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Lymphocyte proliferation stimulated by activated *Cebus apella* macrophages treated with a complex homeopathic immune response modifiers

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Introduction: *Canova* is a complex homeopathic medicine that enhances a specific immunologic responses against several exogenous and endogenous conditions. *Canova* activates macrophages both *in vivo* and *in vitro*.

Aim and method: We evaluated the effects of macrophages activated by *Canova in vivo* and *ex vitro* in the proliferation of lymphocytes. *Canova* was used to activate *Cebus apella* macrophages *in vivo* or *ex vitro* with *Canova*. Lymphocytes were cultured with the macrophage culture medium. The analysis of *Canova* effects in cultured lymphocytes was performed according to the cell cycle phase using flow cytometry. The Interferon gamma and Interleukin-5 cytokines quantification in these lymphocyte culture media was performed by Enzyme-linked immunosorbent assay (ELISA).

Results: We observed that *Canova* activates macrophages *in vivo* and *ex vitro*. The lymphocytes cultured in a supplemented medium with macrophages activated by *Canova* treatment presented a higher number of proliferation cells than lymphocytes not exposed to macrophages activated by *Canova*. The Interferon gamma and Interleukin-5 cytokines were only observed in the medium of lymphocytes exposed to macrophages activated by *Canova*. Thus, *Canova* has potential as a new adjuvant therapy. *Homeopathy* (2012) 101, 74–79.

Keywords: *Canova*; Immune response modification; Cell cycle; Cytokines; Non-human primates; *Cebus apella*

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Introduction

Exposure to foreign substances induces or stimulates the specific immune system. Some immune response modification therapies are directed to specific cells or cytokines that contribute to the immune response.¹ An immune response modifier can be defined as an agent that mimics, augments, or requires participation of host immune cells

for optimal effectiveness.² Immune response modification therapy may aim to enhance the individual's own immunity to favor a particular immunologic response.¹ Immune response modifier agents are either already approved or in pivotal trials for all major cancers and an expanding part of the clinical anticancer armamentarium.²

Macrophages play a crucial role in innate and adaptive immunity in response to microorganisms.³ Macrophage activation is a complex process involving signal transduction events from multiple inflammatory mediators, including exogenous factors, such as pathogen-associated molecules, and endogenous mediators, such as cytokines and chemokines.⁴

Cytokines are major regulators of macrophage activation that fine tune macrophage responses to achieve effective clearance of pathogens, while limiting the amount of inflammation to avoid toxicity and tissue damage. Cytokines also regulate the qualitative nature of macrophage activation to coordinate the most effective innate and acquired immune responses to clear the pathogen.⁴ Among the cytokines, the Interferon gamma (IFN- γ), produced by T helper cell 1 (Th1), is a key endogenous activator of macrophages and also an inhibitor of Th2 cells proliferation.⁴ On the other hand, Th2 cells produce cytokines responsible for inhibition of several macrophage functions. Since activated macrophages stimulate T cells,⁵ there is a direct relationship between macrophage activation and lymphocyte proliferation.

Canova (CA) is a complex homeopathic immune response modifier, that 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 pathological conditions.⁶ CA activates macrophages both *in vivo* and *in vitro*.^{1,6–11} Our research group previously observed that CA treatment leads to an indirect increase of T-lymphocyte proliferation stimulated by phytohemagglutinin *in vitro*.⁷

In the present study, we evaluated the effects of *Cebus apella* (Black-capped Capuchin monkey) macrophages activated by CA *in vivo* and *ex vitro* in the proliferation of lymphocytes. Thus, we analyzed the effects of CA in cultured lymphocytes according to the cell cycle phase by flow cytometry. We also evaluated IFN- γ and Interleukin-5 (IL-5) cytokine quantification in the culture medium exposed to macrophages activated by CA.

Materials and methods

CA

'Canova do Brasil', a Brazilian company, holds the international patent of this medicine (www.canovado brasil.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. Mother tinctures are purchased from suppliers authorized by the Brazilian Health Ministry. The final product contains *Aconitum napellus* dH20, *Apis mellifica* dH19, *Arsenicum album* dH17, *Asa foetida* dH20, *Baryta carbonica* dH20, *Bryonia*

alba dH14, *Calcarea carbonica* dH20, *Conium maculatum* dH16, *Ipecacuanha* dH13, *Lachesis muta* dH18, *Lycopodium clavatum* dH20, *Pulsatilla nigricans* dH13, *Rhus toxicodendrum* dH17, *Ricinus communis* dH14, *Silicea* dH18, *Thuja occidentalis* dH16, *Veratrum album* dH20 and less than 1% ethanol in distilled water. It is an aqueous, colorless and odorless solution. Experiments were performed with commercial CA donated by 'Canova do Brasil'. CA solution was vigorously shaken (succussion) immediately before all treatments.

