Parasitología médica y/o veterinaria: trabajo original

Human toxocariosis: *in vitro* production of soluble mediators against *Toxocara canis* infection

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Summary

Toxocara canis (T. canis) is an intestinal nematode that affects dogs. In humans, this geohelminth induces visceral larva migrans (VLM) syndrome, which is associated with eosinophilia, increased serum IgE and inflammation of the airways. In general, chronic nematode infections induce a polarized TH2 immune response. There are observations that the macrophages response to gastrointestinal nematodes in which the alternative activation pathway plays a more important role in resistance. However, whether or not *Toxocara* infections induce alternative activation of human monocytes/macrophages is still a matter of debate. The objective of this study was to determine the pattern of IL12 and IL10 levels and nitric oxide (NO) production *in vitro* of human adherent cells against antigens from *T. canis*. For this purpose the *in vitro* cytokine production of adherent cells from healthy subjects was examined in response to stimulation with antigens from larval stage of *T. canis*. Cell culture supernatants were removed from antigen stimulated and unstimulated cultures after 24 hours, 15 and 20 days post infection and the concentration of IL-12, IL-10 and NO concentrations were determined. The results revealed high levels of IL-12 at 24 hours post stimulation. After that time, the concentration of this cytoquine declined to undetectable levels. IL-10 revealed a progressive increase from the 24 hours until the 20th day post stimulation. Conversely not detectable levels of NO were found during the study. Therefore, we concluded that during early *T. canis* infection, the activation and suppression of immune processes occur simultaneously and cytokines of adherent cells contribute to the regulation of the immune response.

Introducción

Toxocariosis, caused by infection with larvae of *Toxocara canis (T. canis),* and to a lesser extent by *Toxocara cati (T. cati)* and other ascaridoid species is one of the most widespread public health and economically important zoonotic parasitic infections humans. This neglected disease has been shown through seroprevalence studies to be especially prevalent among children from socio-economically disadvantaged populations both in the tropics and sub-tropics and in industrialised nations (Alcantara-Neves et al. 2014). Human infection occurs by the accidental ingestion of embryonated eggs or larvae from a range of wild and domestic paratenic hosts (Macpherson 2013). The infection is manifested in humans with a range of clinical syndromes including covert or common toxocariosis, visceral and ocular larva migrans and neurotoxocariosis (Desponmier 2003). In general, chronic nematode infections induce a polarized Th2 immune response. However, during its initial phase of infection, a strong pro-inflammatory response is part of the immunological profile and might determine the outcome and/or pathology of the infection (Resende et al. 2015). Previous works have shown that toxocariosis may increase predisposition to the development of allergic diseases, especially in children (Woodhall et al. 2014, Nagy et al. 2012). Several studies have revealed that the expression of cytokines is regulated by different mechanisms. Kuroda and col. (2001) observed that Toxocara-derived antigens can stimulate macrophages to produce IL-6, which enhances the differentiation of Th2 cells. This polarization is associated with the production of IL-5, IL-10 and IL-13 and the secretion of IgE (Turner et al. 2003). Macrophages play a critical role in destroying intracellular pathogens. In this sense, when they are activated by the T helper (Th) 1 cytokines, interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α) produce proinflammatory mediators, such as nitric oxide and IL-12 (Babu et al. 2009); however, macrophages can also be activated by Th2-type cytokines (particularly IL-4 and IL-13) that cause the development of a phenotype, termed "alternative activation," that is distinct from

the classical phenotype (Gordon 2003, Anthony et al. 2006). However, whether or not *Toxocara* infections induce alternative activation of human monocytes/macrophages is still a matter of debate. In fact, the systemic immune response to *T. canis*

infection has been only moderately examined in humans (Malla et al. 2006, Bourke et al. 2010). Therefore, the purpose of this study was to determine the pattern of IL12 and IL10 levels and nitric oxide production *in vitro* of human adherent cells against E/S Ag *Toxocara canis* stimulation.

Material and methods

Blood samples were obtained in sterile conditions (5ml, i.v.) from healthy children who assisted at the Pediatric Allergy and Immunology Department of Reina Fabiola Clinic. All subjects were seronegative for *T. canis* and for parasitological tests in stool samples. All the participants read, understood and signed an informed consent. Ethical approval was granted by the Ethical Committee of Clínica Universitaria Reina Fabiola. Fundación para el Progreso de la Universidad Católica de Córdoba, Argentina.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using a density gradient centrifugation technique and following the protocol provided by Histopaque 1077 (Sigma-Aldrich Inc., Saint. Louis, Missouri, USA) with slight modifications. Undiluted blood was layered 1:1 over Hystopaque. After centrifugation (400x g for 30 min at room temperature), the cells from the interface were aspirated and washed twice with sterile RPMI 1640 medium. The resulting cell pellet was resuspended in a small volume of PBS and the viable cell concentration was determined by microscopic examination using the trypan blue exclusion technique. Viability was >90% in all samples. The cells were suspended in RPMI medium supplemented with 2 mM of glutamine, 100 mg/ml of streptomycin, 100 IU of penicillin and 10% of heat inactivated fetal bovine serum (FBS). Monocyte-macrophage were separated from non adherent cells by incubation for two hours in 5% CO2 and 37 °C. Subsequently, non adherent cells were removed and macrophages were washed and culture with FBS 10% (Natocor, Argentine) and supplemented RPMI (Gibco, Auckland, NZ). Morphology was observed using Giemsa staining procedure. This cells (about 1x106/ml) were cultured within 6 hours of collection and were maintained in a humidified environment of 5% CO2 at 37°C. Adherent cells were stimulated with 10 ug/mL of E/S Ag of *T. canis* for 48 hours at 37º C and 5% CO2. The culture supernatants were aspirated for detection of nitric oxide and cytokines in the presence or absence of *T. canis* antigen and frozen at -20 °C until further use. *T. canis* adults were obtained from the small intestines of puppies by spontaneous elimination. The eggs were isolated from uteri by gentle mechanical maceration, and then purified by straining and cultured to embryonation in 50 mL culture flasks. Isolations were kept in a controlled temperature chamber at 26 ± 1 °C, for 60 days until larvae developed. Embryonation was evaluated microscopically once per week and embryonated eggs were induced to hatch following the physiological method described elsewhere (Ponce-Macotela et al. 2011). Larvae were purified and maintained in RPMI-1640 medium, to collect excretion secretion antigens (E/S) in a tube containing protease inhibitors cocktail. Subsequently they were concentrated by dialysis (10 KDa cut off), and quantified using the Bradford method, aliquoted and stored at −70°C until further use.

