Activation of mononuclear bone marrow cells treated in vitro with a complex homeopathic medication

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Abstract

Canova is a Brazilian homeopathic medication with immunomodulatory properties, recommended for patients where the immune system is depressed. Previous studies demonstrated that Canova induces up-regulation in numbers of leukocytes. The bone marrow microenvironment is composed of growth factors, stromal cells, extracellular matrix and progenitor cells that differentiate into mature blood cells. We now report the effect of in vitro administration of the medication on the mononuclear differentiation of the bone marrow cell. Swiss mice femurs were dissected and cleaned, and the cells of the marrow were flushed. The cells were plated, treated or not, incubated for different times and processed for light, transmission and scanning electron, and confocal microscopy analysis. Bone marrow cells showed an enhanced proliferation in vitro in response to Canova medication and Canova plus M-CSF and an increase was also observed in the numbers of the cell niches and ring-shaped nuclei cells. Confocal and transmission and scanning electron microscopy showed the stages of monocyte maturation, with resting and activated cells. With Canova treatment there was a marked increase in cell size, which is mainly attributable to the augmented cytoplasm, an increase in the number of mitochondria, expansion of the RER and an enlarged Golgi. The response to Canova treatment indicates that it influences mononuclear differentiation and activation of bone marrow progenitor and stromal cells.

Keywords: Bone marrow cells; Stromal cells; Mononuclear cells; Activation; Canova medication

1. Introduction

Great strides in developing western medicine in the last century have been made. Countless diseases were eliminated or controlled through advances in immunology, parasitology, and the discovery of antibiotic drugs and vitamins. Conventional Western, or Allopathic, medicine has achieved significant leaps in preserving both the quality and longevity of life, and it is important to recognize this contribution. However, conventional medicine is a wide label, and not all conventional medicine is beneficial. While in many cases symptoms are relieved, cured or prevented many allopathic medicines can do a lot of harm to the body. The symptoms of a disease, for homeopathy, are the body’s attempt to cure itself. By contrast, conventional medicine often works by suppressing the body’s natural reactions. In the homeopathic method, the immune system of the body is stimulated to support the healing system. This practice accomplishes the equilibrium or homeostasis of the whole body.

Canova (CA) is a Brazilian complex homeopathic medicine produced from Aconitum napellus, Thuya occidentalis, Bryonia alba, Lachesis muta and Arsenicum album. CA is found in homeopathic drugstores and is indicated for patients whose immune system appears to be depressed. Clinical observation of these patients confirmed the success of this treatment. It seems to enhance the individual’s own immunity to trigger a particular immunologic response against several pathological conditions. Previous studies demonstrated that CA activates macrophages both in vivo and in vitro. It was observed that the in vitro production of tumor necrosis factor-α (TNFα) by macrophages is significantly diminished when the medicine is administrated (Piemonte and Buchi, 2002). NADPH oxidase activity was
increased as well as that of inducible nitric oxide synthase (iNOS), consequently producing reactive oxygen species (ROS) and nitric oxide (NO), respectively (De Oliveira et al., 2006). CA stimulates an increase of the endosomal/lysosomal system as well as the phagocytic activity of macrophages when interacted with Saccharomyces cerevisiae and Trypanosoma cruzi epimastigotes (Lopes et al., 2006). The modulatory effects of CA were also observed both in vivo and in vitro in experimental infection by Leishmania amazonensis, controlling infection progression and limiting its dissemination (Pereira et al., 2005). Moreover, it is neither toxic nor mutagenic (Seligmann et al., 2003). Similarly, the improvement in immune response of CA-treated mice was demonstrated in studies with Sarcoma 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 shown. The treatment with CA increased total numbers of leukocytes. Among lymphocytes, T CD4, B and NK cells increased (Sato et al., 2005). These results suggested a direct or indirect action of the CA on hematopoiesis. So the bone marrow cells were treated and processed for light, transmission and indirect action of the CA on hematopoiesis. The adherent cell layers elaborate soluble factors and deposit an extracellular matrix, which in turn influences hematopoietic proliferation and differentiation (Abud et al., 2006).

