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Effect of the injection of an extract of *Ascaris suum* on macrophage activation during the early phase of *Mycobacterium bovis* BCG infection in C57Bl/6 mice

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Abstract

Injection of an *Ascaris suum* extract (Asc) affects both the humoral and cellular immune responses to unrelated antigens when it is co-administered with these antigens. In the present study we evaluated the effect of Asc on macrophage activation in the early phase of *Mycobacterium bovis* BCG (Pasteur strain TMCC 1173) infection in C57Bl/6 mice. C57Bl/6 mice were injected intraperitoneally (*ip*) with 0.1 mg BCG (BCG group) or BCG plus 1 mg Asc (BCG + Asc group). The peritoneal exudates were obtained at 2, 7 and 14 days after infection. The numbers of IFN-γ-secreting cells were assessed by the ELISPOT assay. Nitric oxide (NO) production was measured by the Griess method and by the evaluation of NADPH diaphorase activity in the peritoneal exudates. The administration of Asc extract increased NADPH diaphorase activity (2 days: control = 0, BCG = 7%, BCG + Asc = 13%, and Asc = 4%; 7 days: control = 4, BCG = 13%, BCG + Asc = 21%, and Asc = 4.5%) and TNF-α levels (mean ± SD; 2 days: control = 0, BCG = 169 ± 13, BCG + Asc = 202 ± 37, and Asc = 0; 7 days: control = 0, BCG = 545 ± 15.5, BCG + Asc = 2206 ± 160.6, and Asc = 126 ± 26; 14 days: control = 10 ± 1.45, BCG = 9 ± 1.15, BCG + Asc = 126 ± 18, and Asc = 880 ± 47.67 pg/ml) in the early phase of BCG infection. Low levels of NO production were detected at 2 and 7 days after BCG infection, increasing at 14 days (mean ± SD; 2 days: control = 0, BCG = 3.7 ± 1.59, BCG + Asc = 0.82 ± 0.005, Asc = 0.48 ± 0.33; 7 days: control = 0, BCG = 2.78 ± 1.54, BCG + Asc = 3.07 ± 1.05, Asc = 0; 14 days: control = 0, BCG = 9.05 ± 0.53, BCG + Asc = 9.61 ± 0.81, Asc = 10.5 ± 0.2 (2 x 10^6) cells/ml). Furthermore, we also observed that Asc co-injection induced a decrease of BCG-colony-forming units (CFU) in the spleens of BCG-infected mice during the first week of infection (mean ± SD; 2 days: BCG = 1.13 ± 0.07 and BCG + Asc = 0.798 ± 0.305; 7 days: BCG = 1.375 ± 0.194 and BCG + Asc = 0.548 ± 0.0226; 14 days: BCG = 0.473 ± 0.184 and BCG + Asc = 0.675 ± 0.065 (x 10^2) CFU). The present data suggest that Asc induces the enhancement of the immune response in the early phase of BCG infection.

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- *Mycobacterium bovis*
- *Ascaris suum*
- Macrophage activation
- TNF-α
- NADPH diaphorase

Introduction

The capacity of helminths to modulate host immune responses to unrelated antigens is well documented (1). High dose immunization with adult *Ascaris suum* worm antigen (Asc) induces a marked production of IL-4 and IL-10 by inguinal and periaortical lymph node (LN) cells of DBA/2 mice (2). Asc-injected DBA/2 mice, when simultaneously immunized with ovalbumin (OA), exhibit a marked suppression of both OA-specific cutaneous delayed type hypersensitivity (DTH) and *in vitro* proliferative capacity of LN cells. In addition, OA-induced IL-2, IFN-γ and IL-4 production was diminished in Asc-treated mice and was associated with an impairment of the OA-specific IgG2a, IgG1 and IgE humoral immune response (2). Similarly, Asc induces a significant decrease of *M. bovis*-specific DTH in BCG-infected mice 14 days after infection (3). Since persons in developing countries who are at risk for mycobacterial infections often have *A. suum* co-infection, these observations may have very important public health implications.

Protection against intracellular pathogens operates in two stages, i.e., an early innate nonspecific response followed by acquired immunity with a strong DTH response (4). During the first week of infection, the early innate immune response is the main mechanism controlling mycobacterial proliferation. After two weeks of infection, cellular immunity develops and immature granulomas are formed. After 4 weeks, the cellular immunity reaches its peak and is represented by epithelioid granulomas that are associated with the decrease of mycobacterial load (4-6). In inbred strains of mice, early innate resistance to *M. bovis* BCG infection is controlled (5,7,8) by a single dominant gene designated *Nramp* (natural resistance-associated macrophage protein) that regulates the priming activation of macrophages for nitric oxide (NO) production. For example, mice genetically resistant to *M. bovis* BCG infection (C3H/HeN and A/J) are able to prevent *M. bovis* proliferation without expressing a strong DTH response. In contrast, genetically susceptible mice (C57Bl/6, B10.A and BALB/c) only control mycobacterial proliferation by the acquisition of an effective cellular immune response (5).

