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Immune Functions of *Toxocara canis*-infected Mice

UKI YAMASHITA[1], HONGJIE JIANG[1], HIROKO INOUE[1], YUKIRO MUTOH[2] AND TOSHINORI FURUKAWA[3]

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Abstract

C3H/He mice were infected with *T. canis* by oral administration of embryonated eggs and their immune functions were studied *in vitro*. The spleen cells of infected mice failed to respond to T cell mitogen, Con A, but not to B cell mitogen, LPS. Not only proliferative responses but also the production of IL-2 was impaired in spleens from the infected mice. The spleen cells from the infected mice suppressed the Con A response of spleen cells from normal mice, suggesting that suppressor cells develop in the infected mice. The suppressor cells are macrophages because sephadex G-10-purified spleen cells could not show suppressor activity. On the contrary, B cell activity such as Ig-producing cells was enhanced in spleens from the infected mice. Both IgM- and IgG-producing cells were enhanced. The production of IL-1 by macrophages was also enhanced in spleens from the infected mice. All these results suggest that B cell and macrophage activities are enhanced and T cell activity is suppressed due to the presence of suppressor macrophages in the *T. canis*-infected mice.

Key words: *Toxocara canis,* Immune functions, T cells, B cells, Macrophages

Introduction

The infection of parasites induces a variety of immunological alterations in the hosts. Some of the representative immunological alterations in the infected hosts are the increased level of serum immunoglobulin (Ig)E and eosinophilia (Kojima *et al.*, 1979; Sugane *et al.*, 1984). The increased level of IgE and eosinophilia is considered to be induced by the nonspecific activation of helper T cells and the overproduction of interleukin (IL) such as IL-4 and IL-5 (Yamaguchi *et al.*, 1990; Finkelman *et al.*, 1991). Other remarkable alterations of the immune system are the depression of T cell functions or the polyclonal B cell activation which results in the production of autoantibodies (Corria *et al.*, 1980; Suzuki *et al.*, 1981; Fisher *et al.*, 1981; Takai *et al.*, 1985). However, these alterations are variable by the species of parasites used and the experimental conditions employed. In this paper we attempted to analyze the mechanism of immunological alterations of parasite-infected host by using the combination of mice and *Toxocara (T.) canis*. Recently, the cases of *T. canis* infection in humans are increasing (Inoue and Tsuji, 1989). Humans and mice are paratenic hosts for *T. canis* in which infective larvae migrate through various organs and induce various diseases (Barriga, 1988; Glikman, 1981). Thus, mice can be considered to be suitable animals for the study of immunological functions of parasite-infected hosts. In this paper evidence will be presented that B cells and macrophages are functionally activated and T cell activities are depressed in *T. canis*-infected mice.

[1]*Department of Parasitology, Hiroshima University School of Medicine, Kasumi, Minami-ku, Hiroshima 734, Japan.*
[2]*Nakatsu Gastro-Intestinal Hospital, Miyao, Nakatsu, Oita 810, Japan.*
[3]*Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine, Kasumi, Minami-ku, Hiroshima 734, Japan.*
山下優毅　姜　洪杰　井上洋子（広島大学医学部寄生虫学教室）
武藤征郎（中津胃腸病院）
古川敏紀（広島大学医学部附属動物実験施設）

Materials and Methods

Mice

C3H/HeN mice were obtained from Seiwa Experimental Animal Co. (Yoshitomi, Oita) and maintained on purina rodent chow and water at libitum in our laboratory. Both male and female mice were used at 6 to 8 weeks of age.

T. canis

T. canis adult worms were collected from the intestinal tract of pups at autopsy which were donated from Hiroshima prefecture animal protection center (Hiroshima) and maintained in 1% formaldehyde at 30°C for several weeks to develop the eggs to the second-stage larvae. The eggs were treated with 10% sodium dihydrochloride and kept in 4°C until used. The eggs were orally administered into mice.

