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Activation of Macrophages by *Tetrahymena pyriformis*: Killing of *Toxoplasma gondii in Vitro*

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Introduction

Our previous study (Makioka *et al.*, 1982) has demonstrated that *Tetrahymena pyriformis*, a free-living ciliate, could produce when inoculated into mice an increased nonspecific resistance against a subsequent *Toxoplasma gondii* challenge infection. When compared with BCG, a potent immunostimulant, there were essentially no marked differences in potency against *Toxoplasma* between both antigens.

It was reported that the occurrence of activated macrophages with enhanced microbicidal capacity represents a major mechanism of resistance to *Toxoplasma* in other animal models using some immunostimulants such as Freund's complete adjuvant (Remington *et al.*, 1972) and *Propionibacterium acnes* (old term: *Corynebacterium parvum*) (Swartzberg *et al.*, 1975; Krahenbuhl *et al.*, 1980). If pretreatment of a host with *Tetrahymena* could be shown to activate macrophages and enhance their microbicidal capacity, this potent effector mechanism could underlie the results observed in the *in vivo* study (Makioka *et al.*, 1982).

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The present study was carried out to determine whether macrophages from mice immunized with *Tetrahymena* could elicit toxoplasmacidal activity when tested *in vitro*.

Materials and Methods

Mice: Six- to 8-week-old female outbred ddY mice were used in most experiments. BALB/c derived nu/nu and nu/+ mice, 6- to 8-week-old female, were also used in some experiments. All mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan.

BCG vaccine: Lyophilized viable BCG vaccine of Japanese substrain was obtained from the Japan BCG Laboratory, Tokyo. It was suspended in sterile saline and adjusted to an appropriate concentration before use. Heat-killed BCG was prepared by heating viable organisms for 1 hr at 70 C.

Tetrahymena: The W-strain of *Tetrahymena pyriformis* was obtained from Dr. Y. Watanabe, Institute of Biological Sciences, Tsukuba University, and cultivated as described previously (Makioka *et al.*, 1982). The organisms were harvested by centrifugation at 850×g for 5 min and washed once with inorganic medium. For lysis, the organisms in the inorganic medium were frozen-thawed twice. This

entire preparation was referred to as lysed *Tetrahymena* antigen.

Immunization: Mice were injected intraperitoneally (i.p.) with live *Tetrahymena* or its lysate. Live or heat-killed BCG were injected similarly into mice as positive control. Dose and interval for immunizations of *Tetrahymena* and BCG are described in particular experiments.

In vitro challenge with Toxoplasma: Peritoneal cells were harvested from 4–5 mice each of normal and immunized groups by washing the peritoneal cavity with 5 ml of RPMI-1640 medium (RPMI) (Grand Island Biological Co., Grand Island, N.Y.) and pooled. The spleens from normal and immunized mice were each squeezed in RPMI with a broad forceps. The spleen cells were obtained by passing the tissue homogenate through two layers of gauze. The peritoneal and spleen cells thus prepared were washed twice with RPMI and suspended in RPMI containing 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 20% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) (RPMI-20% FCS). The 4×10^6 peritoneal cells or 5×10^7 spleen cells were each resuspended in 1.5 ml of RPMI-20% FCS. These cell suspensions were seeded into tissue culture tubes (Flat Co., Chiba, Japan) containing coverslips (12×32 mm) and incubated for 2 hr at 37 C in an atmosphere containing 5% CO_2. Thereafter, the tubes were rinsed three times with RPMI to remove non-adherent cells, and 2×10^7 RH strain of *T. gondii*, obtained from the peritoneal exudate of 2 day-infected mice, were placed on the monolayers in a volume of 1.5 ml of RPMI-20% FCS. After 1 hr of incubation, the tubes were rinsed with RPMI thoroughly to remove extracellular organisms and then reincubated with fresh RPMI-20% FCS. One and 20 hr after reincubation, the tubes were washed with phosphate-buffered saline at pH 7.2 and the coverslips were withdrawn from the tubes, and were stained with Giemsa solution after fixation in absolute methanol. The percentage of the adherent cells (tentatively referred to as macrophages in this paper) infected and total number of organisms per 100 macrophages were determined.