The hematopoietic microenvironment influences the growth and differentiation of hematopoietic cells. The adherent cell layers elaborate soluble factors and deposit an extracellular matrix, which in turn influences hematopoietic proliferation and differentiation. The differentiation of mononuclear cells (monoblasts, promonocytes and monocytes) and the differentiation of monocytes into macrophages are supported by macrophage colony-stimulating factor (M-CSF). M-CSF stimulates predominantly the proliferation of progenitors committed to macrophage lineages (Kurosaka et al., 1999; Sweet and Hume, 2003; Hassan et al., 1994; Shima et al., 1995; Imada et al., 2005).

CA medication has been successfully used in clinics and several experimental studies have been carried out to examine the medication’s biological activity and mechanisms of activation of macrophages, although more experiments are necessary to complete the knowledge on the action of Canova. Mononuclear phagocytes, represented by monocytes, tissue macrophages, dendritic cells (DCs), microglia and osteoclasts, maintain tissue homeostasis and provide a first line of defense against invading pathogens. Thus, the aim of this study is further characterize the in vitro effects of CA of mononuclear cultured cells of mice bone marrow.

2. Materials and methods

2.1. Animals

Male Swiss mice from the Rockefeller lineage (8–12 weeks old) were used. The animals had free access to food and water. All recommendations of the National Law (No. 6.638, November, 5, 1979) for scientific management of animals were observed and the Institutional Animal Care Committee of the Federal University of Paraná approved all related practices. Experiments were carried out in the Laboratory of Research in Neoplastic and Inflammatory Cells, which has a management program for residues.

2.2. Canova medication

Canova (CA) is a commercial medicine that represents a new form of immunomodulatory therapy and follows Hahnemann’s ancient homeopathic techniques. Canova is an aqueous, colorless and odorless solution produced and sold in Brazilian authorized drugstores. Mother tinctures are purchased from authorized agencies indicated by the Brazilian Health Ministry. These agencies assure the quality (endotoxin free) and physico-chemical composition of its products. Starting from the original mother tincture (in the case of a plant this is an ethanolic extract) several dinamizations – succussion (shaking) and dilution in distilled water – are performed. Decimal dilutions (dH) are prepared. The final commercial product Canova, is composed of 11 dH Aconitum napellus (Ranunculacea), 19 dH Thuya occidentalis (Cupresacea), 18 dH Bryonia alba (Cucurbitaceae), 19 dH Arsenicum album (arsenic trioxide), 18 dH Lachesis muta (Viperidae) and less than 1% ethanol in distilled water (www.canovadorbrasil.com.br). In our experiments, we used the commercial product purchased from Canova do Brasil. The CA was always vigorously shaken, a process called succussion, immediately before each treatment.

2.3. M-CSF

The L929 cells were seeded in culture bottles (150 cm²), to a density of 1 × 10⁵ cells per bottle, and cultivated in DMEM (Dulbecco’s Modified Eagle’s Medium) rich in glucose (Chem Sigma Co., St Louis, MO, USA), supplemented with 5% of FSB (fetal bovine serum) (Chem Sigma Co.). The conditioned medium was gathered after 7 days, at which period the cells had arrived at confluence. The above mentioned conditioned medium containing the produced M-CSF was centrifuged to eliminate the cells in the conserved suspension and maintained at −20 °C until the moment of its use. It can be conserved under these conditions for more than six months. Once defrosted, the aliquots were preserved at 0 °C to avoid degradation of M-CSF, due to an excess of cycles of freeze-warming.

2.4. Bone marrow preparation

Mice were anesthetized with Ether and cervical dislocation was used to the animal’s euthanasia. Femurs were dissected and cleaned. Epiphyses were removed and the marrow was flushed with DMEM containing 10% FBS with 1 µg/ml ciprofloxacin (Sigma Pharma), and purified by passing 1.077 Ficoll-hypaque (FH) (Chem Sigma Co.). This product is a solution of Ficoll and sodium diatrizoate adjusted density of 1.077. When blood is overlaid on this reagent and the solution is centrifuged,
mononuclear cells concentrate at the plasma-reagent interface (Boyum, 1968). For the FH method, 10 ml of the flushed bone marrow was layered onto 3 ml of the FH solution in a sterile 15 ml centrifuge tube. The tube was capped and then centrifuged in a tabletop centrifuge at 1500 rpm for 40 min at ambient temperature (21 ± 25°C). A diffuse band of leukocytes (mononuclear cells) formed above the erythrocytes and polymorphonuclear cells, which were seen together in pellet. This diffuse band layer of cells was aseptically removed with a pipette and transferred to a sterile 15 ml centrifuge tube. The cells were washed with PBS due toxicity of the Ficoll to cells. Mononuclear cells were counted in a Neubauer chamber and suspended in DMEM with 10% SFB supplemented with 1 µg/ml ciprofloxacin and 4 mM L-glutamine (Chem Sigma Co.).