Our research group previously demonstrated that CA treatment does not induce cytotoxic or genotoxic effects at chromosomal level in lymphocyte cultures.¹²

Animals and treatments

Six male adult *C. apella* (5–7 years old, weight 2.5–3.3 kg) were evaluated. Animals were identified with microchips and were individually housed in Centro Nacional de Primatas, Pará State, Brazil. The animals were fed with a healthy balanced diet not enriched with sodium chloride. Water was given *ad libitum*. This study received the approval of the Ethics Committee of Universidade Federal do Pará (PARECER MED002/2007). According to a basic veterinary examination, all animals were considered healthy at the time of intraperitoneal fluid sampling. This was confirmed by the animals' behavior as judged by the veterinary check.

The animals were assigned randomly by lottery in two groups of three *C. apella* each. One group received CA injections of 1.67 μ l/g commercial CA (CA group) and the other received the same amount of sterile physiological saline solution (negative control group – NC). The groups were of similar median age and weight ($p = 1$, Mann–Whitney test). CA and sterile physiological saline solution were injected by slow infusion in the right femoral vein of *C. apella* in a single dose.¹³ After 72 h of injections, macrophages of NC and CA group were collected by intraperitoneal wash with saline solution.

Macrophage culture

Macrophages from intraperitoneal fluid were counted in a Neubauer chamber. Macrophages were incubated at 37°C under 5% CO₂ for 15 min and nonadherent cells were removed by washing with phosphate buffered saline (PBS). The culture medium consisted of 80% HAM-F10 (Cultilab Mat. Cult. Cel Ltda, Campinas, SP, Brazil), supplemented with 20% heat-inactivated fetal calf serum (GIBCO laboratories, Grand Island, NY, USA), 0.01 mg/mL penicillin (Sigma–Aldrich Co, St. Louis, MO, USA) and 0.005 mg/mL streptomycin (USB, Cleveland, OH, USA). More than 90% of adherent cells were macrophages and the preparation was not further purified.

Additional to macrophages culture of NC and CA groups, we included a positive control of CA (PC group). In this last group, CA was added at 10% of total medium volume after 3 h of culture of macrophages collected from three *C. apella* with no *in vivo* treatment. The macrophage culture was carried out for 48 h *in vitro*. All

experiments were performed in triplicate. The index of macrophage activation was defined considering morphological alterations.¹

Primate lymphocyte proliferation in supplied media

After 120 h of CA and physiological saline solution injections in the studied animals, peripheral blood lymphocytes were collected from the *C. apella* of each group. Short-term lymphocyte cultures were initiated according to a standard protocol.¹⁴ Primate lymphocytes (3×10^5 cells/mL) were seeded on culture plates in the supplemented medium used for the three groups of macrophage cultures (NC, CA and PC), with the addition of 2% phytohemagglutinin. 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).

In this step, we added two lymphocyte culture groups: standard control group (SC) and standard control plus CA (SCCA). In these groups, lymphocytes were cultured as for standard lymphocyte culture (new medium).¹⁴ In SCCA group, CA was added at 10% of total medium volume after 24 h of standard lymphocyte culture. Lymphocytes of health *C. apella* with no treatment were used in SC and SSCA.

Cell cycle analysis

Cell cycle distribution followed the procedure described by Nicoletti *et al.*¹⁵ Lymphocytes were incubated at 37°C for 3 h in the dark in a lysis solution containing 0.1%, citrate, 0.1% triton X-100 and 50 µg/mL propidium iodide. Cell fluorescence was determined by flow cytometry in a Guava® EasyCyte™ Mini System cytometer (Guava Technologies Inc., Hayward, CA, USA), using the CytoSoft 4.1 software. Five thousands events were evaluated per experiment and cellular debris was omitted from the analyses.

