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In Vitro Primary Responses of Mouse T Lymphocytes to *Toxoplasma gondii* Antigens

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ABSTRACT

In general, it has been difficult to stimulate proliferation of non-immunized T lymphocytes in vitro using conventional non-recall antigens, particularly soluble proteins. This limitation has placed severe restrictions on the study of T-cell responses in vitro and the development of T-cell vaccine. A report has indicated that naïve T cells do respond to a non-recall antigen in a novel culture system. Accordingly, this study was designed to establish an in vitro system of responsiveness of naïve mouse T lymphocytes to *Toxoplasma gondii* antigens. The effects of two media, RPMI-1640 and alpha modification of Eagles medium (αMEM), on cellular proliferation were compared. Lymphocytes from *T. gondii*-immunized mice were used as a positive control cells and T-cell mitogen-concanavalin A was used as a positive control stimulant. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide based colorimetric assay was utilized to evaluate cellular response. The results showed that lymphocytes from unimmunized mice responded to *T. gondii* somatic antigen only in αMEM with the addition of menadione. Immune cells responded to *T. gondii* somatic antigen equally well in both culture media. In the presence of menadione, immune cells proliferated significantly better to *T. gondii* somatic antigen in αMEM than in RPMI-1640. Similar results were observed when *T. gondii* excretory/secretory antigen was used. Both immune and non-immune cells responded equally well to concanavalin A in both culture media. However, menadione had more dramatic additive effect on the responsiveness to concanavalin A in αMEM than in RPMI-1640. The deletion of $CD4^+$ T cells or adherent macrophages, but not $CD8^+$ cells, abolished the responsiveness of non-immune cells to *T. gondii* somatic antigen indicating the involvement of $CD4^+$ T cells and antigen-presenting cells in this system.

Key words: in vitro, primary response, T lymphocyte, *T. gondii*, antigen

Introduction

Antigen-specific in vitro T cell responses to recall antigens have been extensively investigated. By contrast, it has been difficult to stimulate proliferation of unsensitized T lymphocytes in vitro using a non-recall antigen. This limitation has placed severe restrictions on the study of T-cell responses in vitro and the development of T-cell vaccine as immunization of human volunteers or animals is not always possible. Nevertheless, a report (Plebanski and Burtles, 1994) has indicated that naïve $CD4^+$ T cells do respond to keyhole limpet haemocyanin, a non-recall antigen in alpha modification of Eagles medium (αMEM). In addition, Young *et al.* (1995) also demonstrated that human peripheral blood mononuclear cells could respond to another non-recall antigen, canine cytochrome c.

The infection of *Toxoplasma gondii*, an obligate intracellular protozoa, is widespread and is of economic and public health importance (Remington and Desmonts, 1983). The occurrence of deadly disseminated toxoplasmosis or toxoplasmic encephalomyelitis in acquired immune deficiency syndrome patients is particularly important (Luft and Remington, 1992; Suzuki and Remington, 1993; Richard *et al.*, 1995). Humans and animals become infected by eating food contaminated with sporulated oocysts, or undercooked meat containing cysts (reviewed in Frenkel, 1988; Dubey, 1994). Studies have shown that cell-mediated immunity is more important than humoral response in controlling toxoplasmosis and this protection is contributed synergistically by both $CD4^+$ and $CD8^+$ T cells (Suzuki and Remington 1988, 1990; Gazzinelli *et al.*, 1991; Herion and Saavedra, 1993). Generally, $CD4^+$ T cells are

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stimulated by exogenous antigens presented by class II major histocompatibility complex molecules on antigen-presenting cells (e.g. macrophages) and $CD8^+$ T cells are activated by endogenous antigens presented by class I major histocompatibility complex molecules on target cells (Kindt *et al.*, 2007).

