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Micron 37 (2006) 277–287

micron

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Phagocytosis, endosomal/lysosomal system and other cellular aspects of macrophage activation by Canova medication

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Received 19 August 2005; accepted 20 August 2005

Abstract

Canova is a homeopathic medication with immunomodulatory properties, recommended for diseases where the immune system is depressed. Our research aims to study the activation of mice peritoneal macrophages when submitted to in vivo and in vitro Canova treatment. Morphological parameters and acid phosphatase activity were analyzed using light and transmission electron microscopy. Differential interference contrast microscopy, including serial time acquisition in living cells, was also performed. The results demonstrated a greater spreading ability in Canova treated macrophages, a higher phagocytic activity of non-infective microorganisms (*Saccharomyces cerevisiae* and *Tripanosoma cruzi* epimastigotes) and a tendency to lower the phagocytic activity of the infective microorganisms *T. cruzi* trypomastigotes and *Leishmania amazonensis,* when compared with control cells. Acid phosphatase activity was analyzed and showed that Canova treatment stimulates an increase of the endosomal/lysosomal system. Treated macrophages that do or do not interact with yeast present a higher number of acid phosphatase marked vesicles compared to control cells. In contrast, the activity of tartrate resistant acid phosphatase (TRAP), is lower in Canova treated macrophages. The net results demonstrate that Canova medication is an effective stimulator of macrophage activity.

Keywords: Macrophage; Activation; Endosomes; Lysosomes; Acid phosphatase; TRAP; Canova; Phagocytosis

1. Introduction

Canova medication (CA) is produced homeopathically 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 has confirmed successful treatment (Buchi & Vecchio, 2002). However, more accurate scientific studies are needed. CA seems to enhance the individual's own immunity to trigger a particular immunologic response against several pathological conditions. The first clinical study with patients carrying the acquired immune deficiency syndrome virus (HIV) demonstrated that CA decreased both viral load and opportunistic diseases (Sasaki, Mariano, Gurgel, & Probst 2001). Similarly, an immune response improvement of CA-treated mice was demonstrated in studies with Sarcoma 180. In this study, a reduction in sarcoma size was shown 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 total regression of the tumor was observed. Besides, among lymphocytes T CD4, T CD8, B and NK cells increased both in normally-treated and S180-treated mice groups (Buchi & Vecchio, 2002; Sato et al., 2005). Neither side effects nor toxicity were detected (Seligmann et al., 2003). Piemonte and Buchi (2002) demonstrated that mice macrophages treated with CA were activated according to morphologic, biochemical and molecular criteria, namely α5-β1 integrins, FC receptors and actin filament distribution were altered and TNF-α production was decreased. These results indicate an activation process (Gordon, 2003), suggesting that CA acts on macrophage pathways, supporting clinical data.

Macrophages play diverse functions including phagocytosis, tumor cytotoxicity, cytokine secretion and antigen presentation. They represent a defense line against pathogens and tumor cells, recognizing and destroying them (Young & Hardy, 1995; Peters, Gieseler, Thiele, & Steinbach, 1996;

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0968-4328/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micron.2005.08.005

Klimp, Vries, Scherphof, & Daemen 2002). Macrophages can be characterized in unstimulated, tissue-resident or fully activated stages. Activated macrophages characteristically demonstrate increased membrane ruffling, increased adhesion and spreading, stimulation of DNA synthesis, modified monokine secretion, increased lysosomal enzyme levels, altered phagocytic activity and increased bactericidal/tumoricidal activity (Cleary, Kelly, & Husband 1999). Phagocytosis is prominent in macrophages and critical for their functions. After closure of the phagosomal cup, the recently created phagosome undergoes sequential fusion events with early, then late endosomes and finally with lysosomes to yield a phagolysosome (Niedergang & Chavrier, 2004). The phagolysosome is an acidic compartment full of hydrolases, where the bulk of degradation of the engulfed contents is thought to occur. Internalized pathogens are killed inside the phagolysosome. In addition, antigen presentation occurs through phagocytosis and endosomal/lysosomal systems (Greenberg & Grinstein, 2002; Lee, Harrison, & Grinstein, 2003; Niedergang & Chavrier, 2004). Thus, endosomal/lysosomal systems are essential for macrophage functions. Acid phosphatase (AcP) is a hydrolase contained inside this system (Robinson & Karnovsky, 1983; Wiese et al., 1996) and is considered to provide cytochemical criteria for evaluation of macrophage activation (Karnovsky & Lazdins, 1978; Matsubara, 2002). In addition to AcP, the lysosomal system of macrophages contains a type-5 tartrate-resistant acid phosphatase (TRAP) (Bune, Hayman, & Evans, 2001). TRAP is critical to bone resorption in osteoclasts and in macrophages. It has been suggested that it plays a role in antigen processing (Janckila, Parthasarathyc, Parthasarathyc, Seelanc, & Yam 2002).

In this paper, we evaluate whether after treatment with CA macrophage functional properties change, such as ability to spread on substrate and the endocytic index of some microorganisms in response to stimuli. Ultrastructural and optical cytochemical reactions of acid phosphatases were also carried out to monitor endosomal/lysosomal systems. Biological response modifiers that are able to activate macrophages have been extensively studied (Klimp et al., 2002; Piemonte & Buchi, 2002) and in this context, Canova, a non-toxic homeopathic medication, represents a good choice for macrophage activation.

2. Materials and methods

2.1. Animals

Male Swiss mice of the Rockefeller lineage (10–12-week-old) were kindly supplied by the Instituto de Tecnologia do Paraná, TECPAR. Animals had free access to food and water. All recommendations of the National Law (No. 6.638, November, 05th 1979) for scientific management of animals were observed and the Institutional Animal Care Committee of the Universidade Federal do Paraná approved all related practices. Experiments were carried out at the Laboratory of Research in Neoplasic and Inflammatory Cells, which has a management program for residues.

2.2. Macrophages

Cells were harvested from peritoneal cavities with Phosphate Buffer Saline (PBS), pH 7.4, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 15 min of incubation, non-adherent cells were washed away and macrophages were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in an humidified atmosphere containing 5% CO_2. Each protocol required a specific number of plated cells. The number of cells was determined using a Neubauer chamber. All experiments were performed at least three times in quadruplicate and with three control groups: (1) cells with no treatment, (2) cells treated with PBS and (3) cells treated with a 0.1% v/v ethanol solution.