Cytokine assay

IFN- γ and IL-5 are the only monkey cytokines detected by commercial kits developed for human cytokine evaluation.¹⁶ The supernatants of the lymphocyte cultures of NC, CA and PC groups were harvested and stored in aliquots at -70°C until use. IFN- γ and IL-5 in the supernatants were detected by capture ELISA using specific kits (Pharmingen, Franklin Lakes, NJ, USA) following the protocols provided by the manufacturer and the procedure described by Garcez *et al.*¹⁶ Each sample was tested in triplicate and cytokine standard solution was simultaneously used in increasing concentrations (from 7.8 to 250 pg/mL) in each plate. The plates were washed, incubated with polyclonal antibody for cytokine detection (50 µl/well) for 1 h at room temperature and washed again. The reaction was developed using tetramethyl-benzidine substrate (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% H₂O₂ in 0.1 M citrate buffer pH 5.5 for 30 min at room temperature, and the reaction was stopped by adding 50 µl 2 NH₂SO₄. The absorbance was measured in a microtitre plate reader (Titertek Instruments – Labsystems Huntsville, AL, USA) at 450 nm.

Statistical analysis

The index of macrophage activation, the distribution of lymphocytes according to the cell cycle phases and the quantification of IFN- γ and IL-5 cytokines were compared between groups using Kruskal–Wallis non-parametric test followed by Games–Howell *post-hoc* test. In all analyses, the confidence interval was of 95% and *p* values less than 0.05 were considered significant. The effect size for Kruskal–Wallis analyses was based on Eta Squared (η^2), in which 0.15 and below was determined as a small effect size; 0.16–0.40, medium effect size; and above 0.40, large effect size. All assays and analyses were performed blind.

Results

Index of macrophage activation

Cells in the NC group were mainly resting macrophages. However, activated macrophages were also present in this group. The median and interquartile range of the index of macrophage activation in NC group was 39% \pm 1.5, in CA group was 67% \pm 2 and in the PC group was 85% \pm 3. The variance of the index of macrophage activation was significantly different between NC, CA and PC groups ($\chi^2 = 7.261$, *df* = 2, *p* = 0.027, by Kruskal–Wallis test; $\eta^2 = 0.908$).

The Games–Howell *post-hoc* analyses demonstrated that the NC group presented a lower index of macrophage activation than CA (*p* < 0.001) and PC groups (*p* = 0.001). We also observed that the index of macrophage activation was higher in PC than CA group (*p* = 0.011).

Cell cycle kinetics in *C. apella* lymphocyte culture

We observed that the NC, SC, SCCA, CA and PC groups were significantly different considering the variances of lymphocytes in Sub-G1 (apoptotic cells, $\chi^2 = 12.233$, *df* = 4, *p* < 0.001, by Kruskal–Wallis test; $\eta^2 = 0.874$), G1 (non cycling cells, $\chi^2 = 12.767$, *df* = 4, *p* < 0.001; $\eta^2 = 0.912$), S (DNA replicating cells, $\chi^2 = 13.033$, *df* = 4, *p* < 0.001; $\eta^2 = 0.931$), and G2/M (dividing cells, $\chi^2 = 12.033$, *df* = 4, *p* < 0.001; $\eta^2 = 0.860$) cell cycle phases.

The Games–Howell *post-hoc* analyses demonstrated that the NC, SC and SCCA groups presented a higher number of lymphocytes in Sub-G1 (*p* = 0.003, *p* = 0.002, *p* = 0.002, respectively) and in G1 (*p* = 0.002, *p* = 0.005, *p* = 0.001, respectively) cell cycle phases than PC group. NC, SC and SCCA groups also presented a significant increase of lymphocytes in G1 phase than CA group (*p* = 0.022, *p* = 0.036, *p* = 0.022, respectively). On the other hand, the groups in which the lymphocytes were cultured in a supplemented medium with macrophages activated by *ex vivo* or *in vivo* CA treatment (PC and CA groups) presented a higher number of cells in S phase than NC (*p* < 0.001 and *p* = 0.024, respectively) and SCCA (*p* = 0.001 and *p* = 0.021, respectively) groups and also a higher number of lymphocytes in G2/M than NC (*p* = 0.004 and *p* = 0.004, respectively), SC (*p* = 0.019 and *p* = 0.018, respectively) and SCCA (*p* = 0.007 and

$p = 0.007$, respectively) groups. Table 1 shows the median and interquartile range of lymphocyte distribution according to the cell cycle phases.