2.5. Liquid culture

Cells were adjusted to a concentration of $2.5 \times 10^5$ ml$^{-1}$, and plated on 24 well culture plates (for adherent and non-adherent cell experiments) with glass cover slips and maintained at 37°C under a 5% CO$_2$ atmosphere for 48, 72 and 96 h. All experiments were performed at least three times in quadruplicate and four treatment groups were examined. The control did not receive any treatment, because our previous results demonstrated no statistical differences between the control group and the ethanolic aqueous solution group. The cells from the Canova group were treated with 20% Canova. The M-CSF group received 30% M-CSF and in the Canova plus M-CSF (CA + M) group the cells were treated with 30% M-CSF and 20% CA. The CA and the CA + M groups also received a 1% CA dose administered daily to the culture, after 24 h of the previous treatment.

2.6. Morphological assay

Cells ($2.5 \times 10^5$) were plated into culture plates with cover slips for morphological analysis (Buchi and Souza, 1992). They were maintained as described above. After 48, 72 and 96 h, they were rinsed with phosphate buffer solution (PBS), fixed in Bouin, stained with Giemsa, dehydrated and mounted with Entellan$^\text{®}$. Adhered cells were observed by light microscopy using a Nikon Eclipse E200 microscope, which detected structural characteristics of lymphocytes, resident macrophages, activated macrophages, cell niches and ring-shaped nuclei cells. For each cover slip, 100 cells were counted from the total cells plated ($2.5 \times 10^5$). Ten cover slips for each treatment and time were observed and counted. Mean data obtained in percentage was transformed as described in the item statistical analysis.

2.7. Transmission electron microscopy

Differences among the groups were mainly found after 96 h of culture. Thus, cells ($2.5 \times 10^5$), cultivated for 96 h, were fixed with 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.2), washed and post-fixed in 1% OsO$_4$ for 30 min in the dark at room temperature (Buchi et al., 1993). After washing, the cells were dehydrated using increasing ethanol concentrations. Cells were CO$_2$ critical point dehydrated, metalized and observed using a JEOL JSM-6360 LV SEM scanning electron microscope in the Electron Microscopy Center at the Federal University of Paraná. A GATAN CCD camera and GATAN digital micrograph software were used to obtain the digital images.

2.8. Scanning electron microscopy

Surface markers for CD 11b were characterized for adherent cells ($2.5 \times 10^5$). All antibodies used were from a mouse lineage panel specific for bone marrow, purchase from BD Pharmingen. As this protocol requires various washing steps, we incubated the cells for 96 h to form a complete monolayer on the plate. This assures that at the end of the process it still had adherent cells. Immunostaining was performed according to standard protocols using commercially available antibodies CD11b (Mac-1) for monocyte/macrophage cells. The cells were maintained on ice, blocked with 1% PBS/BSA (bovine serum albumin) and incubated with 1 µg biotinylated antibody in 1% PBS/BSA for 40 min (Pearce et al., 2004). After washing, they were fixed in 2% paraformaldehyde for 30 min and the aldehyde radicals were blocked with 0.1 M glycine in PBS. The cells were incubated with phycoerythrin (PE) labelled secondary antibody in PBS for 40 min. The nuclei were stained with 300 nM DAPI (4,6-diamidino-2-phenylindole, dihydroxychloride) (Molecular Probes, Eugene, OR, USA), which was added 15 min before cell observation. The cells were washed with PBS, mounted with fluoromont-G and the fluorescence was analyzed with a Radiance 2001 laser scanning confocal microscope (BIO-RAD) coupled to an Eclips E-800 (Nikon).

2.10. Statistical analysis

Percentage data, obtained from light microscopy analysis, were transformed into $\sqrt{x + 0.5}$ consequently presenting a normal distribution. These data were submitted to variance
analysis (ANOVA) with a factorial diagram to determine the statistical significance. The Tukey test was performed when the effects of interaction was significant. The level of significance was taken at (* \( p < 0.05 \), ** \( p < 0.01 \)). Data are representative of three independent experiments.