2.3. Canova medication

CA is a product prepared according to the Hanemannian homeopathic method. *Canova do Brasil* is a Brazilian company that holds the international patent. It is presently registered as a magistral formula, according to Law No. 5.991/73. Mother tinctures are purchased from authorized agencies indicated by the Brazilian Health Ministry. The final product, an aqueous, odorless and colorless solution, contains *Thuya occidentalis* (*Cupresaceae*) extracted from the bark; *Bryonia alba* (*Curcubitaceae*) extracted from fresh roots; *Aconitum napellus* (*Ranunculaceae*) extracted from fresh preparations of the intact plant, including the roots, at the beginning of flowering, combined with *Arsenicum album* (arsenic trioxide) and *Lachesis muta* (*Viperidae*) venom. These matrices have a warranty certificate, which assures quality (endotoxin free) and physic-chemical composition. Experiments were performed with commercial Canova purchased from *Canova do Brasil* (www.canovadobrasil.com.br).

2.4. Animal treatment

2.4.1. In vivo

Mice were treated at daily intervals for 7 days with 7 (l/g of commercial Canova injected subcutaneously. Three controls were performed: animals were injected either with (1) PBS or (2) a 0.1% v/v ethanolic solution (the vehicle of Canova medicine), or (3) were not treated. The animals were then killed with ether, their macrophages were washed from peritoneal cavities, counted (using a Neubauer chamber) and cultured for 24 h as described in Section 2.2. After that, treated and control cells were processed following routine protocols required by each experiment.

2.4.2. In vitro

Macrophages were harvested from untreated mice and cultured for 48 h as described in Section 2.2 and processed according to protocols required by each experiment. After 3 h

of culture, the treated group received 10% of CA and controls received 10% of PBS or 10% of 0.1% v/v ethanolic solution, based on the total medium volume. Controls without any treatment were also analyzed. After 24 h, a fresh dose of 1% CA, as well as control solutions in the control groups, was added without replacing the medium.

2.5. Spread ability

Macrophages from mice treated in vivo were harvested and plated into wells (3×10^5 cells/well) coated (or not) with extracellular matrix compounds (matrigel) in DMEM without FBS. After 120 min, the spread capacity in plastic or matrigel was observed using a phase contrast microscope (Leica), and up to 150–180 cells/sample were counted.

2.6. Microorganisms

Saccharomyces cerevisiae maintained at −20 °C were washed with PBS and resuspended in DMEM without FBS immediately before each experiment (Buchi & De Souza, 1992). *Tripanosoma cruzi* epimastigotes, Dm28c clone (Contreras et al., 1988) were maintained and grown in a liver infusion tryptose (LIT) medium (Camargo, 1964) at 28 °C. To obtain metacyclic trypomastigotes, the epimastigotes in the late exponential growth phase were harvested from the medium by centrifugation and processed according to Ávila et al., 2001. *Leishmania amazonensis* promastigotes (MHOM/BR/73/M2269) (Thomaz-Soccol et al., 1993) were maintained in a Tobie and Evans (Noleto et al., 2002) biphasic medium at 24 °C. Parasites were cultivated in a RPMI 1640 medium supplemented with 20% FBS for 7 days to give rise to multiplication and used in their log phase (Noleto et al., 2002).

2.7. Macrophage-microorganisms interaction and endocytic index

In vitro treated cells were seeded on glass coverslips in 24-well tissue culture plates (1×10^5 cells/well) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 15 min of incubation, non-adherent cells were washed away with PBS (pH 7.4) and macrophages were cultivated in DMEM, 10% FBS. Treated and control groups were established as described in Section 2.4. After 24 h of growth, macrophages were allowed to interact with the microorganisms cited above at a ratio of 10:1 for 2 h in a medium without FBS. The microorganisms were then washed away, the macrophages rinsed with PBS, fixed with Bouin's fixative, stained with Giemsa and mounted with Entellan. For each cover slip, about 100 macrophages were examined with an Olympus BHS microscope using an 100× objective lens.

The percentage of macrophages with ingested microorganisms and the mean number of intracellular particles per macrophages were determined. The endocytic index was calculated by multiplying the percentage of macrophages with ingested microorganisms and the mean number of microorganisms per macrophage (Buchi & De Souza, 1992, 1993).

2.8. Morphological and motion analysis of living macrophages

Analysis of live cells was performed to monitor cellular motion related to phagocytosis. Macrophages were CA treated in vitro and interacted with the yeast as described above. Cover slips were transferred to glass slides in the same culture medium without washing, then mounted as described in Fig. 1. Subsequently, cells were analyzed using DIC (Differential Contrast Microscopy), in a laser scanning confocal microscope (LSCM)—Radiance 2001 (Bio-Rad®) coupled to an Eclypse E-800 (Nikon®)—with a 60×/1.4 NA oil plan-apochromatic objective lens. A 543 nm Green HeNe laser was used and image acquisition was done with an E600LP (long pass) emission filter. Serial time acquisition, at 5 s intervals, during 20–30 min was performed using Bio-Rad Laser Sharp software.

In order to visualize the spreading in living macrophages, cover slips were washed with PBS, mounted on slides as described above using PBS instead of culture medium and observed immediately by DIC.

2.9. Acid phosphatase (AcP) and TRAP cytochemistry

TRAP and AcP detection were carried out according to recommendations of Sigma Diagnostics Acid Phosphatase Kit (386-A). After in vitro treatment, cells were fixed in citrate-acetone solution and AcP was visualized by a capture reaction with naphthol AS-BI phosphate as substrate and Fast Garnet GBC as acceptor. In order to detect total AcP activity, incubations were performed in the absence of sodium tartrate. In this case, staining corresponded to all AcP activities present in the macrophages. With the purpose of detecting only TRAP, sodium tartrate (TRAP inhibitor) was added to the incubation medium. Enzyme controls were made in the absence of substrate and with the addition of fluoride, a classical inhibitor of acid phosphatase activities. Evaluation of the macrophage staining pattern was carried out using light microscopy.

