by PHA. The presence of PHA was fundamental in this study to stimulate T-cell proliferation. CA does not itself induce lymphocyte proliferation in vitro (data not shown).

The MI of lymphocytes cultured in SM was higher than the MI of lymphocytes cultured in CM (control 1–3) and in NM (control 4). Control 1 showed a higher MI than controls 2 and 3. These findings suggest some interference of PBS and ethanol in lymphocyte cultures and emphasize that CA treatment, despite its ethanol content, can indirectly lead to lymphocyte proliferation.

Control 4 (new medium) showed a higher MI than controls 1–3. This finding may be due to nutrient consumption by macrophages and alterations generated during culture time (122 h) in CM groups. Other lymphocyte cultures performed by our research group showed a higher MI (5.4) than that observed in control 4 (median 4.0) using peripheral bleed lymphocytes. This difference may be due to the fact that the patients of

### Table 1 Macrophage activation index of human samples cultured in different media

<table>
<thead>
<tr>
<th>Sample</th>
<th>CA treatment</th>
<th>Control 1</th>
<th>Control 2</th>
<th>Control 3</th>
<th>Control 4</th>
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<td>2.5</td>
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</tr>
</tbody>
</table>

Mean (SD) 88 (2.29) 41.5 (2.59) 46.9 (1.42) 45.2 (2.16)

* Cells with no treatment.
1 Cells treated with 10% PBS.
2 Cells treated with 10% of 0.1% v/v ethanol solution.
this study, who, although they had not had cancer chemo- or radiotherapy, were seriously ill and had ascites. Peripheral blood of controls used in mutagenesis studies are usually collected from healthy donors up to 30 years old and without history of smoking/drinking or chronic drug use.

In conclusion, CA treatment indirectly induced lymphocyte proliferation through activated macrophages. CA has potential as a new adjuvant therapeutic approach to known therapies.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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