Detection of IFN- γ and IL-5 in supernatant of *C. apella* lymphocyte culture

We observed that the CA, PC and NC groups were significantly different considering the variances of IFN- γ ($\chi^2 = 7.448$, $df = 2$, $p = 0.024$, by Kruskal–Wallis test; $\eta^2 = 0.931$) and IL-5 levels ($\chi^2 = 7.448$, $df = 2$, $p = 0.024$; $\eta^2 = 0.931$). PC and CA groups, with macrophages activated by CA *ex vivo* or *in vivo*, presented a significantly increase of IFN- γ ($p = 0.02$ and $p < 0.001$, respectively) and IL-5 ($p < 0.001$ and $p < 0.001$, respectively) levels than NC group. The PC group also presented a significant increase of IFN- γ ($p = 0.001$) and IL-5 ($p < 0.001$) levels than CA group. Table 2 shows the median and interquartile range of IFN- γ and IL-5 cytokine quantification in NC, CA and PC groups.

Discussion

Macrophages play an essential role in host defense against infection and tumoral cells. In this study, we observed that CA activated the macrophages *ex vitro* and *in vivo*. The large effect size observed with the comparison of macrophage activation index, as well as described for other statistical analyses in this study, shows that NC, CA and PC groups constitute real distinct groups despite the small number of samples. Several studies also reported that CA treatment induced macrophage activation *in vivo* and *in vitro*.^{1,6–11} The precise pathway induced by CA is still unknown. de Oliveira *et al.*⁹ suggested that macrophages triggered with CA have an increase in their activity of NAD(P)H oxidase as well as that of iNOS, consequently producing reactive oxygen species (ROS) and nitric oxide (NO) respectively. This action leads to macrophage activation and provides a modification of immune response directed to enhance the individual's own immunity.

This study described that the number of macrophages activated was higher in PC group (*ex vivo*) than CA group (*in vivo*). This result suggests the presence of macrophage

inhibitory cytokines in the healthy animals treated with CA to avoid toxicity and tissue damage due to strong macrophage activation. The percentage of macrophage activation in PC group (about 85%) was similar to the ratio described for macrophages of mice peritoneum culture with 10% of CA (72%).¹ In *C. apella*, we observed that about 67% of macrophages were activated *in vivo* by CA. Da Rocha Piemonte *et al.*¹ described 86% of macrophage activation in mice treated with CA during 7 days. Although we used a higher CA concentration than the previous study, the animals of the present study received a single CA injection.

Abud *et al.*¹¹ evaluated the effect of CA in cultures of bone marrow cells treated with CA and reported the presence of cell niches over the macrophages, which are sites of multiplication and differentiation containing stem cells¹⁷ and/or leukocytes committed with some cellular lineage.¹⁸ The authors observed that CA treatment increased the number of niches presenting several lymphocytes in contact with macrophages. An *in vitro* study demonstrated that a CA-like medicine, Complex Homeopathic Medication, indirectly activates lymphocytes through interaction with macrophages, even without direct cell–cell contact.¹⁹ Sato *et al.*²⁰ also reported that CA treatment increases the number of TCD4 lymphocytes. Our group previously observed that CA induces macrophage activation and indirectly leads to lymphocyte proliferation *in vitro*.⁷ Thus, these findings corroborate our results in which we observed that the lymphocytes cultured in a supplemented medium with macrophages activated by *in vivo* or *ex vivo* CA treatment presented a higher number of proliferation cells than lymphocytes not exposed to CA activated macrophages. Although we did not detect a significant reduction of Sub-G1 lymphocyte population in CA group compared to NC, SC and SCCA groups, the median of Sub-G1 cells in CA group was less than half of that observed in the groups which were not exposed to CA activated macrophages, demonstrating that CA did not induce apoptosis or cell cycle arrest.

Activated macrophages stimulate T cells and, then, initiate signaling events in T cells, which, in the presence of co-stimulatory signals, leads to T cell proliferation. T cells can be subdivided on a functional basis into Th1 cells,

Table 1 Influence of CA on *C. apella* lymphocytes by stage of cell cycle

Groups	DNA content (%)			
	Sub-G ₁	G ₁	S	G ₂ /M
NC	4.58 ± 0.38*	54.04 ± 0.47* [†]	19.24 ± 0.31* [†]	19.19 ± 1.07* [†]
Standard culture	3.69 ± 0.26*	52.69 ± 1.07* [†]	20.82 ± 1.46	21.31 ± 0.38* [†]
Standard culture plus CA	3.98 ± 0.11*	53.57 ± 0.76* [†]	20.1 ± 0.58* [†]	20.83 ± 0.78* [†]
CA treated group	1.73 ± 1.04	47.11 ± 1.41	26.42 ± 1.16	26.53 ± 0.93
PC	1.49 ± 0.23	44.23 ± 0.94	27.43 ± 0.36	27.08 ± 1.04