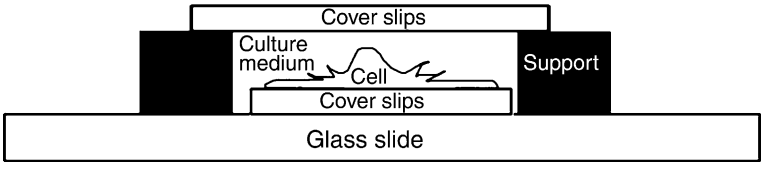


Fig. 1. The schematic glass slides and cover slip arrangement used for living macrophages analysis.

2.10. Ultrastructural cytochemistry of AcP

Peritoneal macrophages (obtained from in vivo treated animals), were prefixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4 °C for 10 min, then rinsed and washed in 0.05 M tris-maleate buffer, pH 5.0. The cells were rinsed and preincubated at 25 °C in a medium containing 2 mM $CeCl_3$, 5% sucrose, 2 mM β-glycerophosphate in 0.05 M tris-maleate buffer, pH 5.0 (Robinson and Karnovsky, 1983). The control cells were incubated in the same medium but without the β-glycerophosphate substrate and containing 10 mM NaF (Sodium Fluoride). After incubation, the cells were rinsed in cacodylate buffer and fixed in 1% glutaraldehyde, 4% paraformaldehyde and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The cells were gently scraped off with a rubber policeman, post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, rinsed in the same buffer, dehydrated in acetone and embedded in Epon. Ultrathin sections were observed without stain using a Jeol 1200 EXII transmission electron microscope. A GATAN image analyzer was used to acquire images.

2.11. Statistical analysis

Results are expressed as the means ± standard deviation (SD). Data were submitted to analyze of variation (ANOVA) and Tukey's test to determine the statistical significance ($*p < 0.05$; $**p < 0.01$) of the intergroup comparisons.

3. Results

3.1. Effect of CA on macrophage morphology, spreading and endocytic index

There are no differences between the in vivo and in vitro controls in all determined parameters (data not shown). The control macrophage morphology vary from rounded cells to characteristically resident macrophages, which exhibited few cytoplasmatic projections and low spreading (Figs. 2(A), (D) and 7(A)). In contrast, Canova treated macrophages show the typical morphology of the activated stage, such as bigger spreading and numerous cellular projections (Fig. 2(B) and (D)), which show enormous remodeling during phagocytosis (Fig. 5(A)–(F); Supplementary movies 1 and 2). Statistical analysis confirms that the spreading ability on plastic or matrigel is significantly higher in cells from the CA-treated group ($p < 0.01$) (Fig. 3).

Phagocytosis is monitored by various methodologies. By Giemsa staining, it is very easy to visualize phagocyted microorganisms, which in most cases are surrounded by a light halo (Fig. 4(A) and (B)). Internalized microorganisms are

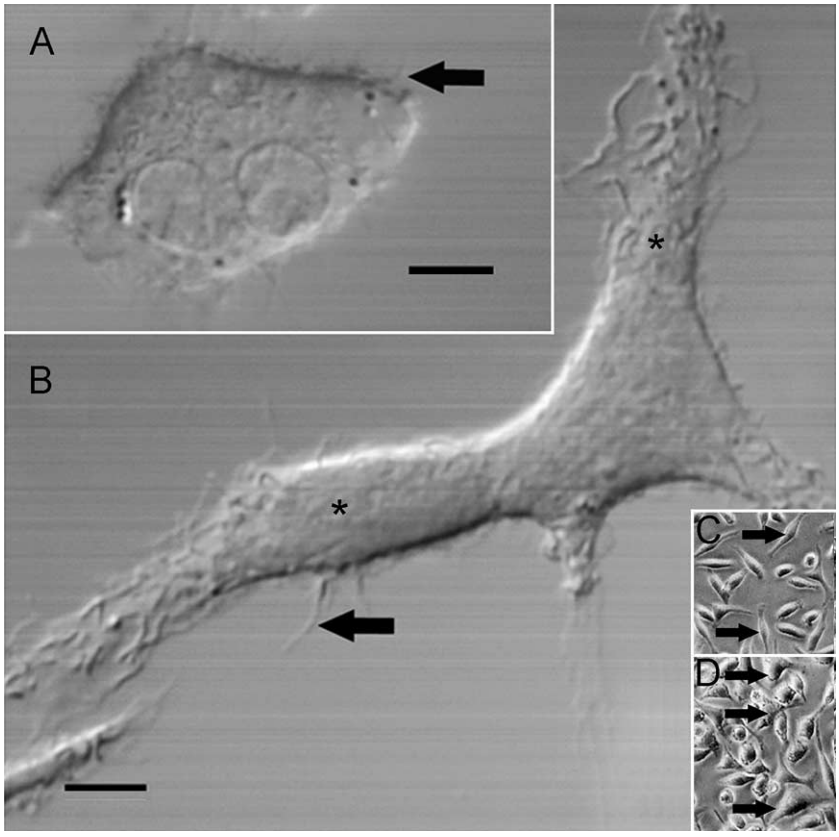


Fig. 2. CA effects on spreading ability. (A) and (B), macrophages analyzed by DIC. (A) Control group shows the characteristic morphology of resident macrophages. Note few cytoplasmatic thin projections (arrow) and poor spreading. (B) Canova-treated macrophages exhibiting morphological characteristics of activated macrophages. Note many large cytoplasmatic projections (arrow), cellular extensions (asterisks) and considerable spreading. (C) and (D) Analysis of macrophage cultures using inverted microscope. (C) Control macrophages show poor spreading (arrows). (D) Canova treatment showing marked spreading (arrows). Bars = 10 μm.