Results

Effect of immunization with various doses of Tetrahymena *on activation of peritoneal macrophages*

To establish the minimum effective dose of *Tetrahymena* for activation of peritoneal macrophages, mice were immunized with different doses of the lysed *Tetrahymena* antigen equivalent to 1×10^5, 1×10^6 and 1×10^7 of original organisms. One week after the immunization, peritoneal cells were harvested and the adherent macrophages were challenged with *Toxoplasma* trophozoites. The mean numbers of the peritoneal cells per mouse were as follows: 6.1×10^6 cells in the non-immunized control, 8.3×10^6 cells in 1×10^5 *Tetrahymena*-immunized mice, 22.2×10^6 cells in 1×10^6 *Tetrahymena*-mice, and 18.0×10^6 cells in 1×10^7 *Tetrahymena*-mice. Those macrophages from mice immunized with *Tetrahymena* at doses more than 1×10^6 were enlarged and rich in cytoplasm. As shown in Table 1, inoculation of mice with 1×10^5 lysed antigen caused little activation of the macrophages. Significant activation was elicited with doses of 1×10^6 and 1×10^7, the latter being more potent. Doses of more than 1×10^8 were not tested because of high viscosity of the inoculum prepared. The size of inoculum for immunization was therefore fixed at 1×10^7 in the following experiments.

*Persistence of anti-*Toxoplasma *activity of peritoneal macrophages from mice immunized with* Tetrahymena

Table 1 Toxoplasmacidal effect of peritoneal macrophages from mice immunized with various doses of lysed *Tetrahymena*

Th dose*	% MØ infected (Tp/100 MØ)†	
	1hr	20hr
	%	%
None	56 (144)	53 (430)
1×10^5	61 (158)	49 (286)
1×10^6	70 (237)	33 (149)
1×10^7	67 (204)	4 (5)

* Mice were injected with 10^5, 10^6 or 10^7 lysed *Tetrahymena* 1 week before harvesting macrophages.
† Number of toxoplasmas found in 100 macrophages including both infected and non-infected cells.

Table 2 Persistence of anti-*Toxoplasma* activity of peritoneal macrophages from mice immunized with lysed *Tetrahymena*

Weeks after immunization*	% MØ infected (Tp/100 MØ)	
	1hr	20hr
	%	%
1 week	57 (118)	3 (6)
3 weeks	55 (102)	3 (7)
4 weeks	48 (68)	41 (256)
control	43 (71)	49 (291)

* Macrophages were harvested 1, 3 or 4 weeks after immunization with 10^7 lysed Th.

Peritoneal cells were harvested from mice 1, 3 and 4 weeks after immunization with lysed *Tetrahymena* antigen and the adherent macrophages were challenged with *Toxoplasma*. The results are shown in Table 2. For at least 3 weeks after immunization, macrophages exhibited strong anti-*Toxoplasma* activity but those harvested at 4 weeks no longer retained the activity.

*Comparison of anti-*Toxoplasma *activities of peritoneal macrophages from mice immunized with* Tetrahymena *and BCG*

The degree of anti-*Toxoplasma* activity of *Tetrahymena*-activated peritoneal macrophages was compared with that of BCG-activated ones. Peritoneal cells were harvested from mice immunized with 1×10^7 *Tetrahymena* antigen or 1×10^7 BCG and then the adherent macrophages were challenged with *Toxoplasma*. For the immunization, live and lysed tetrahymenas and also live and heat-killed BCG were adopted, and injected into mice once or twice at intervals of 14 days. Macrophages from mice non-treated or inoculated with 0.5 mg of glycogen were served as controls. Peritoneal cells were harvested 7 or 4 days after a single or two injections with *Tetrahymena* or BCG. For mice that received glycogen, the harvest was done 1 day after inoculation. The results are shown in Table 3. At 1 hr of incubation, no significant differences were shown between macrophages from non-immunized and immunized groups in regard to both per-

