[Jap. J. Parasit., Vol. 29, No. 6, 535-543, 1980]

Inhibitory Effect of Immune Spleen Lymphocytes on Invasion of *Toxoplasma gondii* Tachyzoites into Cultured L Cells

NOBUYUKI SHINOHARA
Department of Parasitology, School of Medicine, The University of Tokushima, Tokushima, 770 Japan

(Received for publication; August 26, 1980)

Introduction

Mice immunized with low virulent organisms or cell homogenate of *Toxoplasma gondii* can acquire specific resistance to the challenge with homologous parasites (Ruskin *et al.*, 1971; Krahenbuhl *et al.*, 1972; Araujo *et al.*, 1974; Tsuchimoto, 1978). Although transfer of resistance to *T. gondii* has been successful in mice using immune serum (Krahenbuhl *et al.*, 1972), the protection was insufficient as compared with that conferred after infection with a low virulent strain of *T. gondii* (Gardner and Remington, 1978).

On the other hand, immune lymphocytes were also capable of transferring the resistance (Frenkel, 1967). The sort of resistance to this parasite might be mediated mainly by lymphoid cells in the cell-mediated immune system. On the basis of a similar study with *T. gondii*, Nakano (1973) suggested that when experimental hosts were pretreated with immunosuppressive measures, such as X-ray irradiation, cyclophosphamide and 6-mercaptopurine, the entity of resistance to the parasite should be present in spleen-derived lymphocytes. Several workers demonstrated *in vitro* that a fresh anti-*Toxoplasma* serum inhibited the invasion of the parasites into cultured cells (Sekino, 1973; Jones *et al.*, 1975), and some others described a mighty inhibition of the multiplication of *T. gondii* by macrophage in the presence of a product of immune lymphocyte, lymphokine (Jones *et al.*, 1975; Shirahata *et al.*, 1975, 1976). These studies proved the presence of a potent anti-*Toxoplasma* action in antiserum, lymphocytes as well as in macrophages.

We investigated *in vitro* the inhibitory action of immune spleen lymphocytes of mice against invasion of *Toxoplasma* into cultured cells in the presence and absence of immune serum to elucidate the protective role of immune lymphocytes in toxoplasmosis.

Materials and Methods

Parasites: *T. gondii* strain RH was maintained by serial passages through female ddY (22±2 g) mice (Tokushima Experimental Animal Laboratory, Tokushima). The tachyzoites obtained from peritoneal exudates of mice infected with the organisms 3 to 4 days previously were filtered through a gauze and centrifuged to eliminate the host cells and the filtrate was used as an antigen.

Antisera: Rabbit anti-mouse IgG serum and rabbit anti-mouse whole serum serum were purchased from MBL Co., Nagoya. An

anti-*Toxoplasma* serum was obtained from mice immunized with the parasite cell homogenate (Tsuchimoto, 1978), and the antibody titer of the serum after being heat-inactivated at 56 C for 30 min was 1:8,192 when measured by *Toxoplasma* latex agglutination kit (Eiken Kagaku Co., Tokyo). An anti-thymocyte serum of mice (ATS) was obtained from the rabbit immunized with thymocytes of ddY mice at 5 days of age, and treated twice with mouse erythrocytes and acetone-powdered mouse liver to absorb nonspecific antibodies. The antiserum was inactivated by heating at 56 C for 30 min. The titer of ATS was 1:256 when measured by direct agglutination of thymocytes of mice.

Cell cultures: Fibroblasts (L-929) were used as cultured cells (L cells). L cells were incubated in a medium consisting of Eagle's minimal essential medium, 12.5 m*M* N-2-hydroxyethylpiperazine-N'-2-etanesultonic acid and 10% calf serum (MEM-HEPES). The medium contained streptomycin (200 μg/ml) and penicillin (200 IU/ml) and was adjusted to pH 7.2. The cultured cells were scraped off gently with a rubber spatula in their logarithmic phase, dispersed well with a bulb pipette and suspended in medium at a concentration of 5×10^4 cells/ml. Two ml of the suspension was transferred into a test tube containing a cover slip (9×18 mm) and the test tube was put in a 5% CO_2 incubator at 37 C for 3 hr for the cells to adhere to the glass slip.