Thus, the immune mechanisms associated with the regulation of resistance and susceptibility in the innate early phase of BCG infection clearly involve cytokines and NO production (4,9). As mentioned above, injection of *A. suum* extract decreases *M. bovis*-specific immune responses during the stages of infection controlled by cellular immunity (3). To determine whether it also affects early innate responses to these organisms, we studied whether co-injection of *A. suum* extract alters NO production in a model of intraperitoneal (*ip*) BCG infection in C57Bl/6 mice. In this study, we measured NADPH diaphorase activity as a histochemical marker for nitric oxide synthase (iNOS; NOS2), the enzyme which catalyzes oxidation of L-arginine to L-citrulline and the production of NO (10,11). Since several studies have demonstrated that IFN-γ and TNF-α (6,12,13) are important cytokines correlated with the development of NO-mediated nonspecific early host defense, we also measured these cytokines. Surprisingly, our findings suggest that Asc administration in *M. bovis* BCG-infected mice increases both NADPH diaphorase activity and TNF-α production by peritoneal cells and is correlated with a reduction of BCG-colony forming unit (CFU) numbers in the spleen.

Material and Methods

Animals

Female C57Bl/6 mice were obtained from the Reproduction Biology Center, UFJF and were maintained in the animal facilities of

the Biological Sciences Institute, UFJF, where they were housed in plastic cages with unlimited access to food and water. Mice were used in the experiments at 6-8 weeks of age. Three main groups of mice were studied: group BCG - three mice injected *ip* with BCG; group BCG + Asc - three mice injected *ip* with BCG + Asc; group Asc - three mice injected *ip* with Asc only.

M. bovis BCG infection and CFU enumeration

M. bovis BCG, Pasteur TMCC 1173 strain, was thawed, washed in phosphate-buffered saline (PBS) and inoculated at a dose of 0.1 mg in 0.5 ml PBS by *ip* injection. At 2, 7 and 14 days post-infection, BCG-CFU were counted in the spleen. Briefly, spleens were collected and homogenized individually in 1 ml PBS using a teflon-coated tissue homogenizer (Glas-Col Apparatus Co., Terre Haute, IN, USA). Serial ten-fold dilutions of organ homogenates in PBS were prepared and plated onto Middlebrook 7H10 Bacto Agar (Difco Laboratories, Detroit, MI, USA) plates in triplicate. Plates were sealed in plastic bags and incubated at 37°C for 14 days, and the number of bacteria per organ was determined.

Ascaris suum extract preparation and injection

The animals were injected *ip* with 1 mg of adult worm extract from *A. suum*, prepared as described by Macedo and Mota (14). Briefly, live worms obtained from pig intestines were washed with PBS and mixed with an equal volume of borate-buffered saline (BBS), pH 8.0. After homogenization in an Ultra-Turrax apparatus and centrifugation at 10,000 rev/min for 1 h, the precipitate was resuspended in 400 ml of BBS and stirred overnight at 4°C. This suspension was centrifuged again and the supernatant was dialyzed against distilled water overnight in the cold. A clear supernatant obtained after centrifugation at 10,000 rev/min for 2 h was aliquoted and lyophilized.

Diaphorase cytochemistry

Peritoneal cavities were washed with 5 ml of PBS to remove resident macrophages. Cell suspensions were washed once and resuspended to obtain 10^6 cells/ml. Slides were prepared using cytospin (FANEM, São Paulo, SP, Brazil) with 2 x 10^5 cells per well, left to dry at room temperature, fixed in cold acetone for 10 min, wrapped in plastic film and kept at -20°C prior to use. Slides were stained with 50 mM Tris, pH 8, with 0.3 mM nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO, USA), 1 mM NADPH, 0.2% Triton X-100 for 30 min at 37°C, washed, and mounted in glycerol, and dark-blue cells were enumerated using a light microscope at 40X magnitude. The macrophage-like cell line RAW 264-7 (ATCC, Rockville, MD, USA) was stimulated with 1 µg/ml of LPS (Sigma) and used as positive control for NADPH diaphorase activity.