3. Results

3.1. Morphological assay

The mononuclear cells were characterized as lymphocytes, cell niches, ring nuclei cells, and resident and activated macrophages (Fig. 1). To better express the results, all morphological assays are summarized in Table 1. The cell density increased in all groups along the culture time and this increase is higher with M-CSF, CA + M and Canova treatments, respectively.

3.2. Lymphocytes

The lymphocytes were characterized by their classical morphology; rounded small nuclei with condensed chromatin and a thin cytoplasm. A lower cell proliferation was constant in all treatment groups and of culture time. In the CA + M and M-CSF groups, the cultures had a significantly decrease in the numbers of lymphocytes when compared with the control at the end of 96 h treatment. Canova treatment also significantly decreased the number of lymphocytes, but these did not drastically disappear with time, as in the CA + M and M-CSF groups (Table 1; Graph 1).

3.3. Resident macrophages

Resident macrophages were characterized by the classical fibroblast-like morphology with a central nucleus, little

![Fig. 1. Microphotographs of control and treated cultures. After 96 h, mononuclear cells were fixed and stained with Giemsa. (a) Control cells; (b and c) Canova treated cells. In the control group they have a resting cell (RC), mostly round, with fewer membrane extensions, and a classical fibroblast-like aspect. The Canova and M-CSF groups showed activated cell (AC), cell niches (CN) and ring-shaped nuclei cells (RN). Original magnification with 40 objective for (a and b) and 100 objective for (c).]
cytoplasm with few extensions, resulting in an elongated and not so spread appearance. These cells have a small, condensed and "kidney" shaped nuclei. Although the absence of growth factors, after 96 h resident macrophages stayed adhered to the glass coverslip, although their number decreased afterwards (Table 1; Fig. 1).

3.4. Activated macrophages

Their classical morphology, an increased membrane ruffling, increased spreading, and a large and euchromatic nucleus characterized activated macrophages. These morphological characteristics are known to be typical of activated macrophages. In the control group, they were mostly round, cytoplasm with few extensions, resulting in an elongated and not so spread appearance. These cells have a small, condensed and "kidney" shaped nuclei. Although the absence of growth factors, after 96 h resident macrophages stayed adhered to the glass coverslip, although their number decreased afterwards (Table 1; Fig. 1).

Bone marrow cells were differentiated by morphological cell characteristics. The % mean data were analyzed with ANOVA with a factorial diagram and Tukey test. Results are expressed as transformed number, representative of three independent experiments. (CA = Canova; CA + M = Canova plus M-CSF; M = M-CSF). Different treatments were analyzed over different times. Capital letters indicate comparison within the line (time of culture) and small letters within the column (treatments). The same letters indicate that the there is no difference between them and different letters indicate statistical difference between them.