Accordingly, this study was designed to establish an in vitro system of the responsiveness of naïve mouse T lymphocytes to *T. gondii* somatic and excretory/secretory antigens. Because soluble *T. gondii* antigens were given by exogenous way in this study, the involvement of $CD4^+$ T cells and adherent macrophages in this system (Canessa *et al.*, 1988) was also investigated by cell deletion experiments. The non-radioactive colorimetric assay based on the cleavage of the yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) to purple formazan crystals was used to evaluate cellular responses (Mosmann, 1983; Lin, 2006).

Materials and Methods

T. gondii somatic and excretory/secretory antigens

After proliferation in mouse peritoneal cavity, *T. gondii* RH tachyzoites were harvested by peritoneal lavage and washed with phosphate buffered-saline. They were further purified by 3 μm Nuclepore polycarbonate membranes (Costar Corporation, Cambridge, MA). Tachyzoites were resuspended as 10^8 /ml in culture medium and were left at 4^oC overnight allowing the expression of excretory/secretory antigen. Whole culture was then spun to obtain tachyzoites and soluble excretory/secretory antigen. The precipitated tachyzoites were subjected to 3 cycles of freezing-thawing and 10 times of sonication, 35 Watts/30 sec/each, using a sonicator (Heat systems Inc., Farmingdale, NY). The tachyzoite antigen preparation was centrifuged at the speed of 10,000 xg for 40 min at 4^oC and the resulting supernatant was collected as somatic antigen. Both excretory/secretory and somatic antigen solutions were sterilized using 0.22 μm syringe filters and frozen at -70^oC until used. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Lab., Richmond, CA).

Mouse immunization

Each ICR mouse (Laboratory Animal Center, National Taiwan University Medical School) was immunized by inoculating intra-peritoneally (0.1ml) and subcutaneously (0.1ml) with the mixture containing 1 x 10^8/ml dead *T. gondii* tachyzoites plus their excretory/secretory products. Two weeks later, mice received intraperitoneal boost with the same amount of material in incomplete Freund's adjuvant. One week after the second challenge, mice were sacrificed for lymphocyte preparations.

Cells

Lymphocyte preparation. Cervical, axillary, and mesenteric lymph nodes were excised aseptically from *T. gondii*-immunized ICR mice or controls. Single cell suspensions were made by filtering suspensions of the tissues that had been teased with forceps in culture medium through two layers of sterile gauze. Lymphocytes were pooled from three mice for each group. Live cells were counted by trypan blue dye exclusion.

Deletion of $CD4^+$ or $CD8^+$ T cells. Lymphocytes at a concentration of 10^7/ml were incubated with 1:20 diluted monoclonal rat antibody to mouse CD4 or CD8 marker (Serotec, Oxford, England) in the presence of guinea pig complement at 4^oC for 1 hr. Thereafter, the cells were washed and the dead cells were removed by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO). Viable intact cells were separated from lysed cell debris by centrifugation.

Removal of adherent macrophages. Lymph node cells at a concentration of 10^6/ml were incubated in Petri dish at 37^oC for 4 hr. Mostly adherent cells were macrophages. Non-adherent cells, which were mainly lymphocytes, were then harvested by centrifugation.

Culture conditions

Culture media. Two media, RPMI-1640 and αMEM, were compared. The medium was supplemented with gentamicin 100 μg/ml, 2-mercaptoethanol 5 x 10^{-5} M, L-glutamine 2 mM, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] 15 mM, sodium pyruvate 2 mM and 10% fetal calf serum. All reagents were purchased from Gibco BRL, Gaithersburg, MD.

Electron-coupling agent. Menadione (menadione sodium bisulphide; Sigma Chemical Co.) was dissolved in serum-free culture medium

and added to the cell cultures together with MTT in 10 μl amounts resulting in final concentrations of 20 μM (Garn *et al.*, 1994).

