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Experimental infection of murine splenic lymphocytes and granulocytes with *Toxoplasma gondii* RH tachyzoites

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Abstract: *Toxoplasma gondii*, an intracellular protozoan infecting many kinds of eukaryotic cells, has been used to experimentally infect macrophages, epithelial cells, fibroblasts, and various cancer cells, but rarely T and B lymphocytes or granulocytes. The present study was performed to determine the susceptibility of murine (BALB/c or CBA) splenic T and B lymphocytes, and granulocytes to infection with *T. gondii* RH tachyzoites. The ultrastructure of the infected host cells was observed by TEM, and the degree of intracellular parasite proliferation was quantified using ^{3}H-uracil uptake assay. At 24 hrs post-culture, the host cell cytoplasm was found to contain 1 or 2, or a maximum of 7-8 tachyzoites. Infected T lymphocytes demonstrated a peripherally displaced nucleus, a parasitophorous vacuole enveloping the parasite, and an increased number of mitochondria. In B lymphocytes infected with tachyzoites, RER was not well developed compared to uninfected B lymphocytes. Uninfected granulocytes contained many electron-dense granules, but *T. gondii*-infected granulocytes demonstrated a decreased number of granules. Based on the ^{3}H-uracil uptake assay, the susceptibility of T and B lymphocytes, and granulocytes, to infection with *T. gondii* tachyzoites was fairly high irrespective of cell type and strain of mouse. This strongly suggests deterioration in the functioning of infected host immune cells.

Key words: mouse, *Toxoplasma gondii*, T lymphocytes, B lymphocytes, granulocytes, neutrophils, ^{3}H-uracil uptake assay, TEM ultrastructure

INTRODUCTION

Toxoplasma gondii is an intracellular protozoan which can cause significant morbidity and mortality in both man and animals. In immunocompromised hosts the infection is usually more severe (Luft, 1986). *T. gondii* can either invade or be taken into a variety of nucleated host cells, including RE cells (e.g. phagocytes), and epithelial, kidney, and cancer cells (Doran, 1982; Nakao and Konishi, 1991a; Park *et al.*, 1993).

Invasion of *T. gondii* into host immune cells could lead to the destruction and/or functional deterioration of infected cells, and is a suggested reason for immunosuppression in infected hosts. There are, however, few reports on the susceptibility of T and B lymphocytes, or granulocytes, to infection with *T. gondii*

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tachyzoites, and ultrastructural changes in infected cells have not been well studied. Nakao and Konishi (1991a) reported that human neutrophils could be infected with *T. gondii*, but did not quantify the degree of parasite proliferation. A review of the literature has shown that the ability of *T. gondii* to invade T and B lymphocytes has not been studied in detail.

This study was performed to determine the susceptibility of murine splenic T and B lymphocytes, and granulocytes, to infection with *T. gondii* RH tachyzoites. The ultrastructure of infected host cells was observed by transmission electron microscopy (TEM), and the degree of intracellular parasite proliferation was quantified using ^{3}H-uracil uptake assay.

MATERIALS AND METHODS

1. Preparation of *T. gondii* tachyzoites

Virulent RH tachyzoites of *Toxoplasma gondii* were serially passaged in ICR mice every 5 days. Peritoneal exudates were harvested from the mice, suspended in phosphate-buffered saline (PBS), and centrifuged for 5 min at 1,500 rpm. Tachyzoites were isolated from the interlayer between 40% and 50% Percoll (Pharmacia) gradients and resuspended in PBS.

2. Preparation of murine T and B lymphocytes, and granulocytes

Uninfected host cells were obtained from normal male BALB/c and CBA mice weighing 20-30 g. Mice were sacrificed by cervical dislocation and placed in a beaker containing 70% ethanol for wetting. Spleens were removed aseptically, ground on a wire mesh immersed in Hank's balanced salt solution (HBSS), and single cell suspension (1×10^8 cells/ml) was prepared.