NC: lymphocytes cultured in macrophage culture media from animals that received sterile physiological saline solution. Standard culture: lymphocytes from non-treated animals cultured as standard protocol, new culture medium. Standard culture plus CA: lymphocytes from non-treated animals that were cultured with standard culture medium plus 10% of CA. CA treated group: lymphocytes cultured in macrophage culture media from animals that received CA treatment. PC: lymphocytes cultured in the media of macrophage cultures activated *ex vivo* by CA. Data are presented as median values ± interquartile range of five independent experiments performed in triplicate. Five thousand events were analyzed in each experiment. Sub-G1: apoptotic cells; G1: non cycling cells; S: DNA replicating cells; G2/M: dividing cells.

* Significantly different from PC, $p < 0.05$.

[†] Significantly different from the CA group, $p < 0.05$.

Table 2 Influence of CA on IFN- γ and IL-5 (pg/mL) levels in the supernatants of *C. apella* lymphocyte cultures

Cytokine group	IFN- γ Median \pm interquartile range	IL-5 Median \pm interquartile range
PC	17.45 \pm 0.1*	12.43 \pm 0.26*
CA group	14.78 \pm 0.55*, [†]	9.57 \pm 0.26*, [†]
NC	n.d	n.d
Blank	n.d	n.d

PC: medium of lymphocyte culture previously used in the culture of macrophages activated *in vitro* by CA. CA group: medium of lymphocyte culture that was previously used in the culture of macrophages activated *in vivo* by CA. NC: medium of lymphocyte culture that was previously used in the culture of control macrophages. Blank = pure culture medium which was not used in macrophage or lymphocyte culture.

* Significantly different from the NC, $p < 0.05$.

[†] Significantly different from the PC, $p < 0.05$; n.d = not detected; IFN- γ and IL-5 with quantification level below 8.0 pg/mL. Data are presented as median values \pm interquartile range of biological plus technical replicates.

involved in cellular immunity, and Th2, involved in humoral responses. The initial T cell primary response to inflammatory stimuli *in vivo* starts predominantly as a Th1 phenotype and then shifts to a Th2 phenotype. Th2 responses downregulate the cellular inflammatory reactions.²¹

In the present study, we showed that macrophages activated by CA lead to both Th1 and Th2 proliferation stimulated by phytohemagglutinin. We detected the presence of the IFN- γ and IL-5 cytokines in the medium of lymphocyte cultures previously used in CA activated macrophage cultures. The IFN- γ cytokine is produced by Th1 cells and is one of the main endogenous activators of macrophages. IL-5 cytokine is produced by Th2 and plays a role mainly in the production, survival, and granule release of eosinophils.²²

We hypothesized that CA may have a cumulative effect in macrophage activation, stimulating directly the oxidative metabolism⁹ and indirectly the Th1 cells proliferation, which produce IFN- γ . The proliferation of Th2 may be stimulated to regulate the macrophage activation. Further investigations are necessary to understand the production of IL-5 in our *ex vitro* model. However, it has been demonstrated that the profile of mRNA expression of macrophages is highly redundant in response to IL-5 and IL-10, an inhibitor of macrophage function, suggesting a partial overlap with the transcriptional response between these Th2 cytokines.²³

Another study evaluated the capacity of cells treated with CA to elicit cytokines.¹ In this study, the production of IFN- γ did not differ between CA treated and NC groups. Our results did not agree with this previous study using mice cells. However, Da Rocha Piemonte *et al.*¹ evaluated the production of IFN- γ in the supernatant of macrophage cultures. Although we were only able to evaluate the presence of IFN- γ and IL-5 in *C. apella* samples using a human commercial kit, other cytokines may be stimulated by CA in non-human primates.

In conclusion, CA treatment stimulates lymphocyte proliferation through activated macrophages and the lymphocytes indirectly stimulated by CA produce IFN- γ

and IL-5. CA has the potential to be a new adjuvant therapy enhancing the individual's own immunity.

Conflict of interest

All authors declare that they have no conflicts of interest.

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