Measurement of nitrite release

NO release was quantified by the accumulation of nitrite in the supernatants of peritoneal cell cultures after 48 h in culture with 10 µg/ml of BCG antigen, using the standard Griess method (15). Briefly, the supernatant was added to Griess reagent (1:1) freshly prepared prior to use and the preparation was left to stand at room temperature for 5 min. Standards were prepared using sodium nitrite (2-100 µM) and added to each assay plate. Absorbances at 570 nm were read with a spectrophotometer (Titertek Uniskan II, Flow, Inc., Lugano, Switzerland).

Determination of TNF-α levels

Peritoneal cells were collected and cul-

tured for 48 h in RPMI-1640 medium with 10 µg/ml of BCG antigen. Supernatants were collected and TNF-α was quantitated by double sandwich ELISA using 2 µg/ml of purified anti-mouse TNF-α Ab (Pharmingen, San Diego, CA, USA) and 1 µg/ml of biotin rat anti-mouse TNF-α Ab (Pharmingen). Binding of biotinylated antibodies was detected using the streptavidin-biotinylated horseradish peroxidase complex (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), OPD and H_2O_2 and stopped with 50 µl of 0.2 M citric acid. The plates were read at 450 nm with an ELISA reader (Titertek Uniskan II, Flow, Inc.). Samples were quantitated by comparison with standard curves of purified recombinant TNF-α (Pharmingen).

Determination of IFN-γ

The ELISPOT assay was used as previously described (16) to determine the frequency of IFN-γ-producing peritoneal cells. Briefly, milliliter filtration plates (STHA09610, Millipore, Bedford, MA, USA) were coated overnight at 4°C with anti-IFN-γ (R4-6A2, 1 µg/ml). Wells were blocked with 1% BSA-PBS for 2 h at 37°C. Appropriate dilutions of peritoneal cells in 5% FCS-RPMI were added in a volume of 100 µl and incubated with 10 µg/ml of BCG antigen for 16 h at 37°C in 7% CO_2. Plates were thoroughly washed with PBS-Tween 20 (0.05%), before adding aliquots of 100 µl/well of biotinylated anti-IFN-γ mAb (AN-18.17.24, 0.5 µg/ml; kindly provided by Drs. Mahasti Sahihi de Macedo and Ises A. Abrahmsohn, USP, São Paulo). After incubation at 37°C for 2 h, plates were washed with PBS-Tween 20 several times and avidin-alkaline phosphates (Sigma) were added. After 1-h incubation at 37°C, plates were washed 8 times with PBS-Tween 20 and twice with alkaline phosphate buffer, pH 9.5 and the substrate, 5-bromo-4-chloro-3-indoyl phosphate (Fluka, Basel, Switzerland), was added. After 15 min, blue dots had developed and the reaction was stopped with distilled water. Individual spots were counted under a dissecting microscope at 32X magnification.

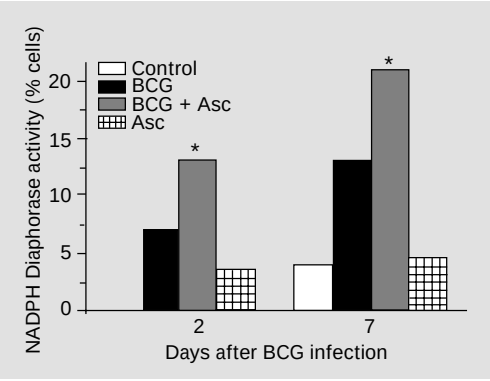

Figure 1 - Effect of *Ascaris suum* extract on NADPH diaphorase activity in cells obtained from C57Bl/6 mice injected with BCG, BCG + Asc or Asc. Results are reported as percent of dark cells indicating NADPH diaphorase activity of one representative experiment with duplicate measurements per data point. *P<0.05 compared to BCG (Student t-test).

Statistical analysis

The Student *t*-test was used to determine significant differences between the different groups of mice, with the level of significance set at $P<0.05$.

Results

***Ascaris suum* extract increased NADPH diaphorase activity**

A high proportion of peritoneal macrophages from BCG-infected animals injected or not with Asc extract were positive by diaphorase cytochemistry. However, BCG-infected mice injected with Asc extract increased 2-fold their capacity to induce diaphorase activity ($P<0.05$). Control and Asc-injected mice showed a reduced percentage of positive cells (Figure 1).

Nitrite production in peritoneal cell cultures

Supernatants of peritoneal cells from animals injected with BCG, BCG + Asc or Asc were collected after 48 h of culture with BCG antigen, and the amount of NO was evaluated (Figure 2). Low levels of NO

production were detected at 2 and 7 days after BCG infection, increasing at 14 days post-infection (pi). No significant difference ($P<0.05$) in NO production by peritoneal cells was observed between BCG-injected mice and BCG + Asc-injected mice. In addition, a high level of NO production was also detected in mice injected only with Asc on the 14th day.

