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Cissampelos sympodialis Eichl (Menispermaceae) leaf extract induces interleukin-10-dependent inhibition of *Trypanosoma cruzi* killing by macrophages

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Abstract

The aqueous fraction of the ethanolic extract (AFL) of *Cissampelos sympodialis* Eichl (Menispermaceae), popularly known as milona, has been shown to have both immunosuppressive and anti-inflammatory effects. In the present study we investigated the modulation of macrophage antimicrobicidal activity by *in vitro* treatment with the extract from *C. sympodialis*. Normal and thioglycolate-elicited mouse peritoneal macrophages were infected *in vitro* with the protozoan *Trypanosoma cruzi* DM28c clone. We observed that the AFL (used at doses ranging from 13 to 100 µg/ml) increased *T. cruzi* growth and induced a 75% reduction in nitric oxide production. This inhibition could be mediated by the stimulation of macrophage interleukin-10 (IL-10) secretion since the *in vitro* treatment with the AFL stimulated IL-10 production by *T. cruzi*-infected macrophages. These results suggest that the anti-inflammatory effect of the AFL from *C. sympodialis* could be, at least in part, mediated by the inhibition of macrophage functions and that the inhibition of macrophage microbicidal activity induced by the *C. sympodialis* extract may be mediated by the decrease in macrophage function mediated by interleukin-10 production.

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Key words
- *Cissampelos sympodialis*
- Macrophage
- *Trypanosoma cruzi*
- Nitric oxide
- Interleukin-10

Introduction

The infusion of *Cissampelos sympodialis* Eichl (Menispermaceae) roots is popularly used in Northeast Brazil for the treatment of asthma, bronchitis and rheumatism, among other inflammatory diseases (1). It was reported (2) that the aqueous fraction obtained from the ethanolic extract of *C. sympodialis* roots reduced the spontaneous tracheal muscle tonus of guinea pigs. The effect of the aqueous fraction of the ethanolic extract of the leaves (AFL) of *C. sympodialis* on isolated smooth muscle was also investigated and this extract was shown to inhibit cyclic nucleotide phosphodiesterase activity and to increase intracellular cyclic adenosine monophosphate (cAMP) synthesis in

intact smooth muscle cell cultures (3). Studies using guinea pig bronchoalveolar leukocytes showed an increase in the intracellular levels of cAMP after *in vitro* treatment (4). The function of lymphoid cells is also modified by the AFL of *C. sympodialis* leaves. The AFL has been reported to induce significant inhibition of BALB/c spleen cell proliferation and to increase the secretion of both interleukin (IL)-10 and IL-4 (5). Phytochemical studies of the *C. sympodialis* species have indicated the presence of alkaloids (warifteine, methylwarifteine, milonine, and laurifolin) (1,6,7). Warifteine was shown to be a component with spasmolytic action (7).

The protozoan *Trypanosoma cruzi* is the causative agent of human Chagas' disease. *T. cruzi* replicates inside a variety of cells, including macrophage (8). The neutralization of cytokines that modify macrophage function has been reported to influence the outcome of *T. cruzi* infection (9). IL-10 was reported to increase macrophage susceptibility to microorganisms (10). This cytokine was also shown to inhibit the *in vitro* interferon-γ (IFN-γ)-induced killing of the parasite protozoan *T. cruzi* by macrophages (11). Nitric oxide (NO) is the critical mechanism controlling *T. cruzi* infection by activated macrophages and the production of NO can be inhibited by IL-10 (12). Besides its effect on macrophage function, IL-10 is also an important regulator of immune and inflammatory function (13). This regulatory role may be very important in the control of responses where secretion of TH1-type cytokines is detected, as is the case for *T. cruzi* infection (13).

Recent evidence has shown that the AFL of *C. sympodialis* may have anti-inflammatory effects (14). In the present study we investigated the effect of the AFL from *C. sympodialis* on an additional aspect of the inflammatory response, i.e., macrophage function. To investigate the macrophage response, we evaluated the antimicrobial activity of the AFL against the protozoan *T. cruzi*. Our data suggest that the *in vitro* treatment with the AFL decreased the microbicidal activity of macrophages and indicated that the decrease in macrophage function may be one of the mechanisms of anti-inflammatory activity of the AFL.