Immunization: Mice were immunized intraperitoneally with *Toxoplasma* cell homogenate in the presence and absence of Freund's complete adjuvant (FCA) and FCA alone. After 10 days the animals were boosted by a second injection of the antigen in the absence of adjuvant. The dose of antigen was equivalent to 2×10^8 tachyzoites per mouse.

Preparation of lymphocytes: Mice immunized with homogenate antigen with FCA were bled to death 20 days after the final injection. Spleens were excised aseptically, cut in pieces with a pair of scissors, ground gently between 2 pieces of frosted slide glasses and suspended in MEM-HEPES. To eliminate adherent cells, the suspension was placed in a glass wool (Ishizu Pharmaceutical Co., Tokyo) column (300×10 mm) and the column was allowed to stand at 37 C for 30 min before running. Then the lymphocytes in the elute were further separated on a lymphocyte separation medium (Bionetics Co., U.S.A.) by centrifugation at 400×g for 30 min (IM・L). Normal lymphocytes were also obtained in the same manner from the respective group of mice which received antigen alone (A・L), FCA alone (FCA・L) and normal mice (Norm・L).

Preparation of thymus-derived lymphocytes (T cells): To examine the interaction between T cells and *T. gondii* under an electron microscope, T cells were separated from spleens of mice using a nylon wool (Julius *et al.*, 1973).

Preparation of soluble factor(s) of lymphocytes: To assess the effect of soluble factor(s) of lymphocytes, the parasite homogenate (equivalent to 2×10^8 cells/ml) was added at a concentration of 10% to an IM・L suspension (5×10^5 cells/ml). The reaction mixture was incubated in 5% CO_2 and 95% air at 37 C for 24 hr, and centrifuged at 1,000 rpm for 10 min. The resulting supernatant was used immediately as a preparation of soluble factor(s).

Treatment of lymphocytes with antisera: Anti-mouse IgG or anti-mouse whole serum serum was added at concentrations of 10, 1 and 0.1% to a test tube in which a lymphocyte-parasite mixture was reacted with a L cell monolayer on a glass slip. The test tube was then incubated by the same method as described above for cell culture. A lymphocyte suspension containing 1×10^9 cells was treated with ATS at a final concentration of 50% and the cells were washed 3 times by centrifugation in MEM-HEPES. The

treated lymphocytes were reacted with guinea pig complement (50 CH_{50}/ml) at 37 C for 30 min and washed by centrifugation before use as a lymphocyte source for the following assay system.

Assay system for invasion and multiplication of parasites: Two ml of a tachyzoite suspension (5×10^5 cells/ml) prepared from the peritoneal fluid of infected mice was transferred into test tubes in which the previously prepared L cell-adhering slip and IM·L (1×10^7 cells) were subsequently placed. In order to allow the tachyzoites to contact well with IM·L, the slips were carefully positioned horizontally in the test tubes and they were incubated in a CO_2 incubator at 37 C. At intervals slips were taken out and examined. The invasion and multiplication of parasites in the cultured cells were measured as follows. The L cell, parasites and IM·L prepared as described above were similarly incubated together at 37 C for 3 hr. The slip was washed gently with MEM-HEPES and fixed in absolute methanol. After being stained with Giemsa, the slip was examined under the light microscope. To measure the invasion rate, the number of L cells that were infected with the parasites (cells with more than one parasite) was counted against a total number of 50 to 75 L cells in 5 fields in 2 preparations. Normal lymphocytes were treated in the same manner as control experiments. During incubation, the slip was also examined for the number of parasites per infected L cells to know the multiplication of the organism in the cells. The generation time was calculated from the plot on a semilogarithmic graph of the number of *Toxoplasma* per vacuole vs. time (Kaufman and Maloney, 1962).

Electron microscopy: Lymphocytes treated with ATS and complement were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. A drop of the fixed material was put on a cover slip which had been previously treated with 0.1% poly-l-lysine. Then the sample was critical point dried, coated with metal gold in a Hitachi sputtering system and then examined under the Hitachi SS-2 scanning electron microscope. Lymphocytes reacted with the parasites were similarly processed for electron microscopic observation.

Statistical analysis: A statistical analysis of the results was made by Student t test and the 0.05-probability level was considered significant.

