ANTIGEN SPECIFIC LYMPHOCYTE PROLIFERATIVE RESPONSE OF PATIENTS WITH ACUTE AND CHRONIC TOXOPLASMOSIS

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ABSTRACT: *In vitro* lymphocyte proliferative response of peripheral blood leucocytes (PBL) to purified *Toxoplasma gondii* antigen were evaluated by 3[H] methyl thymidine incorporation in patients acutely and chronically infected with *Toxoplasma gondii*. PBL from three patients with acute symptomatic toxoplasmosis showed no response to *T. gondii* antigen during the emergence of anti-*Toxoplasma* IgM antibodies and the response returned as the infection became chronic. Lymphocytes of twelve chronically-infected patients responded positively to the antigen. In all patients the lymphocyte proliferative response to the mitogen, Concanavalin A (Con A) was normal. Analysis of *Toxoplasma* proliferative response of PBL from a patient with acute toxoplasmosis showed that $CD8^+$ cells were responsible for induction of suppression while the response during the chronic infection was mediated by $CD4^+$ cells. In human toxoplasmosis there was antigen-specific lymphocyte unresponsiveness during the acute phase of the infection and it appears that the immunesuppression was mediated by $CD8^+$ cells. *(JUMMEC 1999; 1:34-38)*

KEYWORDS: Toxoplasmosis-lymphocyte blastogenesis-antigen specific-$CD4^+$, $CD8^+$

Introduction

Toxoplasma gondii, one of the most common parasites of man all over the world, is known to cause latent infection in man and produce a persistent antigenic stimulus. *T. gondii*, even in asymptomatic humans, is known to stimulate a humoral response (1). In addition, a well-developed cell-mediated immune response against the parasite is characteristic of toxoplasmosis and can be demonstrated by delayed type hypersensitive reactions to specific antigens (2,3). More recently *in vitro* studies of cell-mediated immunity in humans with toxoplasmosis showed that lymphocytes proliferate differentially in response to *T. gondii* during the acute and chronic phases of the disease (4,5,6,7). However due to the scarcity of the number of cases that could be followed-up from the time of initial infection to the latent stage of infection, the dynamic change or pattern in the lymphocyte responsiveness during the course of the disease is yet to be fully delineated.

In this study we analysed the responses of patients lymphocytes to specific *Toxoplasma gondii* antigen and to non-specific mitogen, Con A. We now report the results of lymphocyte transformation in healthy individuals, acute and chronic patients with toxoplasmosis and characterize the cells that may be responsible for the observed results.

Material and Methods

Patients and controls

This study was carried out according to the principles of the Declaration of Helsinki, with patients' consent and Universiti Sains Malaysia Human Experimental Committee approval.

A total of 20 subjects all female, ages ranging from 20 to 35 years were randomly selected through the antenatal clinic. They were divided into three groups; group I consisted of five subjects who were negative for both IgG and IgM *Toxoplasma* antibodies; group II comprised of twelve subjects who had *Toxoplasma*-specific IgG

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antibodies. The third group was three cases of acute infection, of which one was a laboratory acquired infection who presented with acute symptoms for three months. All three acutely infected patients were symptomatic and had high levels of *Toxoplasma*-specific IgM antibodies.

Preparation of Toxoplasma antigen

The RH strain of *Toxoplasma gondii* was collected from two sources, viz. peritoneal exudate of infected mice and *in vitro* cultures of infected nasopharyngeal carcinoma cells. The method of *Toxoplasma* antigen preparation is as described by Rahmah et al. (8). Briefly, the exudate was mixed with RPMI-1640 and fetal calf serum and incubated in plastic petri dishes for 45 minutes to remove macrophages. The suspension was centrifuged at 500 rpm for 5 minutes to remove cellular contaminants. The pellet was discarded and the supernatant recentrifuged at 3000 rpm for 20 minutes. The resulting supernatant was discarded and the pellet was washed with phosphate-buffered saline (PBS) twice by centrifugation at 3000 rpm for 10 min. The final pellet was stored at - 20°C until required.