Material and Methods

Plant material and preparation of extract

Leaves from *C. sympodialis* were collected in João Pessoa, PB, Brazil, in January 1998 from the plant grown in the Botanical Garden of the Federal University of Paraíba and voucher specimens were deposited (code, Agra 1456) in the Botany Department of the Federal University of Paraíba, João Pessoa, PB, Brazil. The leaves were dried at 40ºC and pulverized and the material was successively extracted in a percolator with 70% ethanol (v/v) at room temperature (25-30ºC). Solvents were removed under reduced pressure using a rotary evaporator to obtain a dry extract. The yield at constant weight was 27 g of dry extract per 100 g of pulverized leaves. When required, the dry extract was dissolved in water and filtered and known volumes were dried to determine the final concentration, since only 72% of the powder was water soluble. The concentrations described in the text correspond to the final concentration of the water soluble material. NMR and fast atom bombardment mass spectrometry analysis, performed as previously described (15), revealed no contamination with bacterial lipopolysaccharides (LPS).

Animals

Male and female BALB/c mice (6 to 8 weeks of age) were obtained from the animal facility of Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil, where they were bred and housed according to institutional policies for animal care and usage.

Peritoneal macrophage isolation and cell cultures

Mice were sacrificed by CO_2 inhalation and peritoneal macrophages were rinsed out by injection of 5 ml of Hank's balanced salt solution. The cells obtained were resuspended in RPMI medium supplemented with 10% (v/v) fetal calf serum (FCS; Gibco, Grand Island, NY, USA) and added to 24-well tissue culture plates (3×10^5 cells/well). The cultures were incubated for 3 h at 37ºC in an atmosphere of 5% CO_2. Nonadherent cells were removed by washing with RPMI medium supplemented with FCS as above. One milliliter of DMEM medium (supplemented with 10% FCS (Gibco), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.05 mg/ml gentamicin) was added to each well. Thioglycolate (TG)-elicited macrophages were harvested 3 days after *ip* injection of 2 ml TG medium prepared as previously described (16). All reagents whose suppliers are not indicated were purchased from Sigma, St. Louis, MO, USA.

Determination of microbicidal activity

Peritoneal macrophages were removed and placed on 13-mm round glass coverslips inserted into 24-well tissue culture plates (10^7 cells in 0.1 ml). The cultures were incubated for 12 h at 37ºC in an atmosphere of 5% CO_2. Next, the coverslips were vigorously washed to remove nonadherent cells. The adherent cells were infected overnight with 10^5 chemically induced trypomastigote forms of the DM28c *T. cruzi* clone (17). Extracellular protozoans were removed by washing. Cultures were incubated for the indicated periods of time in the presence of the AFL (50 µg/ml). Some cultures also received 2 ng/ml LPS from *Escherichia coli* 0111:B4 (Difco Laboratories, Detroit, MI, USA) and 40 U/ml murine recombinant IFN-γ (Pharmingen, San Diego, CA, USA). Free supernatant trypomastigote numbers were determined at different time points by hemocytometer counting. The appropriate concentration of both LPS and IFN-γ was determined by previously performed titration experiments (data not shown).

Nitrite measurement

NO synthesis was measured by a microplate assay method that determines the levels of nitrite, as previously described (18). To measure nitrite, 0.1-ml aliquots were removed from culture supernatants and incubated with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. Absorbance at 540 nm was determined with a Titertek Multiskan Reader (Flow Laboratories, North Ride, Australia). Nitrite levels were determined using sodium nitrite as standard.

Assay of IL-10 levels produced in culture

Murine IL-10 concentration was determined by ELISA using paired antibodies and standards obtained from Pharmingen. Briefly, flat-bottomed polystyrene plates (Dynatech, Alexandria, VA, USA) were coated overnight at 4ºC with 50 µl of monoclonal anti-murine IL-10 antibodies. The plates were then washed with phosphate-buffered saline (PBS)/10% Tween 20 and blocked for 60 min at 37ºC with a 10% FCS solution diluted in PBS. Culture supernatant (100 µl) and standard (recombinant IL-10) were added in triplicate to the wells and incubated at 37ºC for 24 h. The plates were then washed and 100 µl of biotinylated anti-mouse IL-10 monoclonal antibody was added to each well for 45 min at 37ºC. Alkaline phosphatase-conjugated streptavidin (Pharmingen) was added and incubated for 30 min at 37ºC. Next, 100 µl of *p*-nitrophenyl-phosphate (Sigma, used at

1 mg/ml) diluted in 0.1 M glycine buffer was added. The plate was then incubated for 30 min at 37ºC in the dark before reading at 405 nm with a Titertek Multiskan Microplate Reader.

Statistical analysis

The results are reported as means ± SEM. The Student *t*-test was used to determine the statistical significance of the response of AFL-treated cultures compared to untreated ones. A P value of less than 0.05 was considered to be significant.