Results

Effect of immune lymphocytes on invasion of parasites: The invasion rate of tachyzoites into cultured L cells in the presence of A·L and Norm·L was 35.0±8.0 and 92.0±6.0%, respectively and that in the presence of IM·L and in the control (FCA·L) was 29.2±7.5 and 90.0±5.5%, respectively (Fig. 1). The difference in both cases was statistically significant ($p<0.05$). The intracellular multiplication of the parasites in the culture was not affected by

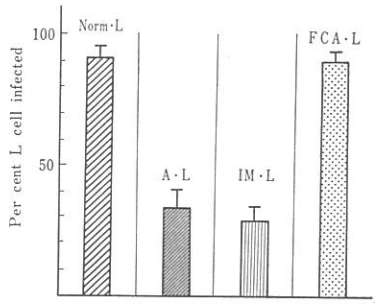

Fig. 1 Effect of spleen lymphocytes from mice immunized with *Toxoplasma* cell homogenate in the presence and absence of Freund's complete adjuvant on *in vitro* invasion of the parasites into L cells.

Norm·L: spleen lymphocytes from untreated mice

FCA·L: spleen lymphocytes from mice treated with FCA alone

IM·L: spleen lymphocytes from mice immunized with cell homogenate and FCA

A·L: spleen lymphocytes from mice immunized with cell homogenate alone

FCA: Freund's complete adjuvant

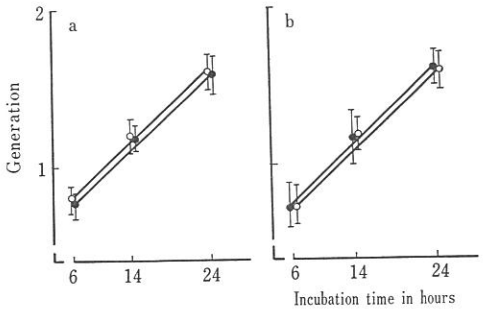

Fig. 2 Growth rate of *Toxoplasma* tachyzoites in L cell cultured in the presence of spleen lymphocytes from mice immunized with *Toxoplasma* cell homogenate in the presence and absence of Freund's complete adjuvant.

a) ○——○: immunization with cell homogenate alone ●——●: immunization with cell homogenate and FCA b) ○——○: immunization with FCA alone ●——●: non-treatment

FCA: Freund's complete adjuvant

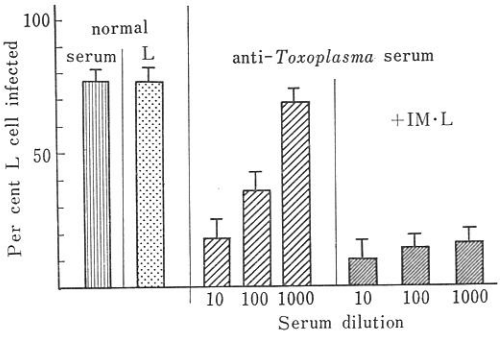

Fig. 3 Effect of anti-*Toxoplasma* serum on *in vitro* invasion of *Toxoplasma* into L cells in the presence and absence of immune mouse spleen lymphocytes.

L: spleen lymphocytes IM·L: spleen lymphocytes from mice immunized with cell homogenate and FCA

FCA used in immunization (Figs. 2a, b).

Effect of anti-*Toxoplasma* mouse serum on the activity of IM·L against invading *Toxoplasma*: The invasion rate of the parasites into cultured L cells in the presence of anti-*Toxoplasma* mouse serum at concentrations of 10, 1.0 and 0.1% were 18.8±7.0, 36.0±7.5 and 68.6±5.1%, respectively and that with normal mouse serum was 75.2±7.5%, showing a dose response relationship between the concentration of the antiserum and the invasion rate. However, addition of IM·L to the system inhibited strongly the invasion of *T. gondii* at low concentrations of antiserum. The values decreased to 10.0~16.0% from 76.0±7.0% in the control, indicating a potent inhibitory effect of IM·L on the invasion regardless of the concentration of the antiserum (Fig. 3). The inhibition was not suppressed by treating the lymphocytes with rabbit anti-mouse IgG serum or rabbit anti-mouse whole serum serum (Fig. 4).