MTT assay

Cells, 2.5 X 10^5 in 75 μl of culture medium, were dispensed into wells of 96-well plates in a triplicate fashion. Lymphocytes from *T. gondii*-immunized mice were used as a positive control cells. Twenty five μl of 2.5 μg *T. gondii* somatic or excretory/secretory antigen, 0.5 μg T-cell mitogen-concanavalin A (ConA; Sigma Chemical Co.), or culture medium alone were then added. Con A was used as a positive control. After 6 days of incubation for *T. gondii* antigens and 3 days of incubation for ConA, 10 μl of the yellow MTT solution (5 mg/ml) were added and the plate was incubated at 37°C for additional 4hr. After this incubation period, purple formazan salt crystals are formed. One hundred μl of solubilization solution were placed into each well to solubilize purple formazan salt crystals. Plate was allowed to stand at 37°C overnight. The optical density of resulting colored solution is correlated well with cell proliferation or cell number (Molinari *et al.*, 2003; Dutta *et al.*, 2005; Noguchi *et al.*, 2005). Absorbance (optical density) of converted dye was measured using a microplate reader (Dynatech Lab., Chantilly, VA) at a wavelength of 570 nm with background subtraction at 690 nm. The experiments were repeated three times.

Data analysis

All comparisons were made by one-way analysis of variance. The difference was considered to be significant for $P<0.05$.

Results

In table 1, lymphocytes from naïve (non-immune cells) or *T. gondii*-immunized mice (immune cells) did not proliferate significantly in the absence of any stimulants (culture medium only) either in RPMI-1640 or αMEM. Both immune and non-immune cells responded equally well to ConA, however, no significant difference in the optical density values was observed when either of the culture media was used. Again, the difference of culture medium did not influence the responsiveness of immune cells to *T. gondii* somatic antigen (0.436 ± 0.034 v.s. 0.443 ± 0.047, $P=0.844$).

In table 2, the addition of electron-coupling agent-menadione increased the background optical density values. There was no significant difference for non-immune or immune cells in either of the culture media without stimulant even when menadione was added. However, in the presence of menadione and *T. gondii* somatic antigen, non-immune cells proliferated significantly better in αMEM than in RPMI-1640 (0.510 ± 0.011 v.s. 0.421 ± 0.054, $P=0.049$). Similar results were observed when immune cells were used (0.820 ± 0.027 v.s. 0.716 ± 0.029, $P=0.01$). In the presence of menadione, both immune and non-immune cells responded to T-cell mitogen ConA equally well. However, menadione significantly enhanced the

Table 1. Responsiveness of mouse lymphocytes to stimulants in RPMI-1640 or α-MEM measured by MTT assay. Lymphocytes were obtained from naïve mice (non-immune cells) or *T. gondii*-immunized mice (immune cells). Stimulants used included *T. gondii* somatic antigen and concanavalin A. After incubation with stimulants, MTT was added. Solubilization solution was then added to each well to solubilize purple formazan salt crystals. Absorbance (optical density) of converted dye was measured by a microplate reader at the wavelength of 570 nm with background subtraction at 690 nm. Lymphocytes were pooled from three mice for each group and the data were evaluated in a triplicate fashion. The experiments were repeated three times and the data were expressed as mean ± standard deviation. Different superscript letters (a, b, c) indicate significant difference ($P < 0.05$).

	MTT assay without the addition of menadione			
	RPMI-1640		α-MEM	
Stimulant	Non-immune cells	Immune cells	Non-immune cells	Immune cells
None	0.249 ± 0.014^a	0.262 ± 0.008^a	0.256 ± 0.008^a	0.265 ± 0.015^a
T. gondii antigen	0.242 ± 0.022^a	0.436 ± 0.034^b	0.252 ± 0.020^a	0.443 ± 0.047^b
Concanavalin A	1.211 ± 0.095^c	1.263 ± 0.064^c	1.288 ± 0.042^c	1.285 ± 0.103^c