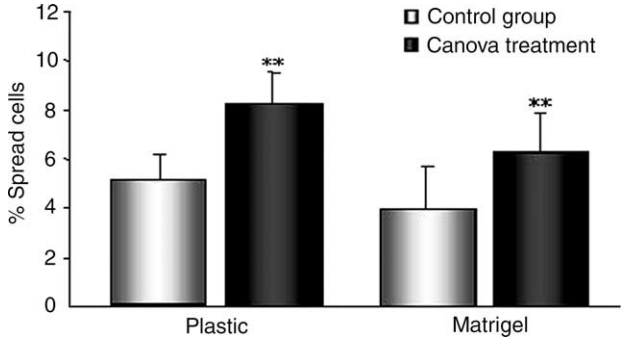


Fig. 3. Statistical analysis of CA effects on spreading ability. Macrophage spreading is analyzed as described in Section 2. Macrophage spreading ability on plastic or matrigel is significantly higher in cells from the CA-treated group ($p < 0.01$).

counted in these preparations and statistical analysis demonstrates that the endocytic index is significantly higher in the Canova treated cells for non-infective forms, *S. cerevisiae* (37%) and *T. cruzi* epimastigote (185%) (Table 1). In respect to *T. cruzi* trypomastigotes (25%) and *L. amazonensis* (16%), although no significant differences are detected for the infective forms (Table 1), there is a tendency for a lower endocytic/infection index in the treated group.

DIC analysis and time series acquisition are used to verify in vivo yeast internalization by macrophages. It becomes clear that endocytosis occurs in at least two different ways, namely macrophage projections attach and pull the yeast near the cell and then internalize it (Fig. 5(A)–(F); Supplementary movies 1 and 2), or they can surround the yeast with pseudopodia (Fig. 5(G)–(I)). As shown in Fig. 5(A)–(F), the CA-treated

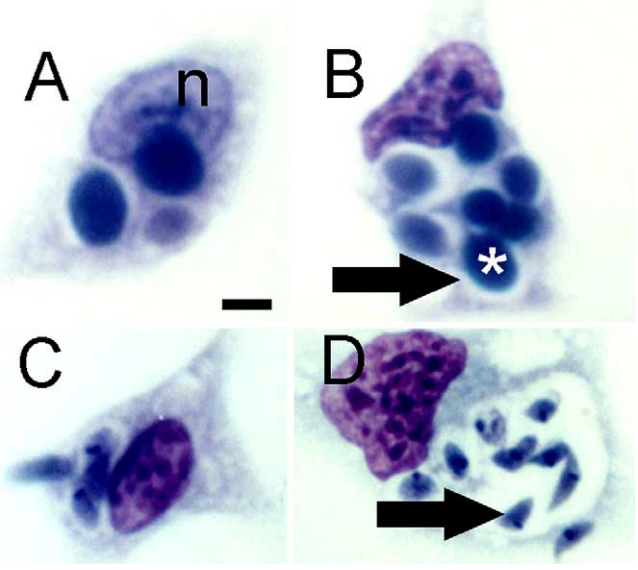


Fig. 4. Phagocytosis analysis by Giemsa staining. (A) Control macrophages contain few internalized yeasts (asterisk). A clear halo between yeast and vacuolar boundaries is shown (arrow). (B) Canova-treated macrophages exhibit numerous internalized yeasts. (C) *T. cruzi* epimastigote inside a control cell. (D) Various *T. cruzi* epimastigotes inside macrophages (arrow). Bars = 5 μm.

macrophages have a high killing potential, even when many yeast are internalized.

3.2. Effect of CA on AcP activity analyzed using transmission electron microscopy (TEM)

The AcP reaction product appears as dark electron dense deposits revealing the site of enzyme activity. No reaction product is present in the enzyme control cells (results not shown). We observe a stronger deposition of the reaction product in macrophages treated with CA as well as a more developed network of marked elements when compared with control cells (Fig. 6(A)–(D)). The reaction product is found inside heterogeneous compartments as a genuine network that varies from tubular to spherical vesicles (Fig. 6(B)–(D)). Patterns of reaction product deposits varied from deposition at the membrane or dispersed in the lumen of spherical vesicles and small tubular structures (Fig. 6(B) and (C)). In some large compartments the electron dense deposits appear to fill the vesicle or tubular lumen (Fig. 6(B) and (C)).

3.3. Effect of CA on acid phosphatase activity by optical cytochemistry

Purple granules of AcP cytochemical stain the entire cytoplasm. Control cells have dispersed AcP marked vesicles (Fig. 7(A)). Numerous vesicles exhibiting higher reaction product level can be seen in CA-treated macrophages (Fig. 7(B)), confirming the results observed by TEM. AcP product deposition in treated cells is difficult to quantify by image analysis because there is a high number of stained vesicles, as shown in Fig. 7(B) (insert—AcP marked vesicles are present even in the extremity of the macrophage extensions). Those patterns are maintained when the macrophages internalize yeasts (Fig. 7(C) and (D)). Besides the granules, the AcP reaction product is localized inside vacuoles containing internalized yeasts, a putative phagolysosome (Fig. 7(C) and (D)). Control macrophages that interacted with yeasts (Fig. 7(C)) present a stronger staining when compared to the control without yeasts (Fig. 7(A)), but a weaker staining when compared to CA-treated macrophages that interacted with yeasts (Fig. 7(D)). These observations are quantified as in Fig. 7(E). These results suggest that AcP marked vesicles fuse with phagosomes. No reaction product is observed in the enzyme control macrophages that interacted or not with yeasts (Fig. 8(E)).

Stained TRAP granules range from brown to brown/purple all over the cytoplasm, cellular extensions and inside phagolysosomes (Fig. 8(A)–(E)). In the controls, a higher proportion of stained granules is observed (Fig. 8(A) and (B)) when compared with CA cells (Fig. 8(C) and (D)). Similarly, control cells that interacted with yeast, demonstrate a higher number of stained granules (Fig. 8(C)) when compared with Canova treated cells (Fig. 8(D)). No reaction product is observed in enzyme control cells and yeasts appear yellow inside the vacuoles (Fig. 8(E)).