Assay of proliferative responses

Spleens were obtained from normal and infected mice and spleen cell suspension was prepared after the treatment with red blood cell lysing buffer. Spleen cells (2×10^5) were cultured with concanavalin A (Con A, 10 μg/ml, EY Laboratories, San Matero, CA) or lipopolysaccharide from *Escherichia coli* (LPS, 10 μg/ml, Sigma Chemical Co. St. Louis, MO) in 0.2 ml of Eagle Hanks Amino Acid (EHAA) medium described by Corradin *et al.* (1977) containing 10% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, NY) in wells of flat-bottomed microtiter culture plates (#3072, Falcon Plastics, Oxnard, CA) at 37°C for 3 days in 5% CO_2 and 95% air. The cells were labeled during the last 18 hours with 0.5 μCi of tritiated thymidine (^{3}H-TdR, specific activity, 6.0 Ci/mmol, Amersham Plc, Buckinghamshire, UK) and were harvested with the aid of an automated cell harvester (Abe Kagaku Co., Chiba). The amount of radioactivity incorporated into DNA in the cells was measured with a beta counter (Harrison *et al.*, 1974). The results were expressed as the mean counts per minute (cpm) of triplicate cultures with the standard error.

Fractionation of spleen cells

Spleen cells from the infected mice were treated with 20× diluted rabbit anti-mouse IgG sera (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) or 100× diluted anti-mouse Thy 1. 2 monoclonal antibody (Olac 1976 Ltd, Blackthorn Biester, Oxon, England) and 6× diluted rabbit complement (Cedarlane Laboratories) at 37°C for 1 hour and T cell and B cell fractions were obtained. Spleen cells were also purified by passing through sephadex G-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden) to deplete macrophages.

Assay of IL-2

Spleen cells (5×10^6/ml) were cultured with or without Con A (10 μg/ml) in EHAA medium containing 10% FCS at 37°C for 48 hours in 5% CO_2 and 95% air. The culture supernatant was recovered by centrifugation and dialyzed against 100 volumes of RPMI 1640 medium (Nissui Seiyaku Co., Tokyo). The IL-2 activity in the culture supernatant was assayed with a murine cytotoxic T cell line (CTLL) (Malek *et al.*, 1984). CTLL (1×10^4) were cultured with serially diluted IL-2 samples in 0.2 ml of EHAA medium containing 10% FCS in flat-bottomed microtiter culture plates (#3072, Falcon Plastics) at 37°C for 48 hours in 5% CO_2 and 95% air. The cells were pulsed with 0.5 μCi of ^{3}H-TdR for the last 12 hours, then harvested and the radioactivity incorporated into CTLL was counted. The results were expressed as units of IL-2 produced by 10^6 spleen cells compared with recombinant human IL-2 (Taniguchi *et al.*, 1983) donated by Dr. J. Hamuro, Ajinomoto Co., Tokyo as a standard.

Production and assay of IL-1

Spleen cells (5×10^6/ml) were cultured in RPMI 1640 medium containing 10% FCS in culture dishes (#3002, Falcon Plastics) at 37°C for 2 hours. Non adherent cells were removed by gentle washing. Adherent cells were obtained by detaching from the dishes using a rubber policeman and cell number was adjusted. The recovered cells (1×10^6/ml) were cultured with LPS (10 μg/ml) in EHAA medium containing 10% FCS in culture dishes (#3002, Falcon

Plastics) at 37°C for 48 hours in 5% CO_2 and 95% air. The culture supernatant was harvested by centrifugation, dialyzed against 100 volumes of RPMI 1640 medium and used as a source of IL-1. This IL-1 source did not contain IL-2 activity detected by CTLL assay as mentioned above. The IL-1 activity of the culture supernatant was assayed using murine thymocytes according to the method of Mizel *et al.* (1978). Thymocytes (1.5×10^6) from C3H/HeN mice in 0.2 ml of EHAA medium containing 10% FCS were cultured with serially diluted IL-1 samples in flat-bottomed microtiter culture plates (#3072, Falcon Plastics) at 37°C for 3 days in 5% CO_2 and 95% air. The cells were labeled during the last 18 hours with 0.5 μCi of ^{3}H-TdR, then harvested and the radioactivity incorporated into thymocytes was counted. The results were expressed as units of IL-1 produced by 1×10^6 macrophages compared with recombinant human IL-1 (Furutani *et al.*, 1986) donated by Dr. M. Yamada, Dainippon Pharmaceutical Co., Osaka) as a standard.