Table 2. Responsiveness of mouse lymphocytes to stimulants in RPMI-1640 or α-MEM measured by MTT assay with the addition of manadione. Lymphocytes were obtained from naïve mice (non-immune cells) or *T. gondii*-immunized mice (immune cells). Stimulants used included *T. gondii* somatic antigen and concanavalin A. After incubation with stimulants, MTT and menadione were added. Solubilization solution was then added to each well to solubilize purple formazan salt crystals. Absorbance (optical density) of converted dye was measured by a microplate reader at the wavelength of 570 nm with background subtraction at 690 nm. Lymphocytes were pooled from three mice for each group. The data were evaluated in a triplicate fashion and expressed as mean ± standard deviation. Different superscript letters (a, b, c, d, e, f) indicate significant difference ($P < 0.05$).

	MTT assay with the addition of menadione			
	RPMI-1640		α-MEM	
Stimulant	Non-immune cells	Immune cells	Non-immune cells	Immune cells
None	0.404 ± 0.017^{a}	0.418 ± 0.031^{a}	0.408 ± 0.027^{a}	0.427 ± 0.035^{a}
T. gondii antigen	0.421 ± 0.054^{a}	0.716 ± 0.029^{b}	0.510 ± 0.011^{c}	0.820 ± 0.027^{d}
Concanavalin A	2.151 ± 0.167^{e}	2.288 ± 0.180^{e}	2.616 ± 0.073^{f}	2.622 ± 0.069^{f}

cellular responsiveness to ConA in αMEM than in RPMI-1640. Similar results were observed in both culture media with or without the addition of menadione when *T. gondii* excretory/secretory antigen was used (data not shown). The responsiveness of non-immune cells to *T. gondii* somatic antigen in αMEM with the presence of menadione (0.510 ± 0.011) was abolished when $CD4^+$ T cells (0.394 ± 0.04, P=0.004) or adherent macrophages (0.401 ± 0.03, P=0.004), but not $CD8^+$ cells (0.483 ± 0.021, P=0.120) were removed.

Discussion

Since its introduction by Mosmann in 1983, the MTT bioassay has been used in a variety of assays to determine the viability and/or the metabolic state of cells. MTT assay has excellent sensitivity among non-radioactive assays for measurement of cellular response (Lin, 2006). Nonetheless, the MTT assay has some drawbacks. Cells with low metabolic activity (e.g. lymphocytes) must be used in high numbers to achieve a measurable MTT reduction. Electron-coupling agent menadione (also known as vitamin K_3) has been used to increase reduction of MTT leading to higher sensitivity in the MTT assay (Garn *et al.*, 1994).

In general, naïve lymphocytes do not respond to non-recall antigens (Lin *et al.*, 1992). In previous (Plebanski and Burtles, 1994) and this study, αMEM offered better T-cell proliferation environment than RPMI-1640. In the culture containing αMEM and menadione, naïve lymphocytes responded to soluble *T. gondii* antigen as indicated by MTT assay, though the degree of responsiveness was much less than that of sensitized immune cells. Similar to previous report (Lin, 1995), no difference of stimulation ability was found between somatic and excretory/secretory antigen in this study. *T. gondii* somatic and excretory/secretory antigens do share some similar protein compositions (Darcy *et al.*, 1988; Duquesne *et al.*, 1990). Menadione also significantly enhanced the cellular responsiveness to T-cell mitogen ConA in αMEM than in RPMI-1640. ConA, used as a positive control, is a lectin which stimulates T-cell proliferation non-specifically.