The cell suspension was washed with complete RPMI 1640 media containing 10% fetal calf serum at 4°C, layered on Histopaque (Sigma) 1.077, and then centrifuged at 2,500 rpm for 10 min at 20°C. A mixture of T and B lymphocytes was harvested from the upper layer and washed with complete RPMI 1640. Further purification of T and B lymphocytes was performed by layering the cell mixture on Percoll density gradients 1.052, 1.063, 1.075, 1.085 and 1.122, and centrifugation at 2,500 rpm for 20 min at 20°C. Cells of the interlayers between 1.063 and 1.075, and 1.075 and 1.085 were used for T lymphocytes, and those between 1.052 and 1.063 for B lymphocytes (Gutierrez *et al.*, 1979).

Granulocytes were purified by layering the spleen cell suspension on Histopaque 1.077 and 1.119 gradients, followed by centrifugation at 3,000 rpm for 10 min at 20°C. The interlayer containing pure granulocytes, over 95% of which were neutrophils, was collected.

3. Exposure of host cells to *T. gondii* tachyzoites

Purified T and B lymphocytes, and granulocytes, were washed with complete RPMI 1640, and the cell concentration adjusted to 6×10^6/ml. One hundred and fifty microliters of cell suspension was placed in each well of a U-bottomed 96-well microtitre plate (Falcon), and incubated at 37°C for 24 hrs in a humidified 5% CO_2 incubator; 7×10^6 or 1.4×10^7 tachyzoites were then inoculated into the culture which was left to stand for 24 hrs.

4. Morphological observations by LM and TEM

The ultrastructure of the infected host cells was observed by transmission electron microscopy (TEM). Light microscopy (LM) was also performed on semithin sections prepared for TEM. Tachyzoite-infected host cells were washed with cacodylate buffer (pH 7.4), and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde at 4°C for 12 hrs. After washing, they were post-fixed with 1% osmium tetroxide for 2 hrs, and then dehydrated in graded series of ethanol. They were embedded in epon, and semithin sections of 1 μm thickness were prepared and stained with toluidine blue for LM observation. To prepare ultrathin sections, semithin sections were further processed, and then stained with uranyl acetate and lead citrate. The stained samples were observed through a TEM (1200 EX-II, JEOL, Japan) at 80 KV.

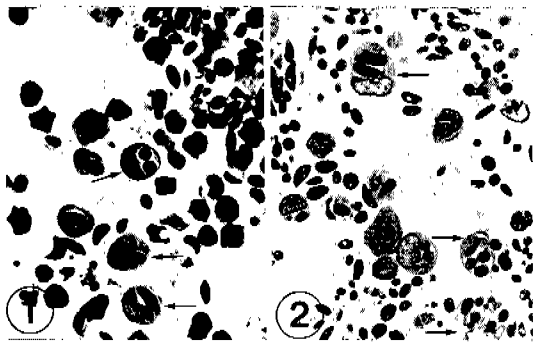

Fig. 1. A thin section of splenic T lymphocytes (arrows) of BALB/c mice experimentally infected *in vitro* with RH tachyzoites of *Toxoplasma gondii*, 24 hrs after infection (× 1,000). **Fig. 2.** Ibid, neutrophils (arrows) isolated from the spleen of BALB/c mice and infected *in vitro* with tachyzoites of *T. gondii*. Neutrophils contain a few to many tachyzoites which presumably divided intracellularly, 24 hrs after infection (× 1,000).

5. ^{3}H-uracil uptake assay

Using the procedure reported by Schmatz *et al.* (1986), the ^{3}H-uracil incorporation by *T. gondii* tachyzoites was quantified. Briefly, purified T and B lymphocytes, and granulocytes, were exposed to *T. gondii* tachyzoites for 24 hrs, washed, and then 10 μl (1 μCi/ml) of ^{3}H-uracil (Amersham) was added to each well. Then the mixture was left to stand for 2-2.5 hrs. Using a multi-channel cell harvester (Skatron, USA), host cells were harvested onto a small filter disk. Individual disks were dried in an oven for 60 min, and placed in scintillation vials; 5 ml of scintillation fluid was added to each vial. The samples were counted by a beta scintillation counter (Beckman, USA) to determine counts/minute (CPM), and data for this were expressed as the mean and standard deviation of values derived from triplicate wells.