Assay of Ig-producing cells

Ig-producing cells in the spleen were detected by a reverse hemolytic plaque forming cells (PFC) assay on slide glasses with protein A (Pharmacia Fine Chemicals)-coated sheep erythrocytes (Pierce *et al.*, 1971). Sheep erythrocyte-specific antibody response was also detected using sheep erythrocytes as target antigens. Both direct and indirect PFC, which were facilitated by rabbit anti-mouse IgG serum, were detected (Gromowize *et al.*, 1976). The results were expressed as the mean numbers and the standard errors of PFC/spleen in triplicate cultures.

Determination of spleen cell number

Viable cell number was determined by a trypan blue dye exclusion test.

Statistical anslysis

Statistical analysis was performed by student's t test between the normal and the infected animals. A confidence level of $P < 0.05$ was considered significant (Zar, 1974).

Results

Proliferative responses of spleen cells from T. canis-infected mice

C3H/He mice were infected with *T. canis* by oral administration of embryonated eggs and immune functions of these mice were studied by culturing spleen cells *in vitro*. As shown in Table 1, spleen cells from normal mice responded well with the stimulation of T cell mitogen, Con A and B cell mitogen, LPS to proliferate. However, Con A-induced proliferative response of spleen cells was markedly impaired in mice infected with *T. canis* 1 to 4 weeks previously. The impairment of the response was most remarkable in spleen cells from mice infected 2 weeks previously. The Con A-response recovered to the normal level in 8 weeks after the infection. Spleen cell number also increased in mice 1 to 4 weeks after the infection and recovered to the normal level in 8 weeks. The time course of the increase of spleen cell number was the same as the suppression of Con A response. The proliferative response induced with LPS was not markedly impaired. Table 2 shows the dose response of the administration of *T. canis* eggs. Although the suppression depended on the number of eggs, 10 eggs were quite enough to suppress Con A response. Not only proliferative response but also IL-2 production was impaired in the infected mice (Table 3).

Suppressor macrophages exist in T. canis-infected spleen

The impaired Con A response of spleen cells from the infected mice may be due to the presence of suppressor cells. In order to study this possibility, we performed mixed culture experiment. As shown in Table 4, the Con A response of spleen cells from infected mice was again markedly impaired. When spleen cells from the infected mice were added to the spleen cells from normal mice, the Con A response of normal spleen cells was suppressed. The treatment of infected spleen cells with anti-Ig antiserum or anti-Thy-1 antiserum and complement gave no effect on the suppressive activity. However, the purification of infected spleen cells by sephadex G-10

Table 1 Proliferative responses of spleen cells from *T. canis*-infected mice

Source of spleen cells from mice after infection*	Recovered spleen cells $\times 10^6$‡	[^{3}H]-thymidine uptake (cpm)† stimulated with 0	Con A	LPS
Normal	86.7± 8.7	4,111±495	53,985±4,907	34,225±2,564
1 week	173.0±16.0	3,694±838	11,687± 669§	25,616±1,251
2 week	146.5±19.5	3,931±524	9,194± 768§	29,667±1,906
4 week	103.5± 0.3	4,536±246	20,875±4,123§	29,643± 981
8 week	80.8± 8.1	5,495±370	60,932±4,468	47,645± 916

*: 100 *T. canis* eggs were orally administered.
†: 2×10^5 spleen cells were cultured 2 days, were labeled with [^{3}H]-thymidine 1 day and were harvested. The results were expressed as the means and standard errors of cpm in triplicate cultures.
‡: Means and standard errors of three mice in each group.
§: Significantly suppressed.