Results

To determine whether the AFL from *C. sympodialis* would modulate macrophage microbicidal activity, we studied the effect of AFL on the growth of *T. cruzi* trypomastigote forms in macrophage cultures. As shown in Figure 1A,B, the addition of the AFL alone increased the number of *T. cruzi* trypomastigote forms in normal peritoneal macrophage cultures. This effect was observed even when the AFL was added 24 h after the beginning of a 48-h culture (Figure 1A). The AFL did not show any potential toxic effect on macrophages in control cultures treated with the AFL alone since the addition of the AFL at doses as high as 100 µg/ml did not reduce macrophage viability (data not shown). The inhibitory effect could not be explained by an increase in the growth of *T. cruzi* forms since we did not observe any increase in the growth of epimastigote forms of *T. cruzi* after addition of the extract (data not shown). We also performed a dose-response study and observed that maximal *T. cruzi* growth could be observed after the addition of 50 µg/ml of the extract (Figure 1B). The protozoan growth was higher when we used long-term cultures (Figure 1B).

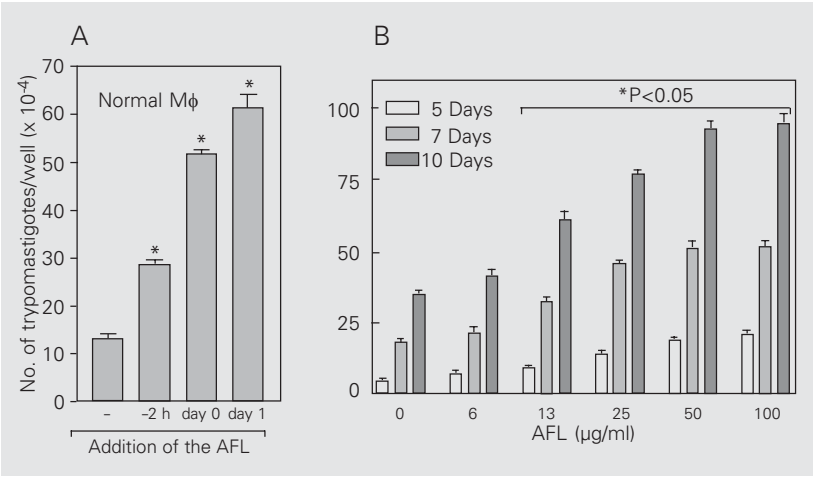

Figure 1. Effect of the aqueous fraction of the ethanolic extract (AFL) of *Cissampelos sympodialis* on the susceptibility of peritoneal macrophages (Mϕ) to infection with *Trypanosoma cruzi*. *A*, Peritoneal macrophages obtained from BALB/c mice were infected with *T. cruzi*. Some cultures were treated with the AFL (50 µg/ml) at the indicated times. The 0 h point refers to the time of macrophage infection. The number of extracellular parasites was determined in culture supernatants after 48 h of *T. cruzi* infection. Data are reported as the mean ± SEM of three independent experiments performed in triplicate. *P<0.05 compared to untreated cultures (Student *t*-test). *B*, Peritoneal macrophages obtained from normal mice were infected with *T. cruzi in vitro*. The AFL (100-6.2 µg/ml) was added to some cultures 24 h after *in vitro* infection. The extracellular parasite number was determined in culture supernatants collected after 5, 7 and 10 days of *in vitro* infection. Data are reported as the mean ± SEM of experiments run in triplicate and are representative of three independent experiments. *P<0.05 compared to untreated cultures (Student *t*-test).

We next investigated whether previous macrophage activation would abolish the inhibitory effect of the AFL on the microbicidal activity of macrophages. As can be seen in Figure 2, the AFL increased the growth of trypomastigote forms of *T. cruzi* when normal or TG-elicited macrophages were used. We also determined the potential inhibitory effect of AFL on the macrophage activity in the presence of stimuli such as IFN-γ and LPS. The inhibitory effect of the AFL on macrophage function was still detectable even in the presence of these potent macrophage activators (Figure 2).

To address the issue of whether the inhibition of the production of endogenous mediators would lead to a decreased macrophage microbicidal activity, we determined the level of NO production in culture after infection with *T. cruzi* and treatment with IFN-γ and LPS either in the absence or in the presence of the AFL. Figure 3 shows that treatment with the AFL inhibited the LPS- and IFN-γ-induced NO production by both

normal and TG-elicited macrophages. We next determined whether the inhibitory effect of the AFL could be related to the modulation of cytokine secretion. Figure 4 shows the levels of IL-10 secreted by either normal or TG-elicited macrophages infected with trypomastigote forms of *T. cruzi* for 48 h in the presence of the AFL. AFL stimulated a significant increase in IL-10 secretion by either normal or infected macrophages. The response was comparable whether normal resident or TG-elicited macrophages were used (Figure 4).