### Table 1
Morphological analysis of adherent cells

<table>
<thead>
<tr>
<th></th>
<th>Treatment/time</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
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<tr>
<td><strong>Lymphocytes</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>0.7979 a (A)</td>
<td>0.7151 ab (B)</td>
<td>0.7177 a (B)</td>
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<td>Canova</td>
<td>0.7679 b (A)</td>
<td>0.7273 a (B)</td>
<td>0.7152 a (B)</td>
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<td>Canova + M-CSF</td>
<td>0.7199 c (A)</td>
<td>0.7204 ab (A)</td>
<td>0.7071 a (A)</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>0.7316 c (A)</td>
<td>0.7107 b (B)</td>
<td>0.7071 a (B)</td>
<td></td>
</tr>
<tr>
<td><strong>Resident macrophages</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.8279 a (A)</td>
<td>0.6873 a (B)</td>
<td></td>
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<tr>
<td>Canova</td>
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<td>0.8520 a (A)</td>
<td>0.5307 b (B)</td>
<td></td>
</tr>
<tr>
<td>Canova + M-CSF</td>
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<td>0.6004 c (AB)</td>
<td>0.5271 b (B)</td>
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<td>M-CSF</td>
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<td>0.7109 b (B)</td>
<td>0.4924 b (B)</td>
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<tr>
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<td>0.1555 b (B)</td>
<td>0.2974 b (A)</td>
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<tr>
<td>Canova</td>
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<td>0.1044 b (B)</td>
<td>0.4488 a (A)</td>
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<tr>
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<td>0.3507 a (B)</td>
<td>0.4491 a (A)</td>
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</tr>
<tr>
<td>M-CSF</td>
<td>0.1709 b (C)</td>
<td>0.2674 a (B)</td>
<td>0.4703 a (A)</td>
<td></td>
</tr>
<tr>
<td><strong>Cell niches</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>0.7071 b (A)</td>
<td>0.7071 b (A)</td>
<td>0.7071 b (A)</td>
<td></td>
</tr>
<tr>
<td>Canova</td>
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<td>0.7085 b (A)</td>
<td>0.7091 b (A)</td>
<td></td>
</tr>
<tr>
<td>Canova + M-CSF</td>
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<tr>
<td>M-CSF</td>
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<td>0.7169 a (B)</td>
<td>0.7283 a (A)</td>
<td></td>
</tr>
<tr>
<td><strong>Ring nuclei cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>0.7107 b (A)</td>
<td>0.7071 b (A)</td>
<td></td>
</tr>
<tr>
<td>Canova</td>
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<td>0.7157 a (AB)</td>
<td>0.7112 ab (B)</td>
<td></td>
</tr>
<tr>
<td>Canova + M-CSF</td>
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<td>0.7265 a (A)</td>
<td>0.7201 a (A)</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>0.7123 b (A)</td>
<td>0.7089 b (A)</td>
<td>0.7119 ab (A)</td>
<td></td>
</tr>
</tbody>
</table>

Graph 1. Number of lymphocytes among adherent cells. Different treatments were analyzed over the different times. Capital letters indicate comparison within the line (time of culture) and small letters within the column (treatments). The same letters indicate that the there is no difference between them and different letters indicate statistical difference between them.

Graph 2. Number of activated macrophages among adherent cells. Different treatments were analyzed over the different times. Capital letters indicate comparison within the line (time of culture) and small letters within the column (treatments). The same letters indicate that the there is no difference between them and different letters indicate statistical difference between them.

3.4. Activated macrophages

Their classical morphology, an increased membrane ruffling, increased spreading, and a large and euchromatic nucleus characterized activated macrophages. These morphological characteristics are known to be typical of activated macrophages. In the control group, they were mostly round,
with fewer membrane extensions. The addition of Canova, M-CSF and CA + M to the cultures increased cell spreading and cell density. CA + M gave the highest number of characteristic activated macrophage morphology, followed by M-CSF, Canova and control group, which had the lowest. At 96 h CA, CA + M and M gave the same amount of activated cells (Table 1; Fig. 1; Graph 2).

3.5. Cell niches

Cell niches were associated with the adherent layer in small foci over the stromal cells. The M-CSF group showed a higher presence of cell niches and it was increased with culture time. In the control group, these clusters were not observed and Canova and CA + M groups evidenced an intermediate number of these cells (Table 1; Fig. 1; Graph 3).

3.6. Ring-shaped nuclei cells

These cells exhibited constricted ring-shaped nuclei with a wide cytoplasmic center. The Canova and CA + M groups had the higher number of these cells. This number decreased with time for all groups except the Canova plus M-CSF treated group (Table 1; Fig. 1).

3.7. Transmission electron microscopy (TEM)

3.7.1. Non-adherent cells

The majority of cells found in the culture supernatant was round and had a large nucleus containing a large nucleolus. There were cells with a resident and activated morphology. The same had a nucleus with condensed chromatin, sparse mitochondria and few organelles. The activated cells had a

![Fig. 2. Transmission electron-micrographs of control and treated cultures. (a) Supernatant control cells with resting morphology, nucleus with abundant heterochromatin and a moderate amount of cytoplasm. (b–d) Supernatant and adherent Canova treated cells with activated morphology, nucleus with many euchromatin, and fingerlike projections, a Golgi apparatus is present in the juxtanuclear region, rough surfaced endoplasmic reticulum and elongated mitochondria. RN, Ring-shaped nucleus; N, nucleus; M, mitochondria; RER, rough endoplasmic reticulum; G, golgi apparatus.](image-url)
A nucleus with abundant euchromatin and an evident nucleolus. Aggregates of free ribosomes and rough endoplasmic reticulum were prominent. Many elongated mitochondria were dispersed in the cytoplasm. Many finger-like projections were seen at the cell surface, some of them contacting other cell. The Golgi apparatus showed great number stack of membranes. All these morphologic characteristics show an intense cell metabolism. These results were found in the all groups (Fig. 2).