Effect of Asc on TNF-α production in peritoneal cell cultures

The kinetics of TNF-α production was measured in supernatant culture during the early phase of BCG infection (Figure 3). TNF-α was detectable in the supernatants at 2, 7 and 14 days pi. At 2 days after BCG infection TNF-α was observed in mice injected with BCG and mice injected with BCG + Asc. However, at day 7 there was a significant increase ($P<0.05$) in TNF-α levels produced by mice injected with BCG + Asc compared with mice injected only with BCG. At the end of the early phase of BCG infection (day 14) there were detectable lower levels of TNF-α in mice injected with BCG + Asc. In mice injected only with Asc, higher TNF-α levels were observed at 14 days after Asc injection.

Presence of IFN-γ-producing cells during intraperitoneal BCG infection

The ELISPOT assay was used for the determination of frequency of IFN-γ-producing peritoneal cells (Table 1). Cells (10^5) were cultured with 10 µg/ml of BCG antigen for 24 h. Numbers of spontaneous IFN-γ spot-forming cells (SFC) were evaluated at 7 days after BCG infection with or without Asc injection. No significant differences were demonstrated between mice injected with BCG or BCG + Asc. In contrast, mice injected with Asc presented significantly ($P<0.05$) lower numbers of SFC when compared with mice injected with BCG or BCG + Asc.

Asc interferes with BCG proliferation in spleen

The number of CFU was investigated in the early phase of BCG infection (Figure 4). A significant reduction in number of BCG-CFU was observed during the first week of BCG infection in mice injected with BCG + Asc when compared with mice injected only with BCG (2 and 7 days pi). However, by the

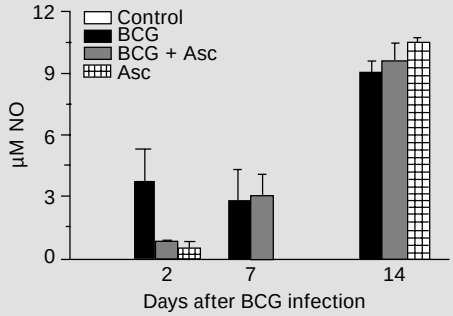

Figure 2 - Effect of *Ascaris suum* extract on NO production in C57Bl/6 mice. NO was measured with the Griess reagent. Peritoneal cells (2 x 10^5) were incubated for 48 h with 10 µg/ml of BCG antigen at 37ºC in 5% CO_2 and absorbance was determined at 540 nm. Results are reported as mean ± SD of one representative experiment with duplicate measurements per data point.

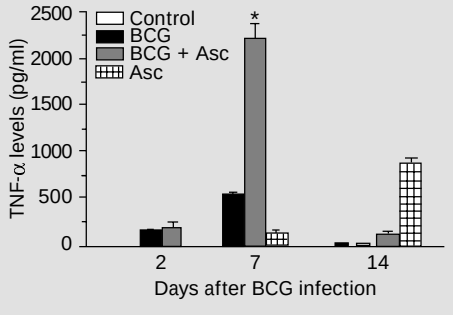

Figure 3 - Effect of *Ascaris suum* extract on TNF-α production in culture of peritoneal cells of C57Bl/6 mice during BCG infection. Supernatants were harvested 48 h after stimulation with 10 µg/ml of BCG antigen and measured by two-sandwich ELISA. Kinetic TNF-α data are expressed as mean ± SD (pg/ml) of one representative experiment with quadruplicate measurements per data point. *P<0.05 compared to BCG and BCG + Asc groups (Student *t*-test).

Table 1 – Frequencies of IFN-γ spot-forming cells (SFC) in C57Bl/6 at 7 days pi.

Results are reported as mean ± SD of one representative experiment with duplicate measurements per data point. *P<0.05 compared to BCG and BCG + Asc (Student *t*-test).

Groups	Number of IFN-γ SFC/10^6 peritoneal cells (mean ± SD)
Control	15 ± 1.2
BCG	706 ± 25.6
BCG + Asc	749 ± 6.2
Asc	400 ± 10.0*

end of the early phase of BCG infection there was no significant difference in number of BCG-CFU between the BCG group and the BCG + Asc group.

