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EFFECT OF TOXOCARA CANIS SECOND STAGE LARVAE ON THE HOST IMMUNE SYSTEM

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Abstract

We have studied functional alteration of immunocytes in mice following inoculation of **Toxocara canis** second stage larvae. Results indicated depression of lymphocyte blastogenesis in response to concanavalin-A, phytohemagglutinin and allogeneic non B-cells; however, B-cell polyclonal activation was not affected as indicated by the production of total IgG. In contrast, frequency of antibody secreting cells was reduced. Similarly, interleukin-1 and interleukin-2 production were not affected by **Toxocara canis** infection. Furthermore, spleen cells of infected mice synthesized more prostaglandin E_2 than uninfected animals. This data suggests that **Toxocara canis** infection induces suppression of both cell mediated and humoral immunity; perhaps due to an increase of prostoglandin E_2 production.

Introduction

Toxocara canis (T. canis) infection in dogs is prevalent world wide and has proven to be of veterinary and medical importance in that it is transmissible to humans especially young children [1,2]. The visceral infection of children with **T. canis** may cause a variety of symptoms that persist for 6 to 24 months [3,4]. Data from several serologic studies suggest that aproximately 7% of apparently healthy individuals in the United States tested had serum antibody to **T. canis** [5]. The percentage of dogs infected with **T. canis**, ranges from 20% to 100% depending on the location of the survey [6]. The high frequency of incidence in dogs imposes a serious health hazard especially among children who are in close contact with the infected animals [5]. In addition to public health concern, persistant infection in puppies and adult dogs [7] suggests possible host immune compromise which leads to secondary infection [8].

There have been numerous studies concerning purification and characterization of **T. canis** secretory component(s) [9-13], surface antigen(s) [14,15] and antigenic alteration of the developing larvae [15] in the last two decades; while there has been only a few studies describing the mechanism(s) of survival of the larvae in the host and its reaction with the immune system [17,18]. The studies reported herein were carried out to determine the effect of **T. canis** second stage larvae on the immune system. We have found that second stage larvae depresses a certain component of the immune system by producing higher levels of prostaglandins E_2.

Key words: *Toxocara Canis, immunocytes*

Materials and Methods

Mice

Ten to twelve week-old outbred Swiss Webster mice were used in this study. Mice were divided randomly into infected and control groups (4 mice in each group).

Embryonation of ova and preparation of second stage larvae

Toxocara canis eggs were collected and embryonated by the method of Cypress **et al** [19]. Briefly adult female **T. canis** worms were collected from the feces of infected puppies following antihelminthic treatment. The eggs were recovered from uteri of the worms then embryonated **in vitro** by incubating them in 2% formalin at room temperature with aeration for 4 to 6 weeks. The embryonated eggs were washed several times to remove the formalin and stored in saline until needed. Before use, the embryonated eggs were decoated with 5.25% sodium hypocholorite then washed with phosphate buffered saline 5 times to remove the bleach. The second stage larvae (L_2) were then resuspended in saline to make a suspension of 2,000 larvae/ml to be used for inoculation of mice.

Experimental Infection

A group of 4 mice for each experiment (3 experiments for each assay) was inoculated. Mice were lightly anesthetized with ether then inoculated with 0.2 ml of L_2 suspension (400 larvae) by gastric intubation. The control group received 0.2 ml of saline. Infection of each mouse was verified by the Baermannization technique [20] using the remainder of the sacrificed animal following removal of the spleen.

Spleen Cell Suspensions

Spleens were removed (from infected and control groups) aseptically and placed in cold RPMI 1640 cell culture media. A single cell suspension was made by teasing the spleen apart and then passing it through a 26 gauge needle. The cells were washed 3 times with cold medium and resuspended in RPMI 1640 medium (5×10^6 cells/ml), supplemented with 5% heat-inactivated fetal calf serum (FCS), (2 mM/ml) L-glutamine, 100 U/ml penicillin, (100 ug/ml) streptomycin (complete medium). All the cultures were incubated at 37°C in a 5% CO_2 incubator unless otherwise stated.