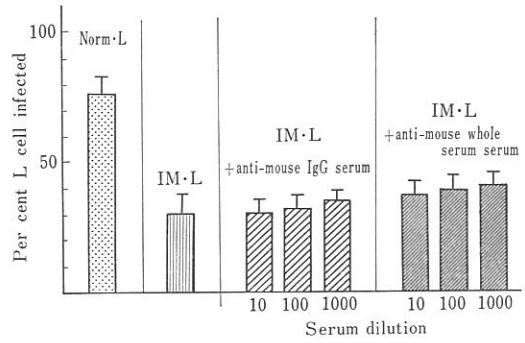

Fig. 4 Effect of immune mouse spleen lymphocytes on *in vitro* invasion of *Toxoplasma* into L cells in the presence of rabbit anti-mouse IgG serum and rabbit anti-mouse whole serum serum.

Norm·L: spleen lymphocytes from untreated mice

IM·L: spleen lymphocytes from mice immunized with cell homogenate and FCA

Effect of soluble factor(s) of lymphocytes: The role of soluble factor(s) of lymphocytes on the *Toxoplasma* invasion was studied in the absence of IM·L. The invasion rate was 90.1±8.9% when the soluble factor(s) of IM·L was present while it was 92.3±7.0% when the factor(s) of normal lymphocytes was present. These values did not differ much from that in the control (93.1±6.4%) (Table 1).

Effect of ATS-treatment on inhibitory activity of IM·L: To know which lympho-

Table 1 Effect of soluble factor(s) of lymphocytes on the invasion of *Toxoplasma gondii* into L cells

Source of soluble factor(s)	Per cent L cell infected
immune lymphocyte	90.1±8.9
normal lymphocyte	92.3±7.0
(lymphocyte-free)	93.1±6.4

±: standard error

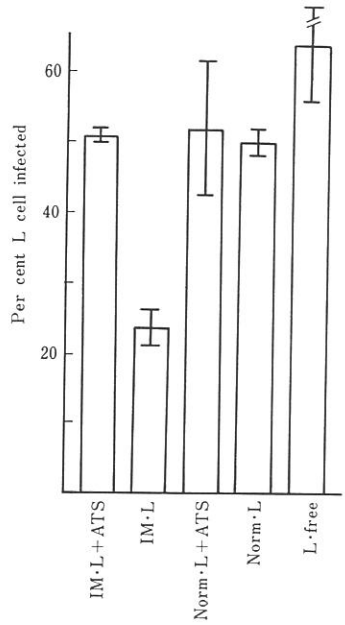

Fig. 5 Effect of anti-thymocyte serum on *in vitro* invasion of *Toxoplasma* into L cells in the presence of immune mouse spleen lymphocytes.
IM·L: spleen lymphocytes from mice immunized with cell homogenate and FCA
Norm·L: spleen lymphocytes from untreated mice
L·free: lymphocyte-free
+ATS: treatment with anti-thymocyte serum

cyte population, T cell or B cell, was involved in the observed inhibition of *Toxoplasma* invasion, IM·L were treated with ATS and complement. Figure 5 shows the invasion rate in the presence of IM·L which had not been treated with ATS was 23.5± 3.0% while that in the presence of ATS-treated IM·L was 51.5±1.7% which was nearly equal to the values in the controls (51.0±2.8 and 52.4±10.5% in the presence of Norm·L and ATS-treated Norm·L, respectively). Cytotoxic damage of lymphocytes after treatment with ATS and complement was examined by scanning electron microscopy. Many cells appeared to be destroyed as shown in Photo. 1e. It was also examined whether there was any direct contact between the parasites and the lymphocytes. When lymphocytes were mixed with the parasites and processed for scanning electron microscopy, many T cells of immune lymphocytes were observed attached with tachyzoites (Photo. 1a) while lymphocytes from normal animals were rarely attached to the parasites (Photo. 1d). Photos. 1b and c show a typical example of this phenomenon in higher magnifications.