Discussion

In the present experiment we investigated the effect of Asc extract during the early phase of *ip* BCG infection in C57Bl/6. The immune response of BCG-infected mice was evaluated on the basis of NO production, NADPH diaphorase activity, numbers of IFN-γ-secreting cells, TNF-α production by peritoneal cells and number of BCG-CFU in the spleen. The data demonstrated that BCG infection (*ip*) induced NADPH diaphorase activity, NO production, TNF-α and IFN-γ production during the development of the early phase of infection (2 weeks pi) in C57Bl/6 mice. The administration of Asc extract (group II) increased NADPH diaphorase activity and TNF-α levels in the early phase of BCG infection. Furthermore, we also observed that Asc injection induced a decrease of BCG-CFU in the spleens of BCG-infected mice during the first week of infection.

BCG administration to BCG-susceptible mice (C57Bl/6, BALB/c and B10.A) generated an efficient protective early response to the challenge with homologous BCG and heterologous pathogens (*Lysteria monocytogenes*) (5). It was also demonstrated that BCG administration restricted *Mycobacterium avium* proliferation and, at the same time, promoted expression of TNF-α mRNA and IFN-γ mRNA in spleen cells, suggesting that these cytokines act in an additive or synergistic fashion in the induction of bacteriostasis (17). *In vitro* TNF-α was produced by macrophages upon stimulation with rIFN-γ and mycobacterial infection and both cytokines are crucial for activation of macrophage functions (18). Our data demonstrate that Asc extract in BCG-susceptible mice (C57Bl/6) is associated with the development of a protective early response to BCG represented by a reduction of BCG-CFU in the spleen. In addition, the present results suggest that the enhancement of TNF-α production and NADPH diaphorase activity is probably associated with the induction of a protective immune response.

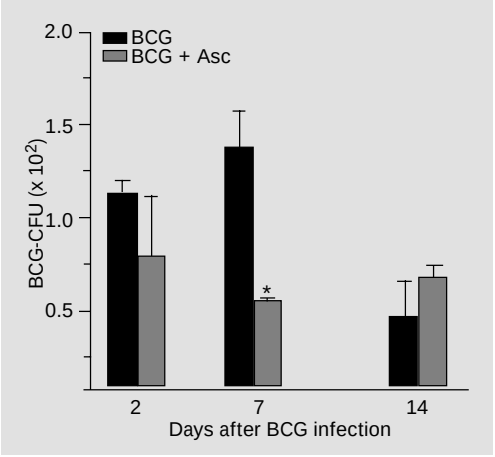

Figure 4 - Effect of *Ascaris suum* extract on the number of BCG-CFU in the spleen of BCG-infected mice. At the indicated periods, spleens were recovered and bacterial counts (BCG-CFU) performed as described. Data represent mean ± SD numbers of BCG-CFU (two animals per data point) from triplicates of one representative experiment. *P<0.05 compared to BCG group (Student *t*-test).

The murine resistance to an intracellular pathogen upon genetic regulation of the *Nramp* gene is correlated with the priming/activation of macrophages linked to TNF-α-dependent production of antimicrobial NO (19). In studies using NADPH diaphorase histochemistry and PCR it was observed that macrophages in the lungs of people with active *Mycobacterium tuberculosis* often express catalytically competent nitric oxide synthase (20). Yoshida et al. (4) showed that C57Bl/B10 mice presented high levels of iNOS protein and mRNA at 3 and 6 days after intravenous BCG infection. Our results for C57Bl/6 mice also demonstrated the participation of iNOS in the early phase of BCG infection. Additionally, the results also support the hypothesis that the production of TNF-α is closely linked to the levels of NADPH diaphorase activity.

In the present study we did not observe significant differences in NO production between group BCG and group BCG + Asc. Some studies have reported the presence of iNOS protein associated with little or no synthesized NO in human (21) and mouse

macrophages. In our experiment it is possible that the nitrite produced in the supernatants of peritoneal cells was utilized by macrophages in their activation during culture.

Many studies have demonstrated that Asc impaired important T cell functions for cell-mediated and humoral responses to other antigens by the induction of a predominantly Th2 type response with increases in IL-4 and IL-10 (2,22,23). Interestingly, it was recently found that the mechanism by which IL-10 inhibits macrophage killing of schistosomes involves suppression of TNF-α production, and can be reversed by the addition of exogenous TNF-α (24). The ability of mice to develop DTH after subcutaneous BCG immunization was inhibited by the administration of Asc extract (3). In the present study, we investigated the effect of Asc injection during the early phase of BCG infection during the development of innate nonspecific host immune response. We observed that the administration of Asc induces increased resistance against BCG infection in a susceptible strain of mice. Therefore, our data suggest that the effect of Asc might be influenced by the stage of the host response developed during BCG infection. Finally, we postulate that Asc has different effects on the innate immune response and acquired immune response in BCG-infected mice and that these responses are correlated.

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