Cytokine Production

Spleen cells were resuspended in complete RPMI 1640 medium to make a 5×10^6 cells/ml cell suspension [for production of interleukin-1 (IL-1), a serum substitute (1% ITS, supplied by Collaborative Research, Inc.)[1] replaced 5% FCS in the complete medium]. One milliliter of the cell suspension was placed in each well of a 24-well tissue culture plate. Cultures received either 2 ug of lipopolysaccharide (LPS) plus 10^{-5} M of indomethacin for production of IL−1, or 1 ug of concanavalin A (Con A), for production of interleukin-2 (IL-2) or 2 ug of LPS only, for production of prostaglandin-E_2 (PGE_2). After 48 hours incubation, the supernatants were recovered following centrifugation and stored at −20°C until needed. For IgG production, spleen cells were resuspended in complete RPMI 1640 containing 10% FCS and 5 ug/ml pokeweed mitogen (PWM) to make a 2×10^6 cells/ml cell suspension. After 3 days incubation at 37°C, cultures were replenished with 0.5 ml of fresh media. The cell free culture supernatants were recovered at day 7 of incubation and stored at −20°C until needed.

Enumeration of Antibody Secreting Cells

Seven days after inoculation with second stage larvae (predetermined duration), mice were injected I.P. with either 100 ug DNP-KLH adsorbed on Bentonite or 2×10^8 sheep red blood cells (SRBC). The number of indirect anti-DNP splenic plaque-forming cells (PFC) was measured 7 days after immunization with DNP-KLH (predetermined duration) using procedures described by McIntosh **et al** [21]. Only indirect PFC were enumerated because direct PFC constitutes a negligible portion of the response. The spleen cells of mice injected with SRBC were assayed for direct PFC 4 days after immunization. The percent of immunosuppression was calculated by comparison of the mean PFC/10^6 spleen cells obtained in the experimental group with the mean PFC/10^6 spleen cells obtained in control mice [22].

Detection of total IgG Production

Total IgG produced in the spleen cell (stimulated with PWM) culture supernatants was measured by an ELISA according to the method of Voller **et al** [23].

Lymphocyte Transformation Assay

The lymphocyte transformation assay was performed as described by Khansari **et al** [24]. Briefly, spleen cells were resuspended in RPMI 1640, containing 5% FCS, to make a 5×10^6 cells/ml cell suspension. One hundred microliters of the cell suspension was placed into each well of a 96-well flat bottom microtiter plate (quadruplicate). Each well received 100 ul of medium containing either 20 ug/ml PHA or 20 ug/ml Con A. Control cultures received 100 ul of the media only. Plates were incubated at 37°C, 5% CO_2 for 48 hours and then pulsed with 0.83 uCi/well ^{3}H-thymidine (^{3}H-TdR). After incubation for an additional 18 hours, cells were harvested onto a fiber glass filter disk using an automatic cell harvester[2], and the radioactivity of the filter disks was measured by a beta-counter[3]. The stimulation index (SI) was determined by the formula:

TABLE 1
Frequency of Antibody Secreting Cells in Spleens

Host Treatment	Antigen[a]	PFC[b]	%Supp.
None	SRBC	976±191	--
T. canis	SRBC	105±11	90
None	DNP-KLH	1,590±377	--
T. canis	DNP-KLH	343±68	79

[a] Mice were immunized 7 days after inoculation of host with second stage larvae.
[b] PCF per 10^6 splenocytes± standard deviation.

$$SI = \frac{\text{Mean CPM (stimulated cells)}}{\text{Mean CPM (control cells)}}$$

Mixed Lymphocyte Culture (MLC)

Mixed lymphocyte culture was performed using the procedure described by Khansari [25]. Briefly, spleen cells from infected and uninfected mice were used as responder cells and spleen cells from an allogeneic mouse served as stimulator cells. Stimulator cells were irradiated with 2,000 rad in a gamma source in order to abolish proliferation. The responder and stimulator cells were resuspeded (each separately) in RPMI 1640 containing 10% FCS to make a 5×10^6 cells/ml cell suspension. Fifty ul of the responder and 50 ul of the stimulator cells were placed in each well of a 96-well flat bottomed microtiter plate. One hundred ul of media was added to each well to bring the volume of each culture to 200 ul total. The control culture wells (background) received 50 ul responder cells plus 150 ul media only. The cultures were incubated at 37°C for 96 hours then pulsed with 0.83 uCi/well ^{3}H-TdR and then were incubated for an additional 18 hours. At the end of the incubation period, cells were harvested onto a fiber glass disk and the radioactivity was measured by a beta-counter[3]. The SI was determined by the formula:

$$SI = \frac{\text{Mean CPM (responder} \pm \text{stimulator cells)}}{\text{Mean CPM (responder cells only)}}$$