Table 2 Dose response of *T. canis* eggs on proliferative response of spleen cells

Number of *T. canis* eggs*	Recovered spleen cells $\times 10^6$	[^{3}H]-thymidine uptake of spleen cells (cpm)† stimulated with 0	Con A	LPS
0	178.0± 6.5	1,216±104	14,966±927	2,253±197
10	142.0± 5.2	1,852±287	4,643±253‡	3,245±107
30	166.0± 6.1	2,078±116	3,764±228‡	3,500±209
100	174.0± 6.4	1,572±140	2,373±302‡	3,053±275
300	272.0±10.0	1,580±130	1,837± 19‡	2,522±219

*: *T. canis* eggs were administered 2 weeks previously.
†: 2×10^5 spleen cells were cultured.
‡: Significantly suppressed.

Table 3 IL-2 production by spleen cells from *T. canis*-infected mice

Spleen cells from mice after infection*	Production of IL-2 (units/10^6 spleen cells†) stimulated with –	Con A
Normal	<1.0	14.4±0.7‡
1 week	<1.0	12.2±0.6
2 week	<1.0	7.6±0.4§
4 week	<1.0	11.0±0.5
8 week	<1.0	14.8±0.7

*: 100 *T. canis* eggs were administered.
†: 5×10^6/ml spleen cells were cultured 2 days in the presence or absence of Con A and the culture supernatant was harvested.
‡: IL-2 activity in the culture supernatant was assayed by CTLL and the results were expressed as units of IL-2 produced by 10^6 spleen cells.
§: Significantly suppressed.

Table 4 Suppressor activity of spleen cells from *T. canis*-infected mice

Spleen cells from	[^{3}H]-thymidine uptake of spleen cells (cpm)* stimulated with 0	Con A
Normal mice	4,640 ± 266	21,379 ± 2850
Infected mice†	3,737 ± 90	4,436 ± 485§
Normal + infected mice‡	5,083 ± 47	9,983 ± 586§
treated with C'	5,753 ± 258	11,082 ± 567§
Anti-Ig + C'	3,836 ± 201	6,022 ± 210§
Anti-Thy-1 + C'	5,152 ± 367	9,672 ± 455§
Sephadex G-10	10,034 ± 521	38,229 ± 569

*: 2×10^5 spleen cells were cultured.
†: 100 *T. canis* eggs were administered 2 weeks previously.
‡: 2×10^5 spleen cells treated as indicated were added.
§: Significantly suppressed.

column depleted the suppressor activity. These results suggest that suppressor cells are induced in the spleen of infected mice and the suppressor cells are macrophages. Then, the next question is whether T cells from the infected mice can respond normally to Con A or not. In order to address this question, T cells were purified by nylon wool column and cultured with or without normal macrophages. As shown in Table 5, spleen cells from the infected mice could not fully respond to Con A. However, after the purification by nylon wool column, T cells from the infected mice could respond to Con A in the presence of normal macrophages as well as normal T cells. Thus, the impaired T cell response in the infected mice is due to suppressor macrophages and T cell activity itself is not impaired.

B cell activity of T. canis-infected mice

Next, we studied B cell activity of *T. canis*-infected mice by detecting Ig-producing activity using reverse PFC assay. As shown in Table 6, Ig-producing cells were markedly enhanced 1 to 4 weeks after *T. canis*-infection. Both IgM- and IgG-producing cells were enhanced. Antigen specific clones such as anti-sheep erythrocyte antibody producing cells were also enhanced by the infection. The time course of the enhancement of Ig-producing cells is about the same as the impairment of Con A response. Table 7 shows the dose response of *T. canis* eggs on the B cell response. Although the enhancement of B cell response depended on the number of eggs, 10 eggs were quite enough for the enhancement of B cell response. Thus, B cell activity is enhanced in the *T. canis*-infected mice.