Table 1
CA influence on the endocytic index

	Percentage of macrophages with ingested microorganisms		Mean number of microorganisms per macrophage		Endocytic index	
	Control	Canova	Control	Canova	Control	Canova
S. cerevisiae	68±15	89±6	2.4±0.18	2.5±0.40	163	223[a]
T. cruzi epimastigote	31±4	46±3	1.1±0.16	2.1±0.19	34	97[a]
T. cruzi trypomastigote	8±1	6±1	1.0±0.01	1.0±0.01	8	6
L. amazonensis	28±3	27±2	1.33±0.35	1.15±0.22	37	31

[a] After treatment with CA the endocytic index significantly increases with non-infective forms, *S. cerevisiae* and *T. cruzi* epimastigotes. *T. cruzi* trypomastigotes and *L. amazonensis* have a lower endocytic/infection index in the CA-treated group, although no significant differences are detected for the infective forms. $^{**}P<0.01$ when compared to respective control.

4. Discussion

The pronounced macrophage spreading observed in CA treated cells is a typical morphological characteristic of activated macrophages (Cleary et al., 1999). These results confirm previous studies where Canova altered the distribution of proteins related to adhesion and spreading—fibronectin, α_5 and β_1 integrins and actin filaments, in treated macrophages (Piemonte & Buchi, 2002). Many important cellular processes such as phagocytosis, cell motility and endocytosis are closely related to cell spreading (Orth & McNiven, 2002). The spreadig phenomena provide morphological evidence of considerable cellular alterations such as cytoskeleton reorganization and changes of membrane protein activities and expression (Wojciak-Stothard et al., 1997; Berton & Lowell, 1999). Analysis of living cells, by serial time acquisition, demonstrate the great phagocytic activity of macrophages as well as the role of cellular extensions. Like a harpoon, as shown in the Supplementary movies 1 and 2, cellular extensions can contract and engulf the yeast until complete internalization.

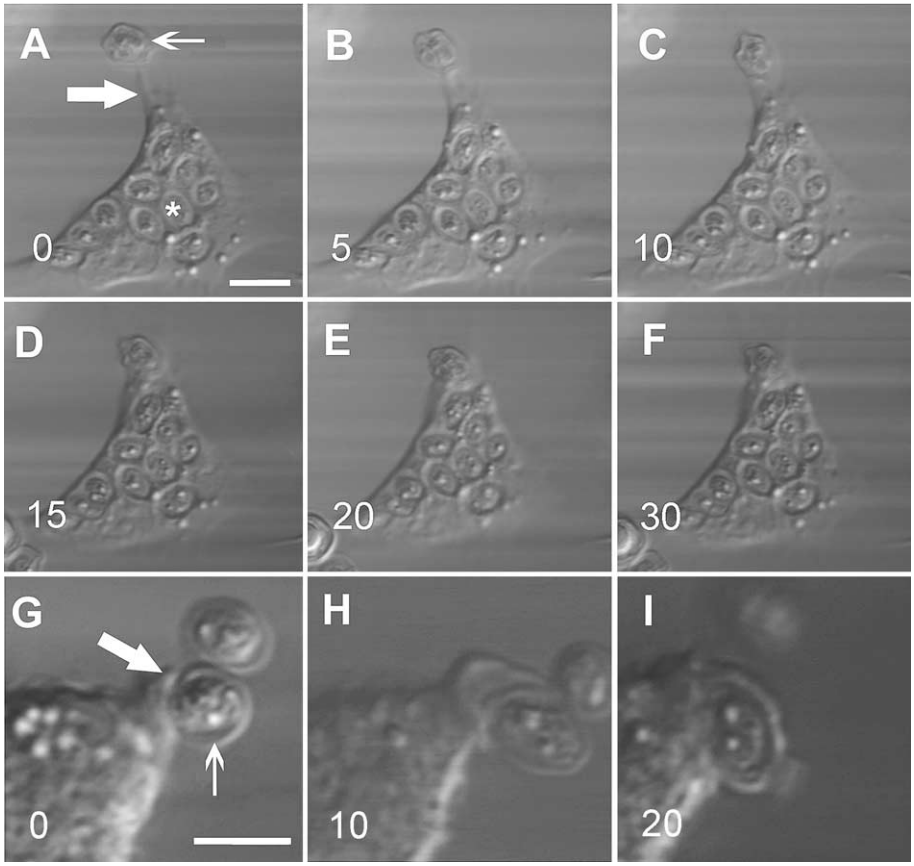


Fig. 5. Phagocytic activity is observed in living CA treated macrophages from serial time acquisitions. (A)–(F) Macrophage 1—pulling and internalization. (A) A yeast (thin arrow) attached to a cellular extension (thick arrow) is shown during initial stages of interaction. (B)–(D) The cellular projection indicated in A is shortened, pulling the yeast into macrophage body. (E) and (F) Yeast engulfment. (G)–(I) Macrophage: typical phagocytosis. (G) Initial stages of pseudopodia (thick arrow) attachment to a yeast (thin arrow). (H) More advanced stages of engulfment. Note the curvature of pseudopodia around the yeast. (I) Final internalization stage. Note that the pseudopodia seem to completely engulf the yeast. Bars = 10 μm. The number at the bottom left in each figure indicates the time (minutes) of serial time acquisition.