Discussion

The immunomodulatory effect of the AFL extract obtained from *C. sympodialis* was recently described. Our group showed that the AFL from *C. sympodialis* induced a significant inhibition of the proliferative response of mouse splenic cells (5). This effect was associated with the inhibition of IL-2 production and with the increase in the culture levels of both IL-10 and IL-4. AFL was also reported to have an anti-inflammatory effect since it depressed the *in vivo*-induced inflammatory response (14). In the present report we have extended these findings concerning AFL activity by describing its inhibitory effect on macrophage microbicidal activity.

We demonstrated that the AFL from *C. sympodialis* completely blocked the microbicidal function of both normal and TG-elicited macrophages. This inhibitory effect was significant despite the addition of the potent macrophage activators LPS and IFN-γ. Our results also suggested that the effect of the AFL could be related to an inhibition of NO synthesis by normal and TG-elicited peritoneal macrophages. This decrease in NO production may possibly be mediated by an autocrine mechanism through the secretion of the inhibitory cytokine IL-10 by the cells treated with the AFL. Actually, it has been shown that IL-10 inhibits the trypanocidal activity of macrophages by blocking NO release (12). We observed that the extract could induce a significant secretion of IL-10 even when added alone. An interesting finding was that the AFL could make even resting macrophages more susceptible to *T. cruzi* infection. We observed that the AFL induces a major increase in IL-10 secretion by these resting cells and this effect

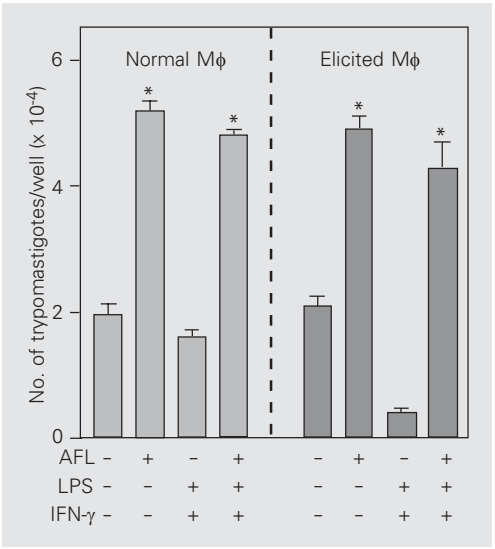

Figure 2. Comparison of the effect of treatment with the aqueous fraction of the ethanolic extract (AFL) of *Cissampelos sympodialis* on survival of trypomastigote forms of *Trypanosoma cruzi* in resident and thioglycolate (TG)-elicited macrophages (Mϕ). Peritoneal macrophages were obtained from either normal or TG-injected mice and infected *in vitro* with *T. cruzi*. Some cultures were stimulated 24 h later with lipopolysaccharide (LPS) (2 ng/ml) and interferon-γ (IFN-γ) (40 U/ml). The AFL was added at 50 µg/ml where indicated. The extracellular parasite number was determined in culture supernatants collected after 7 days of *in vitro* infection. Results are reported as means ± SEM of three independent experiments performed in triplicate. *P<0.05 compared to untreated cultures (Student *t*-test).

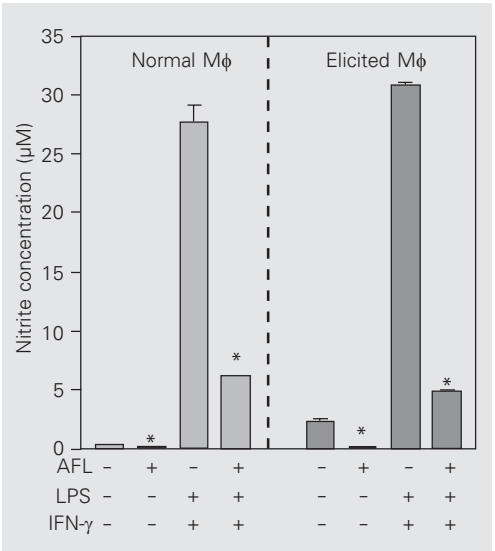

Figure 3. Effect of the aqueous fraction of the ethanolic extract (AFL) of *Cissampelos sympodialis* on nitric oxide production by *Trypanosoma cruzi*-infected macrophages (Mϕ). Peritoneal macrophages obtained from either normal or thioglycolate-treated mice were infected with *T. cruzi in vitro*. These macrophages were activated by 24-h incubation with both lipopolysaccharide (LPS) (2 ng/ml) and interferon-γ (IFN-γ) (40 U/ml) where indicated. Some cultures were also treated with AFL (50 µg/ml). The amount of nitrite released by AFL-treated and -untreated macrophages was measured after 48 h of treatment by the Griess method. Data are reported as the mean ± SEM of three independent experiments run in triplicate. *P<0.05 compared to untreated cultures (Student *t*-test).

of the AFL could be increasing the protozoan growth.