Since soluble *T. gondii* antigen was given by exogenous way, it was expected that $CD4^+$ T cells would be stimulated to proliferate in the presence of antigen presenting cells-macrophages (Canessa *et al.*, 1988; Kindt *et al.*, 2007). By performance of cell deletion experiment, the responsiveness of mouse non-immune lymphocytes to *T. gondii* somatic antigen in αMEM with the addition of menadione was abolished when $CD4^+$ T cells or adherent macrophages, but not $CD8^+$ cells, were removed. The results indicated that $CD4^+$ cells in lymphocyte populations were activated by *T. gondii* antigen in the presence of macrophages in this system. Similarly, primary $CD8^+$ cytotoxic T lymphocytes also can be activated if antigen is given by endogenous way (Plebanski *et al.*, 1995). Since in toxoplasmosis, both $CD4^+$ and $CD8^+$ cells participate synergistically in protective immunity (Suzuki and Remington 1988, 1990; Gazzinelli *et al.*, 1991; Herion and Saavedra, 1993), it is necessary to further establish a system involving activation of $CD8^+$ cells by soluble *T. gondii*

antigens.

In sum, naïve lymphocytes, which are mainly $CD4^+$ T cells, respond to soluble *T. gondii* antigen in vitro provided that culture containing αMEM with electron-coupling agent menadione and macrophages. One exciting speculation would be that ex-vivo activation of autologous naïve pathogen-specific T cells may generate primed effector cells with therapeutic potential when re-introduced into patients.

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References

Canessa A, Pistoia V, Roncella S, Merli A, Melioli G, Terragna A and Ferrarini M. 1988. An in vitro model for *Toxoplasma* infection in man: interaction between $CD4^+$ monoclonal T cells and macrophages results in killing of trophozoites. J. Immunol. 140:3580-3588.

Darcy F, Deslee D, Santoro F, Charif H, Auriault C, Decoster A, Duquesne V and Capron A. 1988. Induction of a protective antibody-dependent response against toxoplasmosis by in vitro excreted/secreted antigens from tachyzoites of *Toxoplasma gondii*. Parasit. Immunol. 10:553-567.

Dubey JP. 1994. Toxoplasmosis. J. Am. Vet. Med. Assoc. 205:1593-1598.

Duquesne V, Auriault C, Darcy F, Decavel JP and Capron A. 1990. Protection of nude rats against *Toxoplasma* infection by excreted-secreted antigen-specific helper T cells. Infect. Immun. 58:2120-2126.

Dutta A, Bandyopadhyay S, Mandal C, Chatterjee M. 2005. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. Parasitol Int. 54:119-122.

Frenkel KK. 1988. Pathophysiology of toxoplasmosis. Parasitol. Today 4:273-278.

Garn H, Krause H, Enzmann V and Drobler K. 1994. An improved MTT assay using the electron-coupling agent menadione. J. Immunol. Meth. 168:253-256.

Gazzinelli RT, Hakim FT, Hieny S, Shearer GM and Sher A. 1991. Synergistic role of CD4 and CD8 T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. J. Immunol. 146:286-292.

Herion P and Saavedra R. 1993. Human T-cell clones as tools for the characterization of the cell-mediated immune response to *Toxoplasma gondii*. Res. Immunol. 144:48-51.

Kindt TJ, Goldsby RA and Osborne BA. 2007. Kuby Immunology. W. H. Freeman and Company, New York.

Lin DS. 1995. Detection of the cellular response to *Toxoplasma gondii* by antigen-coated beads in vitro. Acta Zool. Taiwanica 6:13-24.

Lin DS. 2006. Comparison of three non-radioactive assays for measurement of cell-mediated immunity. BioFormosa 41: 59-66.

Lin DS, Bowman DD and Jacobson RH. 1992. Immunological changes in cats with concurrent *Toxoplasma gondii* and feline immunodeficiency virus infections. J. Clin. Microbiol. 30:17-24.

Luft BJ and Remington JS. 1992. Toxoplasmic encephalitis in AIDS. Clin. Infect. Dis. 15:211-222.

Molinari BL, Tasat DR, Palmieri MA, O'Connor SE and Cabrini RL. 2003. Cell-based quantitative evaluation of the MTT assay. Anal. Quant. Cytol. Histol. 25:254-262.

Mossman T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65:55-63.