RESULTS

1. Morphology of infected host cells

Light Microscopy: As seen on ultrathin sections, T and B lymphocytes, and granulocytes, cocultured with *T. gondii* tachyzoites contained in their cytoplasm 1 or 2, or a maximum of 7-8 tachyzoites (Figs. 1-2). More than half the cells were infected with

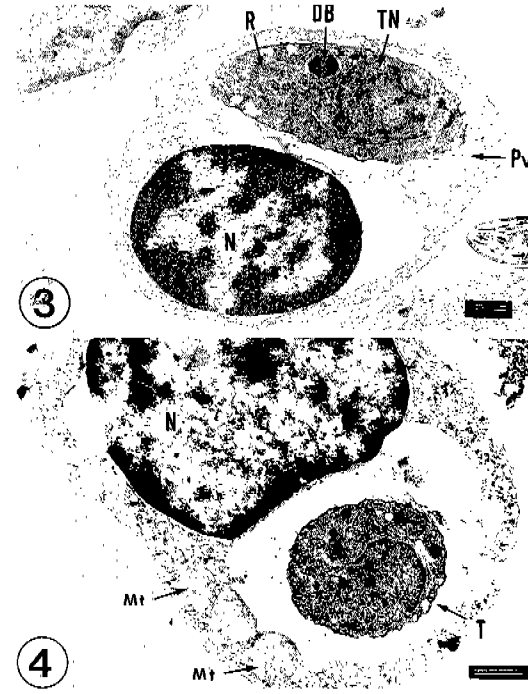

Fig. 3. Electron micrograph of a T lymphocyte isolated from the spleen of a BALB/c mouse and experimentally infected with tachyzoites of *T. gondii in vitro*, 24hrs after infection, characteristically showing the host cell nucleus (N) displaced to the periphery resulting from the parasitophorous vacuole (Pv). The intracellular organelles of the parasite, namely the nucleus (TN), rhoptry (R), and electron-dense body (DB) are observed (white scale: 1 μm). **Fig. 4.** Electron micrograph of a T lymphocyte isolated from the spleen of a CBA mouse and experimentally infected with *T. gondii in vitro*, 24 hrs after infection, showing a tachyzoite (T) surrounded by the parasitophorous vacuole, the slightly displaced host cell nucleus (N) and an increased number of mitochondria (Mt) (white scale: 1 μm).

tachyzoites and there appeared no difference by type of host cells and strain of mice.

Transmission Electron Microscopy: While normal T lymphocytes had a large nucleus which almost filled the entire cytoplasm, those infected with tachyzoites demonstrated a peripherally displaced nucleus, enlarged intracellular space as a result of a parasitophorous vacuole enveloping the parasite, and an increased number of mitochondria (Figs. 3-4). The rough endoplasmic reticulum (RER) was well developed in normal B