IL-1 production by macrophages from T. canis-infected mice

In Table 4, we showed that suppressor macrophages were induced in the spleen of *T. canis*-

Table 5 Responsiveness of T cells from *T. canis*-infected mice

Source of spleen cells	Treated with	Addition of Macrophages‡	[^{3}H]-thymidine uptake of lymphocytes (cpm)* stimulated with 0	Con A
Normal	–	–	11,053 ± 536	103,222 ± 3,292
Normal	Nylon-pass	–	499 ± 86	1,033 ± 48
Normal	Nylon-pass	+	919 ± 34	93,293 ± 7,911
Infected†	–	–	7,325 ± 262	35,203 ± 4,434§
Infected	Nylon-pass	–	1,221 ± 98	3,636 ± 133
Infected	Nylon-pass	+	1,186 ± 148	72,803 ± 9,784

*: 2×10^5 spleen cells were cultured.
†: 100 *T. canis* eggs were administered 2 weeks previously.
‡: 10^5 spleen adherent cells were added after mitomycin C-treatment.
§: Significantly suppressed.

Table 6 Antibody production by spleen cells from *T. canis*-infected mice

Source of spleen cells from mice after infection*	Plaque forming cells/spleen†			
	Protein A-SRBC			SRBC
	IgM	IgG‡	Total	IgM
Normal	6,000± 100	0	6,000± 100	700±150
1 week	34,000±6,000§	0	34,000±6,000§	ND
2 week	29,000±3,000§	10,000±1,000§	39,000±4,000§	4,150±150§
4 week	16,000±1,000§	40,000±8,000§	56,000±9,000§	ND
8 week	2,500± 500	11,500±3,500§	14,000±4,000§	ND

*: 100 *T. canis* eggs were administered.
†: Plaque forming cells (PFC) were detected using SRBC or protein A-coupled SRBC as target.
‡: IgG PFC is total PFC–IgM PFC.
§: Significantly enhanced.
ND: not done.

Table 7 Dose response of *T. canis* eggs on antibody production

Number of *T. canis* eggs*	PFC/spleen†		
	Protein A-SRBC		
	IgM	IgG‡	Total
0	2,500± 1,500	6,000±1,500	8,500± 3,000
10	22,000± 2,000§	14,500±6,000§	36,500± 8,000§
30	27,500±11,200§	15,500±1,000§	43,000±12,200§
100	27,000±11,000§	19,000±3,000§	46,000±14,000§
300	22,100± 6,400§	26,600±6,900§	48,700±13,800§

*: *T. canis* eggs were administered 2 weeks previously.
†: PFC were detected using protein A-coupled SRBC as target.
‡: IgG PFC is total PFC–IgM PFC.
§: Significantly enhanced.

Table 8 IL-1 production by macrophages from *T. canis*-infected mice

Macrophages from mice after infection*	Production of IL-1 (units/10^6 macrophages)† stimulated with	
	–	LPS
Normal	<1.0	12.6±1.2
1 week	<1.0	46.8±1.4‡
2 week	<1.0	63.7±6.0‡
4 week	<1.0	44.4±4.2‡
8 week	<1.0	19.3±1.8‡

*: 100 *T. canis* eggs were administered.
†: 1×10^6 spleen adherent cells were cultured for 2 days in the presence or absence of LPS and the IL-1 activity in the culture supernatant was assayed by murine thymocytes. The results were expressed as the units of IL-1 produced by 10^6 macrophages.
‡: Significantly enhanced.

infected mice. Then, we finally studied the activity of macrophages to produce IL-1. As shown in Table 8, spleen macrophages did not produce IL-1 without stimulation, but the culture of macrophages with LPS induced the production of IL-1. The infection of *T. canis* enhanced the production of IL-1 by macrophages 1 to 4 weeks after the infection. The peak response was observed 2 weeks after the infection and recovered to the normal level in 8 weeks. The time course of the production of IL-1 is the same as the suppression of Con A response.