Noguchi K, Iwahashi M, Tani M, Nakamura M, Nakamori M, Nakatani Y, Ueda K, Ishida K, Naka T, Ojima T, Hotta T, Mizobata S and Yamaue H. 2005. Evaluation of chemosensitivity testing with highly purified tumor cells in 435 patients with gastric carcinoma using an MTT assay. Anticancer Res. 25:931-937.

Plebanski M, Allsopp CE, Aidoo M, Reyburn H and Hill AV. 1995. Induction of peptide-specific primary cytotoxic T lymphocyte responses from human peripheral blood. Eur. J. Immunol. 25:1783-1787.

Plebanski M and Burtles SS. 1994. In vitro primary responses of human T cells to soluble protein antigens. J. Immunol. Meth. 170:15-25.

Remington JS and Desmonts G. 1983. Toxoplasmosis. *In* Remington JS and Klein JO (eds), Infectious Diseases of the Fetus and

Newborn, W.B. Saunders, Philadelphia, pp. 143-263.

Richards FD, Kovacs JA and Luft BJ. 1995. Preventing toxoplasmic encephalitis in persons infected with human immunodeficiency virus. Clin. Infect. Dis. 21(Suppl.):S49-56.

Suzuki Y and Remington JS. 1988. Dual regulation of resistance against *Toxoplasma gondii* infection by Lyt-2^+ and Lyt-1^+, L3T4^+ T cells in mice. J. Immunol. 140:3943-3946.

Suzuki Y and Remington JS. 1990. The effect of anti-IFN-γ antibody on the protective effect of Lyt-2^+ immune T cells against toxoplasmosis in mice. J. Immunol. 144:1954-1956.

Suzuki Y and Remington JS. 1993. Toxoplasmic encephalitis in AIDS patients and experimental models for study of the disease and its treatment. Res. Immunol. 44:66-67.

Young JL, Daser A and Beverley PCL. 1995. In vitro proliferative responses of human peripheral blood mononuclear cells to non-recall antigens. J. Immunol. Meth. 182:177-184.

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小鼠T淋巴細胞在體外對弓蟲抗原之初級反應

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摘　要

一般很難在體外以傳統非回憶性抗原，特別是可溶性蛋白質，刺激未免疫過 T 細胞增生。此限制已嚴重影響到體外 T 細胞反應之研究及 T 細胞疫苗之發展。有報告指出在一特殊的培養系統，未致敏 T 細胞可和非回憶性抗原反應。據此，本研究乃設計以建立體外小鼠未致敏淋巴球對弓蟲抗原之反應系統。使用兩種培養液 [RPMI-1640 及 alpha modification of Eagles medium (MEM)]，並比較其對細胞增生之影響。取自弓蟲免疫過小鼠之淋巴細胞作為陽性對照細胞，而 T 細胞 mitogen-concanavalin A 當作陽性對照刺激物質。使用 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide 呈色試驗作為細胞反應之偵測。結果顯示，未免疫過小鼠之淋巴細胞只在添加 menadione 之 MEM 對弓蟲體抗原有反應。免疫過細胞在兩種培養液對弓蟲體抗原反應一樣好。在添加 menadione 之後，免疫過細胞在 MEM 對弓蟲體抗原反應比在 RPMI-1640 好。當使用弓蟲排泄/分泌抗原時，亦得到相似的結果。免疫和未免疫過細胞在兩種培養液中對 concanavalin A 反應一樣好。然而 menadione 在 MEM 對 concanavalin A 反應的增強效果比在 RPMI-1640 好。去除 $CD4^+$ T 細胞或附著性巨噬細胞，但非 $CD8^+$ 細胞，導致未免疫過細胞對弓蟲體抗原反應喪失，表示本系統有 $CD4^+$ T 細胞及抗原呈獻細胞參與。

關鍵詞：體外、初級反應、T 淋巴細胞、弓蟲、抗原

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