3.7.2. Adherent cells

The adherent cells were characterized by an increase in cell size, with augmented cytoplasm. The characteristics of resident and activated cells are as cited above, as it can be seen in Fig. 2. The activated cells contained numerous electron-dense granules, surrounded by membranes, which were larger than those observed in non-adherent cells. The Golgi apparatus was composed of few stacks of membranes in control cells; in Canova and CA + M groups an enlarged Golgi zone was apparent. The adhered adjacent cells developed elaborate cytoplasm projections, similar to those described for non-adherent cells. The Canova plus M-CSF group and Canova treated group showed more finger-like projections connecting the cells when compared with the Control group. The space between two cells in adhesion areas contained irregularly distributed extra-cellular particles, constituting a septate-like zone (Fig. 3).

3.8. Scanning electron microscopy (SEM) and confocal microscopy

The majority of adhered cells presented macrophages morphological characteristics. This fact was confirmed by the SEM results and almost all cells from the Canova treated group were activated with many projections. The higher cell density in Canova and CA + M groups, constituting a monolayer with a greater number of more spread cells. In the control group, there were fewer and smaller cells (Fig. 4). These observations were confirmed by CD11b positive staining, observed by confocal microscopy. Almost all adherent cells, from all groups, were positively stained with CD11b. Clusters of CD11b receptors were found on the surface of most cells (Fig. 5).

4. Discussion

Abud et al. (2006) showed that the treatment did not modify the expression of the analyzed surface markers, as well as cytokines production. All microscopy techniques showed that the monocytic lineage (CD11b+) and the stromal cells (adherent cells) were activated by treatment. This was expected since adherent cells were mostly macrophages and CA is a macrophage activator (Piemonte and Buchi, 2002; Sato et al., 2005). Each investigator showed that after CA treatment, the total number of leukocytes increased, suggesting a direct or
indirect action of the Canova medication on hematopoiesis. A role for CA in regulating macrophage activation and inflammation was supported by in vitro and in vivo studies, in which CA enhanced macrophage phagocytic activity was showed against non-infective forms (Lopes et al., 2006). An immune response, increasing the number of leukocytes and improving the tumoricidal activity of CA-treated mice, was demonstrated in studies with Sarcoma 180 (Sato et al., 2005). A specific population of cells was now separated from bone marrow. Bone marrow-derived mononuclear cells constitute a

Fig. 4. Scanning electronmicrographs of adherent bone marrow treated cells. (a and b) Give a general view of control cultures with less and smaller cells, with (c and d) a general view of Canova treated cultures with higher cell density, almost all cells being activated with many projections, constituting a monolayer.

Fig. 5. Confocal microscopy of bone marrow cells. The adherent cells were stained with CD11b antibodies (red) and DAPI (blue), fixed and observed using confocal microscopy. The majority of adherent cells was monocyte/macrophage, positive for CD11b. (a) Shows the control culture and (b) the Canova treated group. Bar 30 μm.
population of myeloid and lymphoid cells that can proliferate/differentiate (Bender et al., 2004) in response to specific stimuli, such as M-CSF, which was used as a positive control.

We found few activated macrophages in the control group compared to the CA group (Graph 2). The Canova treatment rapidly differentiated the bone marrow resident macrophages into activated cells. Committed cells are capable to promptly adhere, thus the activated cells found in the control group may be explained by the fact that the differentiated macrophages were not removed as described by others (Lin et al., 2001). The pronounced macrophage spreading observed after 96 h in CA and CA + M groups was a typical morphological characteristic of activated cells, different from that of the control group, which had a small number of adherent cells after 4 days. These results corroborate with these of Dexter et al. (1976), which showed that after 72 h culture of mice bone marrow, the majority of adherent cells was phagocytic mononuclear cells with numerous cytoplasmic extensions, and an overall tendency to spread. Our electron microscopy analysis showed these alterations to occur in macrophages, which increased their cytoplasm volume, the mitochondria number, and the RER, and enlarged the Golgi apparatus. Occasionally, the contact processes of two adjacent macrophages alter the plasma membranes, the cell surfaces and the intercellular regions, as can be seen in Fig. 3. It can be observed that the cytoplasmic extensions modified the contact region, which had septate junctions that possibly function as adhesive elements to maintain the stroma structure. These results confirm previous studies in which Canova medicine altered the distribution of proteins related to adhesion and spreading in treated macrophages (Piemonte and Buchi, 2002).