Discussion

We studied immunological functions of mice infected with *T. canis in vitro*. The T cell response

of spleen detected by Con A-induced proliferative response was impaired in mice infected 1 to 4 weeks previously. The impairment of T cell response was maximum 2 weeks after the infection and was recovered to the normal level in 8 weeks. The spleen cell number and the peripheral eosinophil number also changed by the same time course as the suppression of Con A-response. However, the suppression of T cell response is not due to the change of cell population of the spleen, because the cell population in the infected spleen was not significantly changed from that of the normal spleen (data not shown). Our results are clearly contrast to the reports by Kayes (1984) and Kayes *et al.* (1985) who found that spontaneous and Con A-induced T cell proliferative responses were augmented in mice infected with *T. canis*. The reason why this discrepancy was induced is not clear. The only point of the difference between their and our experimental system is the difference of mouse strains used. They used CBA/J mice, while we used C3H/HeN mice. However, in other publication, they also found that Con A response was impaired in the spleen from *T. canis*-infected C57BL/6 mice (Kayes *et al.*, 1987). We performed the same experiment using C3H/HeJ, BALB/c and DBA/2 mice and always observed Con A response to be impaired in the infected mice. Barriga found that anti-sheep erythrocyte antibody response was impaired in *T. canis*-infected mice and suggested that this impairment was due to the interference of helper T cell function by *T. canis* infection (1988). Thus, we believe that T cell response is impaired in *T. canis*-infected mice. The impairment of T cell response seems to be induced by suppressor macrophages, because spleen cells from the infected mice could suppress the Con A response of normal spleen cells. This suppressor activity was depleted by sephadex G-10 column purification and nylon column-purified T cells from the infected mice could respond normally to Con A in the presence of normal macrophages. The presence of suppressor macrophages are reported by Coulis *et al.* (1978), Corria *et al.* (1980) and Suzuki *et al.* (1981) in the system of *Schistosoma mansoni*-, *Plasmodium chabaudi*- and *Toxoplasma gondii*-infected mice.

In contrast to T cell function, B cell activity detected by Ig-producing cells was enhanced in spleens from *T. canis*-infected mice. The time course is the same as that of the suppression of T cell response. Yamashita reported that antigen-specific antibody response was impaired but total Ig-producing cells were enhanced in the *T. canis*-infected mice (1984). Fisher *et al.* (1981) and Takai *et al.* (1985) also reported that B cells were activated polyclonally in *Schistosoma mansoni*- and *Angiostrongylus cantonensis*-infected mice. Thus, our results are consistent with theirs, and polyclonal B cell activation seems to be a general phenomenon in the parasite-infected hosts. Concerning the mechanism of polyclonal B cell activation, some possibilities can be considered. One of them is a disturbance of immunoregulatory functions of the host by a parasite infection. It may depress suppressor T cell activity which results in the activation of B cells. Alternatively, a parasite infection may activate T cells and macrophages nonspecifically which result in the overproduction of cytokines. In fact we observed in this experiment that T cell proliferative response and IL-2 production were depressed but the IL-1 production by macrophages was enhanced in the *T. canis*-infected mice. Other possibility is that parasites and their products may stimulate B cells directly to proliferate and to differentiate to antibody producing cells. In fact we found that extracts of *T. canis* have a stimulating activity on B cells and macrophages *in vitro* (Yamashita *et al.*, manuscript in preparation). We are now studying the mechanism of B cell activation in parasite-infected hosts from the standpoint of B cell stimulating activity of parasite products.

What is the biological significance of the B cell and macrophage activation in the parasite-infected hosts? We consider that non-specific B cell activation produces large amounts of non-protective antibody which cover the antigenic determinants on the parasites and prevent the access of effector functions to the parasites such as cell mediated immunity. Nonspecific macrophage activation also results in the nonspecific suppression of protective immunity for the hosts

as observed in T cell-mediated immunity. Thus, B cell and macrophage activation by parasites seems to be an escape mechanism of the parasites from the host protective immunity. Other important finding of this experiment is that as few as 10 *T. canis* eggs can disturb the host immune functions: B cell and macrophage functions are augmented and T cell function is depressed. Recently, the cases of human toxocariasis are increasing as the number of dogs increased in close proximity to our living environment (Inoue and Tsuji, 1989).

One of the important diseases caused by *T. canis* is visceral larva migrans such as larval granulomatosis and ocular larva migrans which primarily occurs in toddler-aged children (Glickman and Schantz, 1981). As shown in this experiment, in addition to visceral larva migrans, a further attention should be paid to human toxocariasis from the standpoint of immunological perturbation of the hosts.

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