Table 3 Comparison of anti-*Toxoplasma* activities of peritoneal macrophages from mice immunized with *Tetrahymena* and BCG

Immunization*	% MØ infected (Tp/100 MØ)		
	1hr	20hr	40hr
	%	%	%
Non-immunized	51 (89)	50 (441)	—†
Non-immunized, glycogen-induced‡	54 (116)	40 (342)	—
Th (live), once	52 (77)	6 (11)	10 (25)
Th (lysed), once	52 (81)	5 (8)	10 (21)
BCG (live), once	51 (92)	5 (10)	11 (34)
BCG (heat-killed), once	53 (101)	7 (12)	9 (23)
Th (live), twice§	52 (126)	6 (11)	9 (25)
Th (lysed), twice	53 (104)	6 (13)	9 (39)
BCG (live), twice	53 (119)	7 (10)	10 (25)
BCG (heat-killed), twice	54 (104)	8 (12)	11 (16)

* Immunizing dose was 1×10^7 organisms for both Th and BCG. Macrophages were harvested 7 or 4 days after immunization.
† Observation could not be made because of rupture of macrophages.
‡ 0.5 ml of 0.1% glycogen was injected i.p. into mice 1 day before harvesting macrophages.
§ Interval between two immunizations was 14 days.

Table 4 Comparison of anti-*Toxoplasma* activities between peritoneal- and splenic macrophages from mice immunized with lysed *Tetrahymena*

Expt.	Immunization*	% Peritoneal MØ infected (Tp/100 MØ)		% Splenic MØ infected (Tp/100 MØ)	
		1hr	20hr	1hr	20hr
		%	%	%	%
1	None	58 (132)	56 (500)	46 (75)	47 (286)
	Th (lysed)	64 (146)	6 (19)	44 (79)	46 (232)
2	None	57 (141)	56 (388)	45 (65)	47 (306)
	Th (lysed)	66 (139)	5 (16)	50 (76)	45 (387)

* Mice were injected with 10^7 lysed Th 1 week before harvesting macrophages.

centage of the infected cells and number of *Toxoplasma* per 100 macrophages. After 20 hr, however, there was observed a marked difference between non-immunized and immunized groups; Macrophages from *Tetrahymena*- and BCG-immunized mice revealed sharp reduction of parasitization as shown by the percentage of infected cells and number of toxoplasmas in the cells. In this respect, macrophages from mice immunized with *Tetrahymena* antigens exhibited almost the same ability to kill *Toxoplasma* as those from BCG-immunized mice. Also there was no significant difference in capacity between the groups of macrophages from mice with a single injection and with two injections of *Tetrahymena* or BCG. Although, by 40 hr, almost all macrophages from normal- and glycogen-injected mice were ruptured by multiplication of *Toxoplasma*, macrophages from immunized groups of mice remained intact and still inhibited proliferation of *Toxoplasma*.

*Comparison of anti-*Toxoplasma *activities between peritoneal- and splenic macrophages from mice immunized with* Tetrahymena

Anti-*Toxoplasma* activities of peritoneal- and splenic macrophages from mice inoculated 1 week earlier with the lysed *Tetrahymena* antigen were compared. At 1 hr of incubation, both peritoneal- and splenic macrophages from immunized mice revealed almost similar percentages of infected cells as compared with those from control mice. At 20 hr, splenic macrophages still held the same level of infection, whereas peritoneal macrophages exhibited marked reduction of the infection (Table 4).

*Anti-*Toxoplasma *activity of peritoneal macrophages from nude mice immunized with* Tetrahymena

To determine whether macrophage activation by *Tetrahymena* is dependent on T lymphocytes, nu/nu and nu/+ mice were inoculated each with the lysed *Tetrahymena* antigen. One week later peritoneal cells were harvested from 5 mice of each experimental group and pooled. The adherent macrophages were challenged with *Toxoplasma*. The results showed that macrophages from nude mice immunized with lysed antigen exhibited almost the same capacity to kill *Toxoplasma* as those from nu/+ mice (Table 5).