Discussion and Conclusion

In the *in vitro* experiments to see the activity of immune lymphocytes against invasion into cultured L cells and subsequent multiplication of *T. gondii,* IM·L exerted an inhibitory effect on the invasion in the absence of specific antiserum (Fig. 1). It is unlikely in this system that monocytes or granulocytes are involved in the activity since they were not observed at all in the stained smear of the lymphocyte preparation. The immune lymphocytes gave no effects on the intracellular multiplication (Fig. 2) favoring the result shown in Kaufman and Maloney's studies with high virulent RH strain organisms and MK cells. Furthermore, an anti-*Toxoplasma* serum showed an inhibitory activity on the invasion of the parasites into the cultured cells, but the effect was not additional when the antiserum was added with immune lymphocytes (Fig. 3). In this regard, a possible involvement of cytophilic antibodies can still not be excluded although several centrifugal washings were performed during

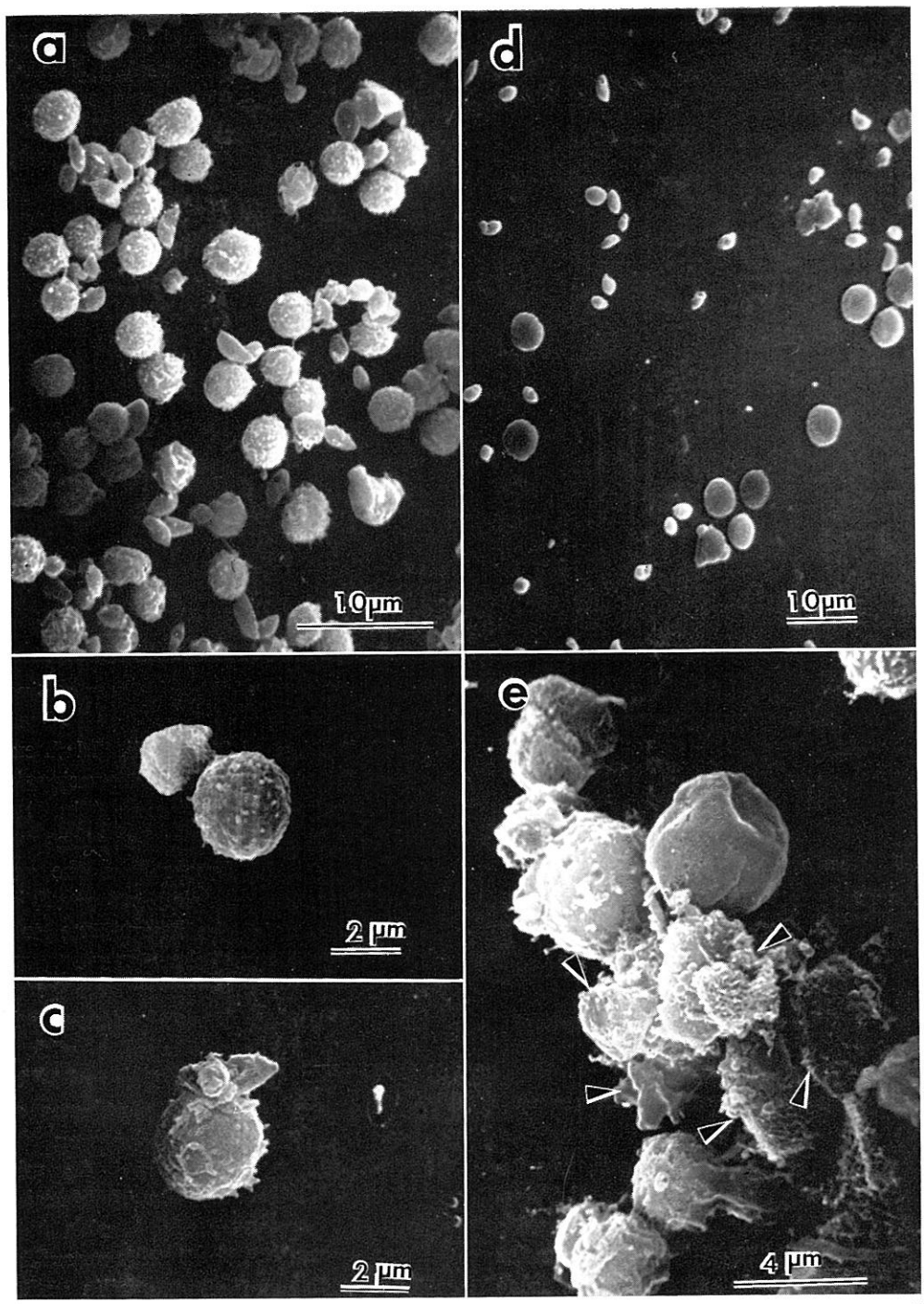

Photo. 1 Scanning electron micrographs showing adhesions between immune spleen lymphocytes (T cells) and *Toxoplasma* tachyzoites (a, b, c and d (control)) and lymphocytes treated with rabbit anti-thymocyte serum (e; arrows).