Isolation of lymphocyte population

Twelve ml of blood was drawn into a venoject containing 100 U of heparin (Upjohn Co., Kalamazoo, Mich., USA) and mixed with an equal volume of sterile 0.15 M Na Cl_2. Twelve ml of diluted blood was carefully layered over 3ml of a mixture of Ficoll and sodium diatrizoate (Hypaque; Pharmacia, Sweden) in 15ml plastic centrifuge tubes (Costar, USA). The tubes were then centrifuged at 1300 rpm for 45 min at room temperature. The layer of mononuclear cells at the plasma Hypaque interface was removed and suspended in culture medium RPMI 1640 with 100U/ml penicillin, 50ug/ml streptomycin and 2mM fresh glutamine. The cell suspension was washed three times in serum free medium and resuspended at a concentration of 4×10^6 cells/ml in culture medium with 10% heat inactivated autologous plasma. Lymphocytes represented more than 90% of white cells recovered.

Lymphocyte Transformation Assay (LTT)

Mitogen-induced in vitro lymphocyte blastogenesis was performed in flat-bottomed microtiter plates (Nunc, USA) while antigen-induced lymphocyte blastogenesis was performed in round-bottomed microtier plates (Nunc, USA). Each well received 100 ml of 4×10^5 cells in RPMI-1640 supplemented with 3% penicillin-streptomycin and 10% autologous plasma. For smitogen-LTT, experimental wells were stimulated with 100 ml of optimal concentration of Con A, 64 ug/ml. For antigen-LTT, experimental wells received 100 ml of culture medium. The plates were incubated in a 5% CO_2-in air atmosphere for 72 hr for mitogen experiments and 120 hr for antigen experiments.

All cultures received a terminal 8-10hr pulse of 1C/well of tritiated thymidine (3H-TdR; Amersham, USA). Cultures were harvested on glass fiber filters using a Minimash 2000 cell harvester (Dynatech, England). To each filter paper disc, 3 ml of scintillation cocktail (Ready Protein, Beckman, USA) was added and the samples were then counted in an LS Counter 9800 (Beckman, USA). Results of triplicate cultures were expressed as mean counts per minute (CPM) ± standard error of the mean (SEM) or as a stimulation index (S.I.) which is the ratio of CPM of stimulated cultures to the CPM of unstimulated cultures.

Subpopulation of Peripheral Blood Lymphocytes

Monoclonal antibodies OKT against T cell subsets were purchased from Behringwerke, Germany. Lymphocyte suspension of 4×10^6 cells/ml was incubated with the appropriate monoclonal antibodies of the appropriate kind (OKT4 or OKT8) for 20 minutes at 4°C. The cells were then washed in RPMI 1640, followed by treatment with 100 ml of 1 : 50 diluted rabbit complement and incubated for one hour at 37°C. After three washings, the cells were resuspended in complete culture medium. Lymphocyte blastogenesis experiments were then performed as described above.

Serological techniques

IgG antibody titres were determined by the immunofluorescense antibody test and titres were expressed in two fold dilutions as previously described (9) using commercially available reagents (Behringwerke, Germany). IgM antibodies were determined by the specific and sensitive immunosorbent agglutination assay, ISAGA (Bio Merieux, France) which utilizes monoclonal antibodies as the solid phase.

Results

Lymphocyte proliferative response to Mitogen and Antigen

The individuals were divided into three groups on the basis of clinical and serological criteria. Individuals with negative serology for *T. gondii* antibody were treated as the control group (Group I), while individuals known to have positive serology for IgG were considered to have latent (chronic) infection (Group II). Group III consisted of acutely-infected patients.

The response of lymphocytes to the mitogen (Con A) and SA in the various groups is as shown in Table 1. Lymphocyte blastogenic response to the mitogen was present in all groups. The lymphocytes were capable of undergoing blast transformation at all times as indicated by their good response to the T-cell mitogen. PBL from

patients chronically-infected with *T. gondii* were found to be very responsive to *in-vitro* stimulation with *T. gondii*

antigen as shown by the high incorporation of tritiated thymidine. On the other hand, none of the control patients responded to *Toxoplasma* antigen. The patient who was accidentally infected with *T. gondii* presented with acute clinical symptoms of fever, lymphadenopathy, flu-like syndrome, abdominal discomfort, and a state of confusion. Prior to the infection, the patient was negative for toxoplasma serology and antigen-specific lymphocyte blastogenesis. After the infection her PBL were still capable of undergoing blast transformation to T-cell mitogen (Con A) at the time the lymphocytes were not responding to the specific *T. gondii* antigen. Thus unresponsiveness of antigen dependent (*T. gondii* specific) T-cell proliferation was observed in this patients. A time-course study of *Toxoplasma* - specific proliferative response of the accidentally-infected patient showed that her PBL failed to demonstrate lymphocyte proliferation to SA for as long as three months, which coincided with the period of her clinical symptoms. At four months after onset of the infection, her lymphocytes transformed to SA and the response continued to be positive thereafter. Therefore the positive cell-mediated immune response of this patient inversely correlated to the presence of her clinical symptoms. The time of onset of clinical