Discussion

The results obtained showed that peritoneal macrophages from mice immunized with *Tetrahymena* were activated to kill *Toxoplasma in vitro*. Several characteristics of macrophage activation by lysed *Tetrahymena* antigen were clarified. The activation was elicited when mice were inoculated with 1×10^6 or more of *Tetra-*

Table 5 Anti-*Toxoplasma* activity of peritoneal macrophages from nude mice immunized with lysed *Tetrahymena*

Mice	Immunization*	% MØ infected (Tp/100 MØ)	
		1hr	20hr
		%	%
Athymic nu/nu	None	61 (163)	56 (634)
	Th (lysed)	63 (172)	8 (32)
Littermate nu/+	None	63 (136)	65 (757)
	Th (lysed)	62 (133)	7 (22)

* 10^7 lysed Th was injected into each of 5 mice 1 week before harvesting macrophages. Batches of peritoneal macrophages were pooled and subjected to the study.

hymena and observed for at least 3 weeks after immunization. These results seem to correlate the conditions for the elicitation of the protective effect in our *in vivo* study in which the minimum effective dose was 1×10^6 organisms for *Tetrahymena* antigens and that the protective effect persisted for 2–3 weeks with decrease afterward. I.p. inoculation of *Tetrahymena* activated peritoneal macrophages to kill *Toxoplasma* but failed to do so for splenic ones. Similar results have been reported by Ghaffar *et al.* (1974), in which the peritoneal exudate cells from *P. acnes*-treated mice were extremely effective in inhibiting tumor growth whereas the spleen and peripheral blood cells were effective only moderately. In this respect, it is possible that macrophage activation may largely depend on the route of administration of immunostimulant as demonstrated by Krahenbuhl *et al.* (1976) using *P. acnes*. This point should be examined upon establishment of appropriate experimental system using much smaller amount of active *Tetrahymena* antigen. Another possibility may be due to difference in proportion of different macrophage subpopulations between the peritoneal exudate and spleen. It was demonstrated in mice that approximately 85% of peritoneal macrophages do not express Ia antigens, while more than 50% of splenic macrophages are Ia positive (Cowing *et al.*, 1978). It remains to be clarified how the proportion of these macrophage subpopulations in different tissues is affected by injection of *Tetrahymena*.

Tetrahymena antigens behaved differently from inert substance like glycogen for macrophage activation. There was demonstrated no difference between *Tetrahymena* and BCG as immunostimulant in regard to *Toxoplasma*-killing capacity of macrophages. It was also clear from the results that two injections with *Tetrahymena* as well as BCG did not enhance activation of macrophages as compared with a single immunization. This may also correlate to the results obtained from our *in vivo* study for the protective effect against *Toxoplasma* as assayed by mortality of mice (Makioka *et al.*, 1982). While the results presented here indicated that macrophages from mice inoculated with live or heat-killed BCG were effective in killing of *Toxoplasma*, Krahenbuhl *et al.* (1981) demonstrated that treatment of mice with the synthetic adjuvant muramyl dipeptide (MDP) afforded substantial protection to *Toxoplasma* infection but failed to enhance the microbicidal capacity of peritoneal macrophages for *Toxoplasma*.

Of interest are the results obtained from our experiment on macrophages from nude mice immunized with *Tetrahymena*.