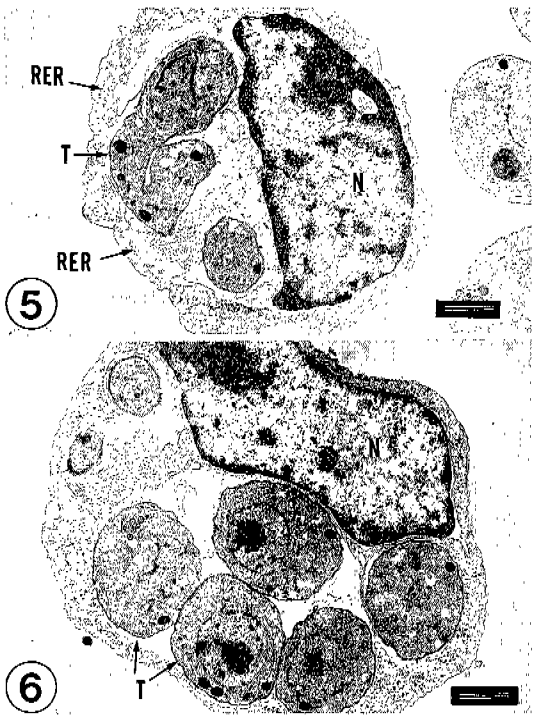

Fig. 5. Electron micrograph of a B lymphocyte isolated from the spleen of a CBA mouse, 24 hrs after infection with tachyzoites of *T. gondii*. Illustrated are the nucleus (N), rough endoplasmic reticulum (RER), and *T. gondii* tachyzoites (T) enveloped by the parasitophorous vacuole (white scale: 1 μm). **Fig. 6.** Electron micrograph of a B lymphocyte isolated from the spleen of a CBA mouse, 24 hrs after infection with tachyzoites of *T. gondii*, showing the host cell nucleus (N) and several divided tachyzoites (T) (white scale: 1 μm).

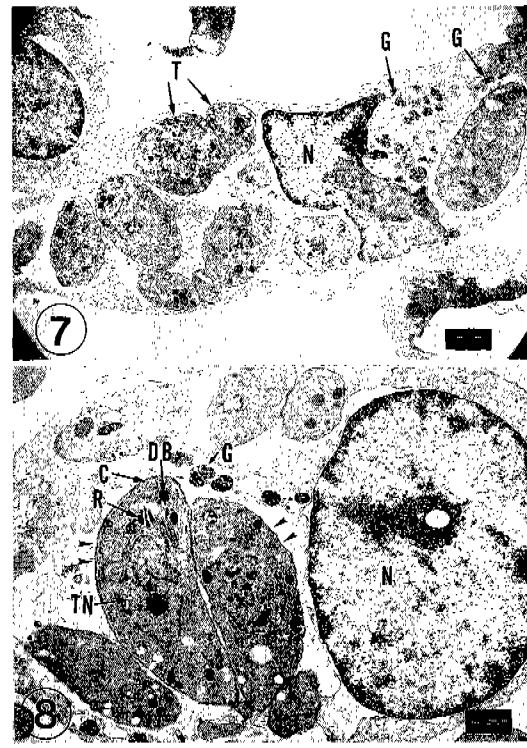

Fig. 7. Electron micrograph of a neutrophil isolated from the spleen of a BALB/c mouse, 24 hrs after infection with tachyzoites of *T. gondii*, showing the host cell nucleus (N) and cytoplasmic granules (G). Many of the tachyzoites (T) divided and occupy almost all the intracellular space of the cell (white scale: 1 μm). **Fig. 8.** Electron micrograph of a neutrophil isolated from the spleen of a CBA mouse, 24 hrs after infection with tachyzoites of *T. gondii*, showing the nucleus (N) and granules (G). Within the cytoplasm, 7-8 tachyzoites of *T. gondii* are seen, and two (arrow heads) are in their endodyogenic phase. The nucleus (TN), conoid (C), rhoptry (R), and electron-dense body (DB) of this parasite are seen (white scale: 0.5 μm).

lymphocytes, but not in cells infected with tachyzoites (Figs. 5-6).

Most ultrathin sectioned granulocytes were neutrophils. Normal neutrophils contained different kinds of electron-dense granules which are believed to be secreted when they are stimulated by foreign material. However, those cells infected with tachyzoites demonstrated decreased numbers of cytoplasmic granules. Nevertheless, the intracellular parasites were not killed and seen to have actively proliferated. Most infected neutrophils contained more than four tachyzoites, which in some, were in their characteristic stage of endodyogeny (Figs. 7-8). Tachyzoites showed their typical cytoplasmic organelles, including conoids, rhoptries, and electron-dense bodies. The host and parasitic ultrastructural features were similar for the two strains of mice.