It was suggested that M-CSF is a requirement for normal macrophage function (Sweet and Hume, 2003). This raises an important issue with regard to the many in vitro studies that have been performed on this cell type. Since, it is normally present in vivo and because its absence has gross effects on normal cell functions, M-CSF should be present in the culture medium during in vitro studies on macrophage function. Thus, the Canova group, despite not having received the M-CSF, had these characteristics, namely, a higher number of macrophages with an active morphology. As macrophages can produce a large amount of cytokines and growth factors and since these cells are the targets of Canova, a partial supply of the necessary molecules may be occurring with this treatment, indicating that the treated stromal cells supplied the needed factors necessary for survival.

Initially, the CA + M group presented the lower number of resident macrophages, probably because of the differentiation process of activated macrophages. It seems that the medicine and the M-CSF acted with synergy accelerating the differentiation process, in such a way that the M-CSF and the medicine alone took more time to give the same effort.

Although, there was a reduction of lymphocytes number in the control group, it was further reduced by the addition of M-CSF or CA + M. The CA group also significantly decreased the lymphocyte number, but these cells did not drastically disappear, as seeing in the CA + M and M-CSF groups. At the same time when there was a decrease in the lymphocytes number, it increased the number of cell niches in the M-CSF group. In contrast, it was not observed in the CA + M group (Graph 3). Considering that stromal cells produce many cytokines that mediate lymphocyte binding, inducing adhesion between them and the stromal cells (Miyake et al., 1991), we can suggest that the set of cytokines produced by M-CSF and by CA are different. We found an increase in cellular niches in the M-CSF group, and in the Ca group and Ca + M, the formation of niches was diminished suggesting that the soluble factors induced by CA are not promoting this type of adhesion in the stromal cells. On the other hand, the ring shaped nuclei cells that can be considered precursor cells (Biermann et al., 1999), were present and maintained their number in the culture in the Ca + M group (Table 1) suggesting therefore that the treatment stimulates the maintenance and proliferation of precursor cells of the myeloid lineage.

The fundamental role of the marrow microenvironment in hematopoietic regulation has been demonstrated by several in vitro and in vivo studies, which showed that marrow stromaL components can regulate and influence the production of young and mature hematopoietic cells (Holyoake, 1996; Long et al., 1990). Some cells with ring-shaped nuclei were described these cell types in mouse bone marrow as monocytes-myeloid precursor cells (Biermann et al., 1999). Studies indicated that these unusually shaped nuclei are more deformable than spheroid nuclei, facilitating passage through the blood vessel endothelial lining and a rapid migration through tissue interstitial spaces (Olin and Olin, 2005).

We now present evidence for an effect of Canova on expansion of mononuclear murine cell bone marrow, when cultured with or without M-CSF, emphasising that Canova is a non-toxic medication. Human therapeutic applications of living cells generated by cell culture are of great current interest in a number of clinical settings. One such application that has just been tested clinically is the ex vivo production of hematopoietic cells for treatment of patients after high-dose chemotherapy and/or radiotherapy. Because of cell plasticity, bone marrow cells have the ability to repopulate injured livers, promote angiogenesis, affect cardiac and skeletal muscles, and neurons (Dahlke et al., 2006; Yokoyama et al., 2006; Orlic et al., 2001; Ferrari et al., 1998; Mezey et al., 2000). Current bone marrow transplantation practices are generally effective for restoring the hematopoietic system after chemotherapy, but suffer from an extremely high cost, limited donor availability, and high patient morbidity and mortality. The feasibility of collection, ex vivo culture-expansion and intravenous infusion of human bone marrow-derived progenitor stromal cells, has therefore been proposed to augment or replace life-saving stem cell transplantation procedures. Thus far, much effort has been focused on the development of biological processes, which can generate potentially therapeutic cells.

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