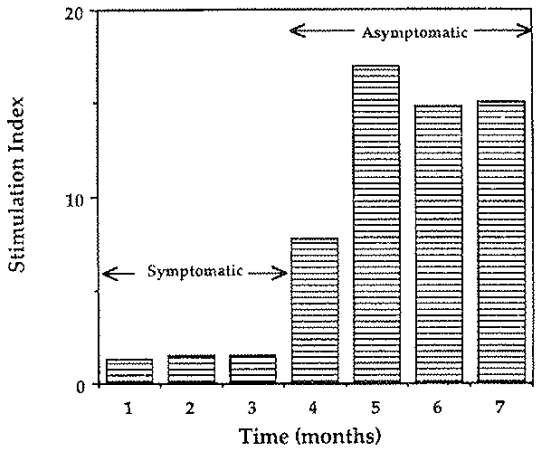

Figure 1. Longitudinal Studies of lymphocyte proliferative responsiveness to *Toxoplasma gondii* antigen of a patient with infection

symptoms of the other two acutely infected patients were not clearly defined.

Characterization of Toxoplasma induced suppressor and inducer T-cells

To characterise the cells responsible for suppression and responsiveness of proliferation of lymphocytes from

Table 1. Serology and lymphocyte proliferate response of P BL to *Toxoplasma gondii* antigen from healthy controls, chronically and acutely infected subjects.

Group	Subject	Toxoplasma antigen (Stimulation index)	Concanavalin A (Stimulation Index)	Antibody titres IgG	IgM
Group I	1	0.96	43.8	Neg	Neg
Control	2	1.30	35.2	Neg	Neg
	3	1.90	28.6	Neg	Neg
	4	1.60	38.1	Neg	Neg
	5	1.25	40.9	Neg	Neg
Group II	1	10.1	46.7	1:1024	Neg
latent infection	2	15.8	39.6	1:1024	Neg
	3	11.7	40.1	1:2048	Borderline
	4	14.9	33.7	1:512	Borderline
	5	17.5	42.9	1:256	Neg
	6	26.1	28.4	1:128	Neg
	7	9.9	30.5	1:128	Neg
	8	18.6	41.3	1:128	Neg
	9	39.1	38.8	1:128	Neg
	10	9.8	34.6	1:128	Neg
	11	12.3	30.2	1:128	Neg
	12	14.6	45.2	1:128	Neg
Group III	1	0.9	32.6	<1:64	12+
acute infection	2	1.4	45.5	<1:64	12+
	3	1.3	40.3	<1:128	12+

Isaga results one read as follow :
0 - 5 = Negative, 6 - 8 = Border line (BL), 9 - 12 = Positive

patients with Toxoplasmosis, the cells were treated with monoclonal antibodies and complement. The monoclonal antibodies used were OKT4 (IgG2b) which characterizes CD4 antigen and defines the helper T lymphocytes, and OKT8 (IgG2a) which characterises CD8 antigen and defines the suppressor T lymphocytes. The effect of treatment with monoclonal antibody and complement on the ability of PBL to proliferate to *Toxoplasma* antigen was determined by the incorporation of ^{3}[H] TdR. The results of the experiments are shown in Table II. Treatment of cells from an acutely infected patient, with anti-CD4 and complement or complement alone did not alter the *Toxoplasma* specific unresponsiveness. However when the cells were treated with anti-CD8 plus complement the response returned, indicating that the CD8 positive cells were responsible for the induction of suppression during the acute phase of infection. In a separate experiment, the effect of monoclonal antibody against CD4 and CD8 was assesed on cells from a chronically infected patient. When the cells were treated with anti-CD8 plus complement or complement alone, the antigen specific response was not affected, but treatment with anti - CD4 eliminated *Toxoplasma* specific proliferative response. Thus these experiments show that *Toxoplasma* specific non-responsiveness during acute phase of infection was mediated by CD8 cells, namely the suppressor T cells, and the responder cells in both acute and chronic phase of the infection are the helper T cells.