2. ^{3}H-uracil uptake

The susceptibility of T and B lymphocytes, and granulocytes, to infection with *T. gondii* tachyzoites was high irrespective of cell type and strain of mouse (Table 1). The β-ray background from normal splenic T

Table 1. ^{3}H-uracil uptake by RH tachyzoites of *T. gondii* in murine splenic T and B lymphocytes, and granulocytes

Host Cell type	Tachyzoite number inoculated /well	^{3}H-uracil uptake(count per minute)[a]			
		BALB/c		CBA	
		control[b][c]	infected culture[c]	control[b][c]	infected culture[c]
T lymphocytes	7×10^6	121 ± 18	5,793 ± 1,210	102 ± 45	4,572 ± 774
	1.4×10^7	121 ± 18	5,694 ± 211	102 ± 45	3,236 ± 321
B lymphocytes	7×10^6	132 ± 44	2,601 ± 222	151 ± 28	4,125 ± 434
	1.4×10^7	132 ± 44	3,299 ± 9	151 ± 28	3,709 ± 251
Granulocytes	7×10^6	86 ± 21	3,886 ± 154	130 ± 42	5,632 ± 496
	1.4×10^7	86 ± 21	3,572 ± 379	130 ± 42	5,201 ± 910

[a]Values represent the mean of triplicate wells (with standard deviation) in a representative experiment, 24 hrs after infection. Experiments were repeated 3 times with similar results.

[b]Control culture only with host cells

[c]In all cell types tested the difference in CPM between control and infected culture was statistically significant ($p < 0.01$).

lymphocytes from BALB/c mice was 121 ± 18, but increased 47 to 48-fold to 5,793 ± 1,210 and 5,694 ± 211 when 7×10^6 and 1.4×10^7 tachyzoites per well, respectively, were inoculated. In CBA mice, the CPM was 102 ± 45 for normal T lymphocytes, while for those which were infected, the figures were 4,572 ± 774 and 3,236 ± 321, respectively, a 32 to 45-fold increase.

For B lymphocytes, the CPM of uninfected cells from BALB/c mice was 132 ± 44, but in infected cells, the CPM increased 20 to 25-fold, to 2,601 ± 222 and 3,299 ± 9, respectively. The CPM of normal splenic B lymphocytes of CBA mice was 151 ± 28, but in infected cells, a 25 to 27-fold increase to 4,125 ± 434 and 3,709 ± 251, respectively, was seen.

Normal granulocytes from BALB/c mice showed CPM of only 86 ± 21, but in those infected with tachyzoites, this increased to 3,886 ± 154 and 3,572 ± 379, respectively (42-45 fold increase). In CBA control mice, the CPM was 130 ± 42, and in infected granulocytes, this increased to 5,632 ± 496 and 5,201 ± 910, respectively (40-43 fold increase).

DISCUSSION

T. gondii has been shown to induce immunosuppression in hosts (Haque *et al.*, 1995; Khan *et al.*, 1995; Channon and Kasper, 1996), but few studies have reported the reasons for this. Channon and Kasper (1996) considered that a soluble factor down-regulating INF-γ, derived from parasites, was responsible for *T. gondii*-induced immunosuppression, but other factors are thought to be involved. Since *T. gondii* invades RE cells, including various kinds of leucocytes, rupture and/or functional deterioration of infected leucocytes could be a reason for the immunosuppression. However, the susceptibility of T and B lymphocytes and granulocytes, to *T. gondii* infection has seldom been studied in detail using quantitative assays.

In the present study, morphological observation and a quantitative assay showed that irrespective of cell type and strain of mouse, murine splenic T and B lymphocytes, and granulocytes (mostly neutrophils), were highly susceptible to infection with *T. gondii* tachyzoites. In view of the amount of uracil uptake, T lymphocytes and neutrophils appeared more susceptible than B lymphocytes; however the possibility that infected B lymphocytes were disrupted earlier than other two kinds of cells, due to the proliferation of tachyzoites, should be ruled out.