IL-1 and IL-2 Activities Determination

The IL-1 activities of the spleen cell culture supernatants were determined using the method described by Conlon [26]. Briefly, LBRM-33-1A5[4] cells were treated with mitomycin-C to abolish DNA synthesis [27]. The cells were then cultured in a 96-well microtiter plate in the presence of 0.5 ug/ml (final concentration) PHA and various dilutions of supernatants recovered from LPS stimulated spleen cells. Control wells received either PHA only or media only. After 24 hours incubation at 37°C, 50 ul of a 8×10^4 cells/ml CTLL-2[4] (an IL-2 dependent cell line) cell suspension was added to each well. Cultures were incubated at 37°C for an additional 20 hours then were pulsed with 0.83 uCi ^{3}H-TdR for 4 hours. At the end of incubation, cells were harvested onto fiber glass filter disks and radioctivity was measured by a beta-counter[3].

The IL-2 activities of supernatants recovered from Con A stimulated spleen cells was determined by the method described by Gillis **et al** [28]. Briefly, 4×10^4 CTLL-2[4] cells (in 100 ul RPMI 1640) were cultured in each well of a 96-well microtiter plate in the presence of various dilutions of con A stimulated culture supernatant. Cultures were incubated for 20 hours then pulsed with 0.83 uCi of ^{3}H-TdR for 4 hours and harvested onto fiber glass filter disks. The radioactivity of the disks was measured by a beta-counter[3].

Prostaglandin E_2 (PGE_2) Assay

The PGE_2 content of the LPS stimulated spleen cell culture supernatants was assessed using a commercially available kit[5] as described by Khansari **et al** [29].

Results

Immunosuppression was apparent 7 days post inoculation and it lasted through the duration of the experiment (20 days). Data presented herein are those representing 7 days post inoculation experiments unless otherwise stated.

Frequency of Antibody Secreting Splenocytes

Enumeration of PFC from spleens of infected mice, which were immunized with either SRBC or DNP-KLH seven days post inoculation, showed fewer numbers of antibody secreting cells than that of non-infected control mice (Table 1). This suggests

that the presence of larvae leads to suppression of the humoral immune response.

Cytokine Production by Splenocytes

Spleen cells of infected mice and non-infected controls were stimulated with various mitogens 7 days post second stage larvae inoculations. As Table 2 shows, there was no change in productior immunoregulatory cytokines IL-1 and IL-2. However, production of prostaglandin E_2 was higher in the infected animals. Total IgG production of the spleen cells in response to a polyclonal activator, PWM, was enhanced in the infected animals in contrast to a decrease in the number of specific IgG producing cells (Table 1).

Mitogen Induced proliferative Response of Spleen cells

Phytohemaglutinin and Concanavalin A both are polyclonal T-cells activators for murine splenocytes. This activation is indicative of T-cell ability to respond to antigens and/or mitogens; therefore, it is called the initial or first stage of the host immune response to stimuli especially in the case of cell mediated immunity. We studied the effect of second stage larvae on T-cell activation in response to both PHA and Con A in infected mice. As is shown in Table 3, our data indicate that spleen cells of infected animals do not respond well to T-cell mitogens (60% less than control).

Proliferative Response of Spleen Cells by Allogeneic Cells

Splenocytes can be stimulated by allogeneic non-T cells. The activated cells (proliferated cells) are a subpopulation of T-Cells which are responsible for killing tumor cells, virus infected cells or attacking invading tissue parasites; therefore; (cytotoxic T-cells) thus this assay measures a specific function of cell mediated immunity. We assessed the stimulation index of splenocytes from infected mice in response to gamma-irradiated allogeneic non T-cells. As shown in Table 3, stimulation of spleen cells from infected animals was half of that of the non infected control.