Discussion

Lymphocyte transformation in *T. gondii* infection was first demonstrated histologically in sensitised rabbits by culturing lymphocytes *in vitro* in the presence of *Toxoplasma* antigen (10). Tremonti *et al.* (11) studied lymphocyte blastogenesis in humans with serological evidence of infection with *T. gondii*. They showed that *Toxoplasma* antigen stimulated blastogenesis in both seropositive and seronegative individuals, therefore demonstrating that antigen derived from *Toxoplasma* acted as a mitogen. Our study shows that asymptomatic patients with serological evidence of *Toxoplasma gondii* infection were positive for lymphocyte transformation to *Toxoplasma* antigen. On the other hand, individuals who were seronegative for *Toxoplasma* antibodies showed no such response of lymphocytes to the mitogen, Con A, thus indicating that the ablity of their lymphocytes to respond to non-specific antigen was intact.

The patient who was accidentally infected with tachyzoites from ascitic fluid of *T. gondii* infected mice showed acute symptoms for about twelve weeks post - infection. During this period, lymphocyte transformation to *Toxoplasma* antigen was not demonstrable. However during this period of depressed response to the specific antigen, her lymphocytes responded normally to Con A. Therefore this demonstrated that her general T-cell immunity was intact and that the unresponsiveness was antigen specific. The two other patients who were acutely infected showed similar findings expect that the lymphocyte responses to *Toxoplasma* antigen were positive much earlier. Yano *et al.* (12), Krahenbuhl *et al.* (13) and (14) also described similar lymphocyte unresponsiveness to specific *Toxoplasma* antigen, but not to T-cell mitogens, in patients with acute symptomatic toxoplasmosis. Other investigators have also documented that in some immunologically normal adults with acute acquired toxoplasmosis, positive lymphocyte blastogenesis to *T. gondii* antigen developed only after a lapse of several months post-infection (4,6).

Table 2. Analysis of Toxoplasma specific proliferative response in PBL of patients with Toxoplasmosis using monoclonal antibodies specific to human T-cell subsets.

	Treatment of PBL	Medium	Antigen-Specific Proliferation Response of PBL[a] TOXO Extract (ΔCPM[b])	ConA (ΔCPM)
Acute	Normal		984	25,325
	Deplete MØ[c]	466±102	906	19,247
	C Alone		837	17,188
	OKT4 + C		972	5,421
	OKT8 + C		3,973	17,249
Chronic	Normal	391±96	5,032	ND
	C Alone		4,492	ND
	OKT4 + C[d]		546	ND
	OKT8 + C		4,113	ND

[a] peripheral blood lymphocytes
[b] counts per minute
[c] macrophage cells
[d] guinea pig complement

The mechanism of lymphocyte unresponsiveness to *Toxoplasma* antigen is unclear. An analysis of the T-cell subpopulations of the accidentally infected patient revealed that there was an absolute increase in the number of T 8 cells resulting in a depressed T4/T8 ratio (15). Luft *et al.* (14), Sklenar *et al.* (16) and De Waele *et al.* (17) also reported increase in absolute numbers of suppressor-cytotoxic T-cells in patients with symptomatic acute toxoplasmosis. On the other hand other investigators have reported that during acute infection of toxoplasmosis the T4/T8 ratio remains unchanged (18). Further analysis of her lymphocytes during the acute period of infection showed that the $CD8^+$ cells were responsible for the suppression and that the removal of $CD8^+$ cells reconstituted the blast transformation. Yano *et al.* (12) postulated that the mechanism of unresponsiveness could be operating through induction of suppression through suppressor

cells. In murine toxoplasmosis, immunosuppression has been postulated to be caused by T-cells (19); antigenic competition (20); activation of suppressor macrophages (21); and production of soluble factors (22, 23). These findings together with our finding further support the idea that transient immunosuppression during the acute phase of toxoplasma infection may be operating through interactions of multiple mechanisms.

This study concluded that in acute symptomatic phase of *Toxoplasma* infection, there was a delayed response to *Toxoplasma* antigen, whereas the response to mitogen was unaltered. Lymphocytes of patients who were not symptomatic but showed positive *Toxoplasma* serology responded to *Toxoplasma* antigen as well as to the mitogen. We also demonstrated that the suppression of response during acute symptomatic infection could be attributed to $CD8^+$ cytotoxic/suppressor cells; while the responder cells during the chronic phase of *Toxoplasma* infection were the $CD4^+$ cells.

Further studies on the unresponsiveness during the acute symptomatic phase with larger number of patients would cast more insights into the anergy of the subjects.

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