The finding that *T. gondii* can invade murine splenic T and B lymphocytes, and neutrophils, and proliferate in these cells, may explain

immunosuppression in infected hosts, which is thought to be very likely due to either the host cell rupturing or functional deterioration of the infected cells. RER within the cytoplasm of B lymphocytes, where immunoglobulins are produced, was less in *T. gondii*-infected cells than in those that were not infected, and so it may be assumed that immunoglobulin production is seriously affected.

T lymphocytes and NK cells are known to be macrophage disrupters or immune response regulators rather than host cells of this parasite (Hakim *et al.*, 1991; Subauste *et al.*, 1991; Sher *et al.*, 1993). Experimental infection of these cells with *T. gondii* tachyzoites has therefore seldom been tried. In the present study, however, T lymphocytes were easily infected with tachyzoites as shown by TEM ultrastructure and uracil uptake assay. This strongly suggests that the capacity of infected T cells to produce cytokines is reduced, though further verification is needed.

The interaction of human neutrophils and *T. gondii* tachyzoites was reported by Nakao and Konishi (1991a, 1991b). They (1991a) observed that in the absence of antibody, parasites proliferated within neutrophils, but did not kill them; and suggested that during the initial phase of infection, until antibody is produced, neutrophils were responsible for the dissemination of tachyzoites. They (1991b) further reported that neutrophil chemotactic factors are secreted from *T. gondii*. On the other hand, Holland and Sleamaker (1970) reported that neutrophils easily phagocytosed *T. gondii* and induced lysis of this parasite. Wilson and Remington (1979) also found that 50% of neutrophils rapidly destroyed *T. gondii*. In the present study, the proliferation of tachyzoites in the cytoplasm of murine neutrophils was verified by TEM ultrastructure and uracil uptake assay in the absence of antibody until 24 hrs post-infection.

The mechanism of host cell penetration by *T. gondii* is controversial, with some reports demonstrating active penetration, and others presenting evidence to support phagocytosis (Doran, 1982). The present study showed that since lymphocytes are nonphagocytic cells, *T. gondii* can actively invade. To more adequately explain the mechanisms of host-parasite relationships in toxoplasmosis, further *in vitro* and *in vivo* studies are in any case needed.

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=초록=

마우스 비장 림프구 및 과립구에 대한 톡소포자충 RH tachyzoite 감염 실험

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톡소포자충의 숙주-기생충 상호관계 연구의 일환으로 마우스 비장에서 분리한 T 림프구, B 림프구 및 과립구(대부분 호중구로 구성)에 톡소포자충의 tachyzoites를 감염시킨 후 감염된 림프구와 호중구의 미세형태 변화를 관찰하는 한편, 각 세포의 충체 감염에 대한 감수성을 동위원소 흡수시험법을 이용하여 정량화하였다. 충체는 병원성이 강한 RH 주를 사용하였고, 각 세포는 BALB/c와 CBA 마우스의 비장에서 분리하여 사용하였다. 감염 후 24시간에 관찰한 결과, T 림프구, B 림프구 및 호중구는 마우스 주에 상관없이 세포질 내에 tachyzoites가 한 개, 두 개 또는 7~8개까지 관찰되었다. 감염된 T 림프구는 충체 주변에 형성된 parasitophorous vacuole로 인해 핵이 한 쪽으로 밀리며, 미토콘드리아의 수가 증가하였다. 감염된 B 림프구는 조내형질세망(RER)이 대조군에 비해 발달하지 않았으며 감염된 호중구는 과립의 수가 현저히 감소하였다. 림프구와 호중구의 톡소포자충 감염에 대한 감수성을 ^{3}H-uracil 흡수량으로 정량화한 결과, 마우스 주에 따른 차이는 없었고 모든 종류의 세포 내에서 충체가 활발히 증식함이 확인되었다. 이상의 결과로 볼 때, BALB/c와 CBA 마우스의 비장 T 림프구, B 림프구 및 호중구는 모두 톡소포자충의 tachyzoites 감염에 대해 감수성이 높음을 알 수 있었고, 감염된 면역세포는 그 기능이 저하될 것으로 추측된다.

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