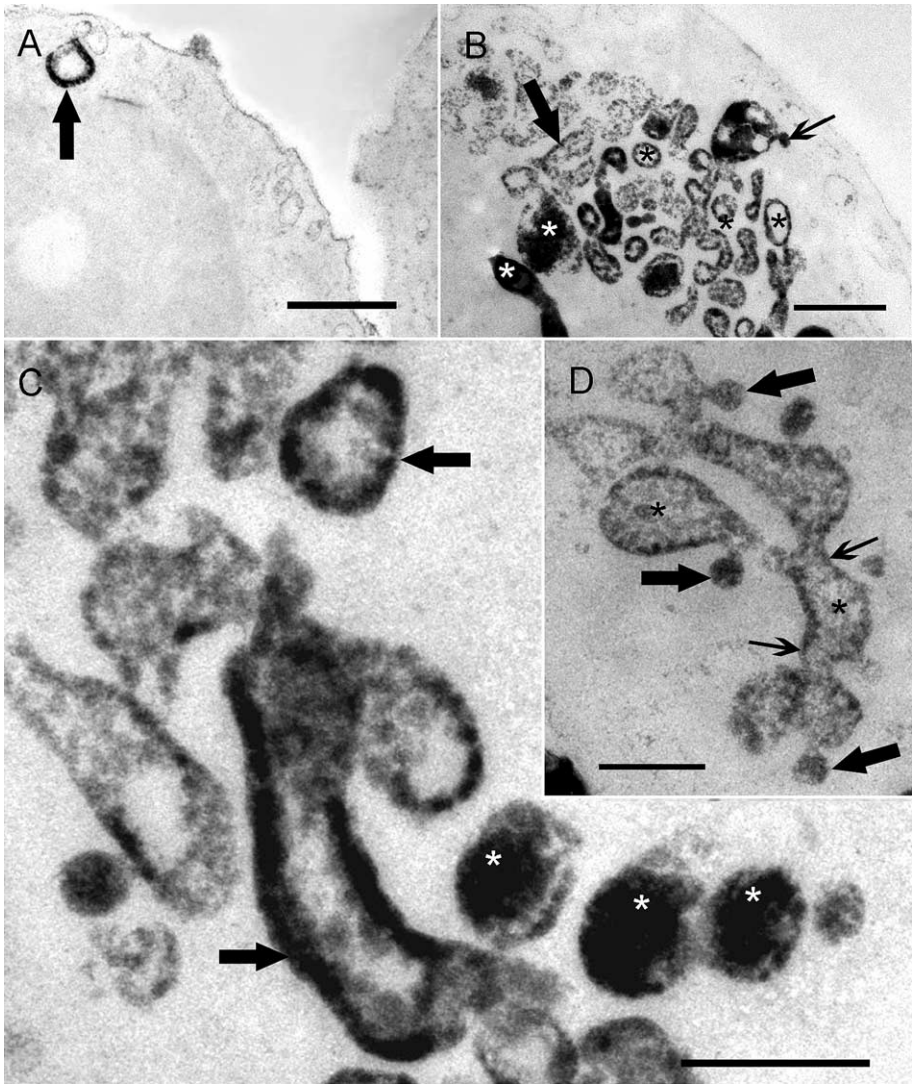
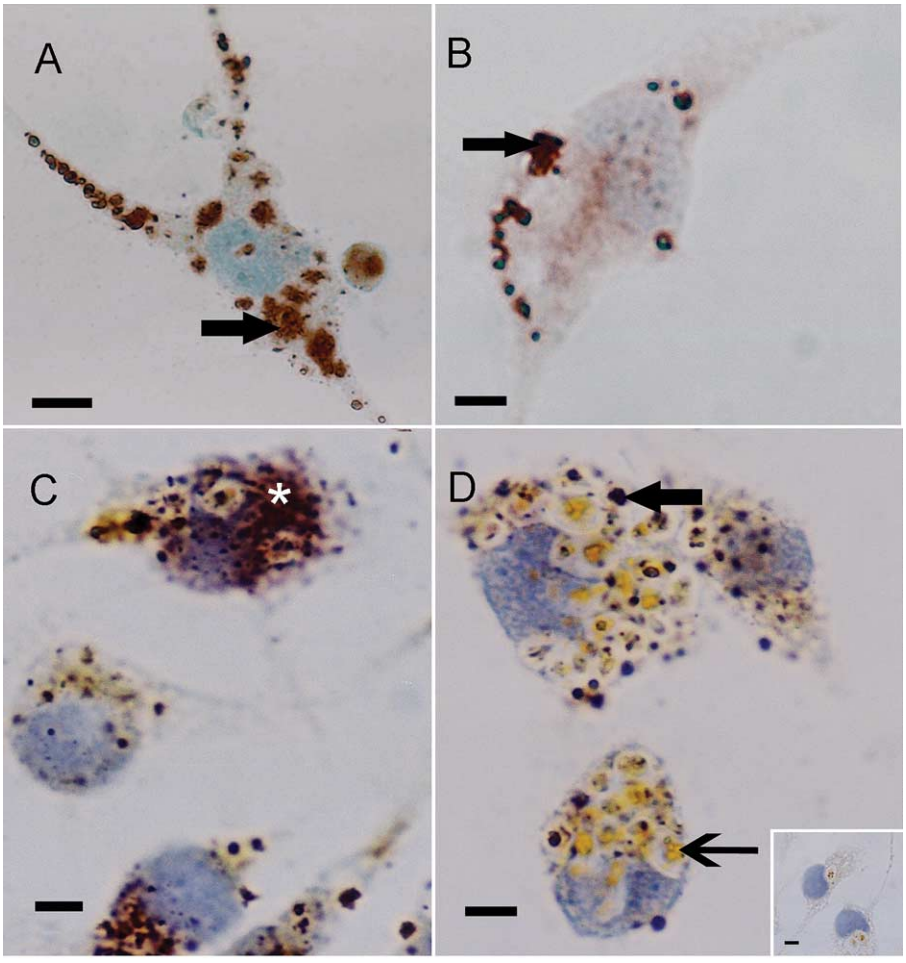


Fig. 6. The influence of CA on the acid AcP activity and the endosomal/lysosomal system shown by TEM. (A) Control macrophages showing few marked vesicles (arrow). Bar = 500 nm. (B) Canova-treated macrophage. A strong reaction product deposition as well as a highly developed network of marked elements is shown. The reaction product can be seen to fill the tubular and rounded shaped compartments (white asterisks) or in association with the vacuolar surface of tubular (thick arrow) and spherical vesicles (black asterisks). Note a putative fusion process (thin arrow). Bar = 500 nm. (C) Canova-treated macrophage. Higher magnification shows reaction deposition on the internal surface of vacuolar membrane in the compartments that exhibit various morphologies (arrows). Spherical vesicles are filled with much electron dense deposit (with asterisk). Bar = 250 nm. (D) Canova-treated macrophage. A network of tubular/dilated structures is shown containing small vesicles (thick arrow) that may be budding or fusing within the network. The reaction product is associated with membranes (thin arrows) and dispersed within the lumen (black asterisks). Bar = 250 nm.

Therefore, the higher endocytic index observed in CA treated macrophages is coordinated with spreading. These parameters thus indicate that CA is an efficient substance to activate macrophages.