Macrophages derived from immunized nu/nu mice were found to be activated to kill *Toxoplasma* to the same level as those from nu/+ mice immunized similarly. This suggests that those activated macrophages were generated independently of T cells, although it would not necessarily be ruled out that immature T precursor cells might have participated. Numerous reports have been presented with respect to T cell dependency of macrophage activation by immunostimulants. Mackaness (1969) demonstrated that normal macrophages became activated to kill *Listeria* by a process which depends on the specific interaction between the immune lymphoid cells from BCG-infected mice and the organism or its antigenic products. Similar results have been reported using BCG (Evans and Alexander, 1972; Ruco and Meltzer, 1977) and *P. acnes* (Christie and Bomford, 1975; Bomford and Christie, 1975) in murine tumor models. On the other hand, Kaplan *et al.* (1974) have demonstrated that activated macrophages with cytotoxic activity for tumor cells could be recovered from T cell-deprived mice inoculated with pyran copolymer. Also, Madraso and Cheers (1978) have reported that polyadenylic acid-polyuridylic acid (poly A:U) can directly activate macrophages *in vitro* as assayed by macrophage spreading. Furthermore, it has been reported that *P. acnes* can activate macrophages to have the anti-tumor activity through T cell-independent mechanism (Christie and Bomford, 1975; Bomford and Christie, 1975; Ghaffar *et al.*, 1975; Woodruff and Warner, 1977). Thus it appears that the process for macrophage activation differs depending on the experimental conditions even if the same immunostimulant is used. Therefore, it would not necessarily be excluded a possibility of participation of T cell-mediated system for macrophage activation by *Tetrahymena* when examined in other experimental system.

With respect to *Toxoplasma* infection in nude mice, it has been demonstrated that nude mice were not more susceptible to *Toxoplasma* than normal control mice and that treatment of mice with sulfadiazine protected both mouse groups against acute fatal toxoplasmosis but after termination of the treatment the nude mice gradually died, whereas the normal hairy mice survived (Emmerling *et al.*, 1976; Lindberg and Frenkel, 1977). Although macrophage activation by *Tetrahymena* injection was elicited to almost the same degree in both nu/nu and nu/+ mice, the nu/nu mice failed to exhibit reduced mortality while the nu/+ mice could elicit resistance to *Toxoplasma* in the *in vivo* test (Makioka *et al.*, 1982). These results suggest that only the occurrence of activated macrophages is not sufficient for nude mice to outcome *Toxoplasma* infection

Studies are now in progress to make clear the mechanism of killing of *Toxoplasma* in the activated macrophages with or without T cell participation.

Summary

Experiments were carried out to determine whether immunization of mice with *Tetrahymena pyriformis*, a free-living ciliate, activates macrophages to be able to kill *Toxoplasma gondii in vitro*. Macrophage activation was elicited when mice were injected 1×10^6 or more of *Tetrahymena* and persisted within 3 weeks after immunization. Intraperitoneal immunization with *Tetrahymena* could activate peritoneal macrophages but could not splenic ones. Peritoneal macrophages from mice immunized with live *Tetrahymena* or its lysed antigen exhibited almost the same capacity to kill *Toxoplasma* as those from mice that received BCG, and no difference in the degree of macrophage activation

between these antigens was demonstrated. Peritoneal macrophages from nude (nu/nu) mice immunized with *Tetrahymena* were activated to kill *Toxoplasma* as effectively as were those from control (nu/+) mice immunized similarly.

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Tetrahymena pyriformis によるマクロファージの活性化: *in vitro* における *Toxoplasma gondii* の殺滅

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自由生活性繊毛虫 *Tetrahymena pyriformis* (Th) をマウスに接種することにより，マクロファージ (MØ) が著しく活性化され，これらの活性化 MØ は *in vitro* の系において，*Toxoplasma* (Tp) を殺滅する能力があることが判明した．Th による MØ の活性化には 1×10^6 個以上の Th を接種（腹腔内）することが必要であり，免疫後 3 週間はその活性が維持された．腹腔内接種により，腹腔 MØ は活性化されたが，脾臓 MØ は活性化されなかった．また，Th 接種による活性化 MØ の Tp 殺滅能力は，BCG 活性化 MØ のそれとほぼ同等であることが示された．さらに，Th 接種をうけたヌード (nu/nu) マウス由来の腹腔 MØ は，同様に免疫された対照 (nu/+) マウス由来 MØ と同等の殺 Tp 能を有することが判明した．