Production of nitric oxide (NO) was measured indirectly at different times post estimulation by assaying nitrites in the supernatant of macrophage culture using the Griess reaction technique. Supernatants were collected and mixed with equal volume (100 ul) of Griess reactive (Sigma-Aldrich Inc., Saint. Louis, Missouri, USA). Optical density (540 nm) measurements were converted to micromoles of nitrites (uM) using a standard curve of sodium nitrite. For determination of IL-12 and IL-10 levels commercial sandwich enzyme linked immunosorbent assay (ELISA) kits were used (Thermo Scientific, Pierce Technology, USA). The detection limits in pg/mL for IL-12 and IL-10 were 3 pg/ml. The cytokine concentrations (pg/ mL) for each sample were calculated by interpolation from a standard curve. Software GraphPad Prism software, version 6 was used for the statistical analysis. Statistical significance between mean cytokine responses ($p < 0.05$) was analyzed using Mann-Whitney's test.

Results and Discussion

At 24 hours post stimulation a peak production of IL-12 occurred (Table 1).

Time post infection	n	ELISA (pg/ml)	
		IL-12	IL-10
basal	**10**	**3,8 ± 1,3**	**3,4 ± 0,8**
24 hours p.i	**10**	**11,8 ± 3,3**	**3,6 ± 1,3***
15 days p.i	**10**	**0,5 ± 0,01**	**5,5 ± 3,1**
20 days p.i	**10**	**0,5 ± 0,02**	**15,6 ± 4,4***

p.i: post infection

*** p≤ 0,04**

Tabla 1. Levels of Cytokines in culture supernatans of adherent cells.

After that time, the concentration dropped to low levels. The results related to IL-10 revealed a progressive increase in this cytokine from the 24 hours until the 20th day post stimulation. Conversely not detectable levels of nitric oxide were found during the study. Similar results were obtained when adherent cells were cultured with larval stage (L2/L3) of *T. canis* (IL 12 at 24 hours post stimulation: 15 ± 5 pg/ml). The IL 10 levels (pg/ml) were: 24 hs: 6 ± 3; 15 days: 7 ± 2; 20 days: 17 ± 4 (p= 0,04).

Cells activation and Th-subset polarization following infection with parasites reflects the influence of the initial local cytokine environment. The study of these soluble immune modulators reveales the important role that they play in mounting effective as well as detrimental immune responses to pathogens. Examining the cytokine responses of macrophages and lymphocytes in culture can provide important understanding of how immune responses to pathogens are orchestrated. The assessment of cytokine levels in various parasitic infections has shown that infectious agents may trigger the production of pro-inflammatory cytokines and, in this way, modulate the course of a disease (De Avila et al. 2016).

In the present work we observed a pro-inflammatory response mediated by IL-12 production in the early phase of the stimulation related to a first encounter between the host cells and the parasite. Studies conducted on *Trichinella spiralis* and *Nippostrongylus brasiliensis* suggest that in the early phase of infection, the Th1 response, rather than the Th2 response, is induced, and there is a polarization of the response from Th1 to Th2 (Malla et al. 2006). In accordance with these authors our data showed a significant increase of IL-10 levels at the end of the study suggesting a modulation of the immune system triggered by *T. canis* antigens. Therefore, in this way we can explain the viability of larval stage at 20th day post interaction with adherent cells observed in other cultures conducted with the same experimental conditions. On the other hand, it can explain the elevated levels of IL-10 previously observed in serum from chronic infected patients compared with IL-10 concentrations in serum from healthy patients suggesting a sistemic immunomodulation during the parasite infection (data not shown).

With respect to NO, macrophages recognize antigen microorganisms through their different

receptors and trigger the production of inflammatory mediators inducing the activity of the inducible nitric oxide synthase enzyme (NOSi2). Important amounts of NO are synthesized, causing modifications in the cellular microenvironment (Gutierrez et al. 2009). However, an excess of NO has harmful effects on the host's tissues (Silva 2010). In our work, the abscence of NO could be associated to a modulation triggered by the parasite in order to evade the host immune response. On the other hand, NO production probably does not have a predominant role in eliminating this parasite because *T. canis* is an extracelular microorganism.

In accordance with Valli and col. (2010) we consider that while the *in vitro* study of PBMC cytokine responses cannot be directly extrapolated to antigens *in vivo* responses, the results do highlight the dynamic and fluctuating nature of cytokine production. Therefore, immune responses in helminth infections, depends on the extent of the parasite load, the timing and duration of the infection and the status of the host immune system.

It is important to highlight that this study was performed with human adherent cells and these findings, taken together, encourage the continuation of studies tending to reveal other cellular and molecular components of the innate immune response involved in the pathogenic mechanisms triggered during *T. canis* infection in paratenic hosts.

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