Discussion

The mechanism(s) by which parasites evade host immune responses is not well understood. One possible mechanism is by induction of immunosuppression which has been shown in many parasitic infections [30-35]. We have investigated the effect of **T. canis** second stage larvae on the cell mediated and humoral immune responses in the murine system in order to elucidate the mechanism(s) of host-parasite interaction. Our results indicate that immunosuppression of the host is detectable when larvae have penetrated various tissues of the host (4 to 7 days following larvae inoculation). The functional alteration of immunocytes seems to be selective since it does not affect all of the immunocytes' functions. For instance, production of IL-1 by macrophages, IL-2 by T-cells, and polyclonal activation of B-cells (non-specific IgG production) were not depressed. These findings indicate that the induction of immunosuppression is an active process rather than a non-specific phenomenon. The effect of the **T. canis** larvae on the host immune system is rather unique in that while it does not depress polyclonal activation of B-cells (it rather enhances it, see Table 2), it depresses the generation of antibody secreting cells (Table 1). Furthermore, both PHA, Con A and allogeneic non-B-cell induced blast transformation were depressed (Table 3). Similarly, in mixed lymphocyte reaction, cytotoxic T-cells are stimulated; therefore, our data implies that cell mediated immunity in general and some function of T-cells in particular (cytotoxic T-cell generation) is affected by the presence of second stage larvae. In contrast to our finding, Kayes [36] has not noticed any suppression in either humoral or cell mediated immunity. However, Kayes [36] did not investigate the effect of **T. canis** on various immunocyte function but only in blast transformation assays.

Even though our data suggest a direct suppressive effect may be exerted by **T. canis** second stage larvae in the host, enhancement of PGE_2 production in **T. canis** infected mice (Table 2) may also have an important role in the survival of **T. canis** larvae in tissue. PGE_2 is produced by macrophages and granulocytes in response to invading microorganisms or LPS. It has been thought that PGE_2 is responsible for most inflammatory symptoms [37,38]. Whatever the true role of PGE_2 is in inflammation, its suppressor effect on most if not all functions of immunocytes is well known [39-40]; therefore, it is quite possible that, at least one mechanism, for the immunosupressive effect of **T. canis** second stage larvae is due to an increased production of PGE_2. Whether there are other mechanisms which protect the larvae from host immune defenses remain to be elucidated.

ABBREVIATIONS

L_2	Second stage larvae
FCS	Fetal calf serum
IL−1	Interleukin−1
LPS	Lipopolysaccharide
IL-2	Interleukin-2
CON A	Concanavalin A

TABLE 2
Cytokine Production by Splenocytes

Cytokine	Non-Infected Splenocyte	Infected Splenocyte
IL-1	19,440[a]±1,393	21,070[a]±889
IL-2	1,052[a]±207	1,407[a]±568
PGE_2	31[b]±14	60[b]±12
IgG	35[c]±31	63[c]±21

[a] Count per minute±standard deviation
[b] pg/ml/10^6 cells±standard deviation.
[c] ng/ml/10^6 cells±standard deviation.

TABLE 3
Blastogenesis[a] of splenocytes in Response to Various Stimuli

Experiment[b]	PHA Non-Infect	PHA Infect	Con A Non-Infect	Con A Infect	Allogeneic Non-Infect	Allogeneic Infect
1	16	7	7	2	2.6	1.2
2	30	9	4	1	5.3	1.8
3	32	13	19	5	2.2	2.3
Mean±SD	26±7	10±2	10±6	3±1.7	3.4±1.4	1.8±0.45

[a] Stimulation Index.
[b] Four mice in each group, four days after **T.Canis** larvae inoculation.

PGE_2	Prostaglandin E2
PWM	Pokeweed mitogen
SRB	Sheep red blood cells
PFC	Plaque forming cells
ELISA	Enzyme linked immunosorbent assay

FOOTNOTES

[1] Bedford, MA.
[2] PHD cell harvester, Cambridge Technology, Cambridge, MA.
[3] Beckman Instrument, Inc., Palo Alto, CA.
[4] American Type Tissue Culture Collection, Rockville, MD.
[5] New England Nuclear, Co., Boston, MA.

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