Phagocytosis is the mechanism by which cells internalize, degrade and eventually present peptides derived from particulate antigens, in order to defend the body (Niedergang & Chavrier, 2004). Thus, enhanced endocytic index of non-infective forms *S. cerevisiae* and *T. cruzi* epimastigotes also indicates macrophage activation. On the other hand, the endocytic/infection index of *T. cruzi* trypomastigotes and *L. amazonensis* in the treated group tend to diminish. This is an interesting fact since they are obligatory intracellular parasites in mammals (Dedet et al., 1999; Ritting & Bogdan, 2000), and lead us to think that CA somehow informs the macrophages to distinguish between infective and non-infective forms. Pereira reported a CA modulatory effect on in vivo and in vitro experimental infection by *L. amazonensis* (Pereira et al., 2004). An infective pathogen needs necessarily to be internalized by a macrophage to multiply itself. This must happen before pathogen opsonisation by antibodies. If opsonisation occurs the complex antigen/antibody will attach to Fc receptors triggering a pathway that ends in antigen destruction. Thus, the decrease of endocytic index of infective pathogen partially explains the clinical and experimental improvements once they are more slowly internalized. The pathogenicity of a microorganism is defined by its ligands in the glycocalix, which are recognized by specific macrophage receptors. The



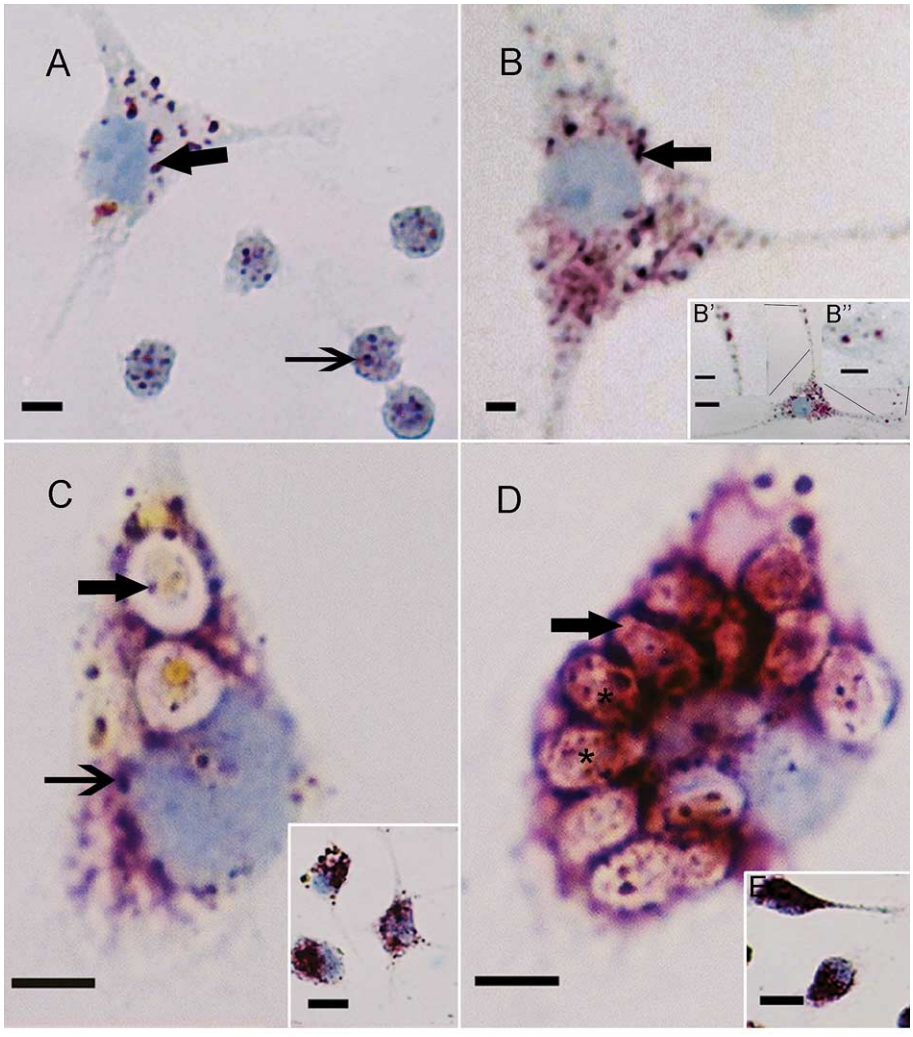
E

Without yeast		Interact with yeast	
Control	Canova treated	Control	Canova Treated
++	+	+++	+

Fig. 7. The influence of CA on AcP cytochemical staining using light microscopy. (A) Control cells reveal AcP stained vesicles dispersed inside the cytoplasm of resident macrophages (thick arrow) and spherical shaped cells (thin arrow). (B) Canova-treated macrophages with numerous overlapping vesicles containing reaction product (arrow). Insert: lower magnifications of the same cell showing stained vesicles in inside extremities of the cellular extensions, shown at higher magnification in B′ and B″. (C) A control macrophage show yeasts (thick arrow) inside phagosomes. Note the large number of AcP stained vesicles (thin arrow). Insert: lower magnification of control cells. (D) A Canova-treated macrophage with numerous phagocytosed yeasts, which frequently exhibit a fragmentory appearance (asterisks). A dispersed homogeneous staining of reaction product is present within the phagosomal lumen (arrow). Insert: a lower magnifications showing heavily stained cells. E. Table shows a comparison between staining intensity based on visual observation. Bars = 10 μm.

expression of these receptors depends on activation. Thus, CA, at no time 'informs' the macrophages. What probably happens is that CA simply activates the macrophage, allowing the expression of certain specific receptors and inhibiting others.