Previous studies have shown that the AFL increased cAMP synthesis by intact smooth muscle cells and bronchoalveolar leukocytes (3,4). Also, our recent studies have shown that the AFL also increased cAMP levels in murine B cells (Alexandre-Moreira MS, Piuvezam MR and Peçanha LMT, unpublished results). Recent findings have shown that cAMP mimetic or activating reagents inhibit secretion of both tumor necrosis factor-α (TNF-α) and IL-12 by activated peritoneal macrophages. This inhibitory effect was shown to be mediated by an increase in IL-10 secretion (19). Based on these findings, it is possible to suggest that AFL-induced IL-10 production may occur via an increase in intracellular cAMP levels.

Cytokines facilitate cell communication within the immune system and play a key role in host resistance against pathogens. Some cytokines (such as IL-1ß and IL-6) show proinflammatory effects, while others (like IL-10) behave as anti-inflammatory modulators. IL-10 was described to inhibit the synthesis of NO, TNF-α, and IL-12 by macrophages and the secretion of IL-2 and IFN-γ by T lymphocytes (19,20). The important immunomodulatory effect of the endogenous production of IL-10 was further exemplified by experiments performed in IL-10 knockout mice that are highly susceptible to LPS-induced endotoxemia due to increased TNF-α production (21).

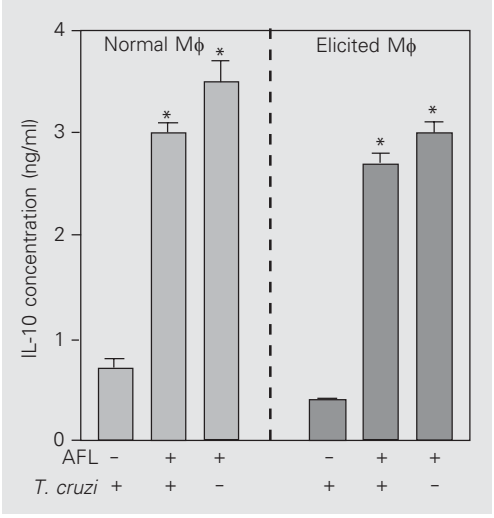

Figure 4. Production of IL-10 by *Trypanosoma cruzi*-infected peritoneal macrophages (Mϕ) treated with aqueous fraction of the ethanolic extract (AFL) of *Cissampelos sympodialis*. Peritoneal macrophages obtained from either normal or thioglycolate-treated mice were infected with *T. cruzi in vitro* (as indicated by a "+" symbol). After 24 h of infection, the macrophages were treated with AFL (50 µg/ml) where indicated. The levels of IL-10 released were measured 48 h later by ELISA. Data are reported as the mean ± SEM of two independent experiments run in triplicate. *P<0.05 compared to untreated cultures (Student *t*-test).

The generation of reactive nitrogen intermediates is one of the major effector mechanism in the antiparasitic function of macrophage (22). IL-10 has been described to decrease the production of both NO and NO synthase by IFN-γ-activated macrophages and thereby to decrease their ability to control parasitic infections (23). It was suggested that macrophages activated by IFN-γ are very effective in limiting *T. cruzi* replication *in vitro* (11). This activity has been associated, at least in part, with an increase in the oxidative metabolism due to cytokine stimulation (24).

The outcome of *T. cruzi* infection is closely controlled by cytokines involved in macrophage activation (10). Studies performed either *in vivo* or *in vitro* have demonstrated a regulatory effect of both transforming growth factor-ß (TGF-ß) (9) and IL-10 (25) in the control of *T. cruzi* infection. Also, the combination of (TGF-ß) and IL-10 has been shown to present a synergistic inhibitory effect on NO synthesis by activated macrophages (12).

We observed here that the AFL increased *T. cruzi* growth by the reduction of NO production. This inhibition of macrophage function may be mediated by an autocrine mechanism that depends on the secretion of IL-10 by macrophages. Taken together, our results indicate that one of the mechanisms by which the AFL would show an anti-inflammatory effect could be related to a decrease in macrophage activity.

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