The enhancement of spreading and the endocytic index of non-infective forms are consistent with the increase in the endosomal system and AcP activities, monitored by TEM and optical cytochemistry. TEM showed the heterogeneous morphology of structures containing the AcP reaction product. These products revealed the different sizes of cisternal, tubular and vesicular structures, as well as a characteristic network being consistent with endosomes, lysosomes and lysosome-related organelles (Gruenberg, 2001; Eskelinen et al., 2003). It is accepted that newly synthesized acidic hydrolases are transported to endosomes and then to lysosomes (Gruenberg, 2001; Eskelinen et al., 2003). A precursor of lysosomal AcP (LAP) is synthesized as a membrane glycoprotein, which is delivered from the TGN (Trans Golgi Network) to lysosomes, traveling along the endocytic pathway. After arrival into dense lysosomes, the luminal domain of LAP is released into the lysosomal matrix (Peters & Figura, 1994). Thus, acid phosphatase linked to the membrane is included as a marker for tubular endosomes (Wiese et al., 1996). In this context, we can speculate that the AcP positive vesicles, exhibiting reaction product deposition only on the membrane, may be representing endosomes. In the same way, the compartments filled with reaction product correspond to more mature lysosomal related vesicles and lysosomes. AcP cytochemistry by itself is insufficient to precisely draw the line between endosomes, lysosome and



F

Without yeasts		Interacted with yeast	
Control	Canova treated	Control	Canova Treated
+	+++	+++	+++++

Fig. 8. CA influence on TRAP cytochemical staining using light microscopy. (A) Control resident macrophages with a high number of stained granules (arrow). (B) A Canova treated macrophage exhibiting fewer TRAP-stained vesicles (arrow). (C) Control cells interacting with yeast. Note the great number of TRAP-stained vesicles (asterisk). (D) Canova-treated cells interacted with yeast. TRAP-stained granules are present inside the cytoplasm (thick arrow). Note that the yeast displays a yellow coloration (thin arrow). (E) TRAP control of the enzyme. No reaction product is present. (F) Table shows a comparison between staining intensity, based on visual observation. Bars = 10 μm.

lysosome-related organelles, although it has been used for a long time as a single parameter for lysosome identification (Karnovsky & Lazdins, 1978; Robinson & Karnovsky, 1983; Niedergang & Chavrier, 2004). Actually, even lysosomes and endosomes are both implicated in macrophage killing ability, regardless of their precise caracterization (Tjelle et al., 1996). AcP detection is an useful parameter to characterize the endosomal/lysosomal system, although this enzyme can be used alone as a measure of for macrophage activation (Karnovsky & Lazdins, 1978; Cleary et al., 1999; Matsubara, 2002; Tooker & Coussens, 2004).

Our results show that AcP stained compartments containing yeasts are consistent with phagosomal maturation. Phagosomes formed after the engulfment of either microbes or cancer cells, readly mature into the phagolysosome, a lytic compartment (Brunet et al., 2003; Niedergang & Chavrier, 2004). The phagolysosome formation occurs through a series of fusion events between the phagosome and organelles in the endocytic pathway (Niedergang & Chavrier, 2004). These events provide the insertion of lysosomal enzymes into phagolysosome, which becomes competent to degrade of foreign material (Garin et al., 2001; Brunet et al., 2003). In addition, besides their key role in the degradation of macromolecules and particles, macrophages contain peptides generated from the degradation of the internalized antigens mediated through endosome/lysosome organelles (Greenberg & Grinstein, 2002; Aderem, 2003). Therefore, part of the AcP positive compartments can represent specialized lysosome-related organelles that play a role in

antigen presentation, a process essential for lymphocyte activation (Brunet et al., 2003).

The TRAP-positive structures observed probably correspond to lysosomes and lysosome-related organelles. In human macrophages, TRAP co-localizes with the lysosomal membrane marker, LAMP-1 (Bune et al., 2001), and in osteoclasts gold markers are observed mainly in vesicular structures, which are interpreted as lysosomes (Reinholt et al., 1990). The decrease of TRAP staining in the yeast and/or Canova activated macrophages may be a result of the secretion process. This hypothesis is consistent with findings that showed two closely related isoforms of TRAP secreted in human serum, namely macrophage-derived TRAP-5a (Janckila et al., 2002) and osteoclast-derived TRAP-5b (Halleen et al., 2000). TRAP 5a-like activity is also found in the conditioned medium from cells, as a secreted product, while TRAP 5b-like activity is retained almost exclusively intracellularly (Janckila et al., 2002). In osteoclasts TRAP 5b is secreted and dephosphorylates bone matrix phosphoproteins in the resorption lacunae (Ek-Rylander et al., 1994) and degrade bone matrix proteins within endocytic vesicles (Halleen et al., 1999). TRAP function in macrophages has only recently begun to receive attention. Mice lacking TRAP (generated by targeted disruption of the murine *Acp 5* gene in embryonic stem cells) reveal a skeletal phenotype, disordered macrophage inflammatory responses, a significantly enhanced pro-inflammatory cytokine secretion, and reduced clearance of the pathogen *Staphylococcus aureus* (Bune et al., 2001). These authors suggested that TRAP may affect the effector pathways of phagocytic activation or antigen presentation, perhaps by affecting phosphorylation of strategic components that mediate cell signaling.

All parameters analyzed in the present work indicate macrophage activation by Canova. Ingesting microbial pathogens, the activated macrophages play a crucial immune function, initiating a microbial killing pathway inside phagolysosomes (Brunet et al., 2003). Clinical recovery observed in patients with cancer and AIDS may be explained in part by macrophage activation. Several clinical investigations address activation and recruitment of macrophages/monocytes as a means to eliminate tumor cells using biological response modifiers and autologous activated monocytes (Hana et al., 2001; Klimp et al., 2002). However, many adverse biological responses hinder these strategies (Klimp et al., 2002). In this context, the CA macrophage activation, a method without harmful effects, provides a system to further investigate the Canova action mechanism and potential applications in the treatment of a range of diseases.

5. Conclusion

The results with CA treatment suggest an improvement in resistance against infectious and foreign agents and show an enhanced immunity favoring a specific immunological response to these microorganisms through the phagocytic pathway. The treatment induces morphological alterations of macrophages, including an increase in their spreading areas. This can be a key event for the enhancement of phagocytic ability of non-infective forms observed. To-date, several potent biological response modifiers, which are able to activate macrophages, have been extensively studied. Canova, a homeopathic medication with no side effects, provides to a good alternative for macrophage activation.

Acknowledgements

We thank PR TECNOLOGIA and CAPES for financial support.

Appendix. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micron.2005.08.005

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