the preparative process of IM·L to eliminate serum antibodies as well as cytophilic antibodies. For this reason, we attempted to neutralize these antibodies with anti-mouse whole serum serum or anti-mouse IgG. In fact, the result (Fig. 4) showed that both serum and cytophilic antibodies were not interfering with the inhibitory action of IM·L against *Toxoplasma* invasion.

T cells are known to account for 25~45% in the spleen lymphocytes (Greaves *et al.*, 1974). To know which lymphocyte population, T cells or B cells, can inhibit *Toxoplasma* invasion into the cultured cells, IM·L were treated with ATS and complement. The inhibitory action of IM·L against *Toxoplasma* invasion was diminished to a great extent by this treatment suggesting that the action of IM·L should be attributed to the T cell population of lymphocytes. Since Mackaness *et al.* (1974) reported an enhancing effect of BCG-component in FCA on the immunological induction of T cells, it was possible that this adjuvant alone was participated to a certain degree in the inhibitory activity of lymphocytes in this experimental system. However, the invasion of the organisms into the cultured cells was not affected by addition of lymphocytes isolated from mice which had been treated with FCA alone (Fig. 1).

These results are to be summerized: (1) IM·L showed an inhibitory action against *Toxoplasma* invasion into cultured L cells, (2) supplement of antiserum was not required for the inhibitory action, (3) the inhibitory action was not synergetic with the activity of antiserum, (4) the inhibitory action was not affected by incubating the L cells and the parasites with soluble factor(s) released from IM·L after antigenic stimulation, and (5) the inhibitory action was evidently diminished by ATS plus complement treatment. These five points would lead to the presence of direct cytotoxic T cells. In fact, adhesions of immune T cells to *Toxoplasma* was demonstrated by scanning electron microscopy. In addition, many more immune T lymphocytes attaching to the parasites were observed as compared with the number of normal lymphocytes attached to the organism (Photos. 1a, b).

The presence of direct cytotoxic T cells has been verified in viral infections and tumors by Zweerink *et al.* (1977) and Cerottini and Brunner (1974), respectively, but not in cases of parasite infections (Mitchell, 1979). Ito *et al.* (1975) studied *in vitro* adherence of immune lymphocytes to *Trichomonas foetus* under the electron microscope, and several damaged spots were found on the membrane of the parasite but not on the corresponding part of the membrane of the immune lymphocyte.

The present study has indicated an inhibitory effect of IM·L on *Toxoplasma* invasion into cultured cells, and suggested involvement of T cells in the activity, but the mode of action of the direct cytotoxic T cells which would play a major role in this inhibition remains to be made clear.

Summary

Attempts were made of an inhibitory effect of mouse immune spleen lymphocytes (IM·L) on the invasion and multiplication of *Toxoplasma gondii* in cultured L cells.

Lines of evidence presented are: (1) The isolated IM·L had an inhibitory action against invasion of *Toxoplasma* tachyzoites into cultured L cells and this action is ascertained both in the presence and absence of Freund's complete adjuvant in immunization, (2) the inhibitory action of IM·L was not related to anti-*Toxoplasma* serum nor affected by anti-mouse serum or anti-mouse IgG, (3) the inhibitory action of IM·L decreased markedly by treating IM·L with anti-thymocyte serum and complement, (4) soluble factor(s) of IM·L showed no effect on the invasion of the parasites and (5) the *in vitro* adhering phenomenon of

IM·L to *Toxoplasma* was demonstrated under the scanning electron microscope. The presence of direct cytotoxic T cells against *Toxoplasma gondii* has been suggested in toxoplasmosis.

Acknowledgement

The author wishes to thank Professors Humio Osaki, Department of Parasitology, School of Medicine, The University of Tokushima and Yoshikazu Oka, Department of Health Science, Faculty of Education, The University of Tokushima for valuable discussions and suggestions during this work and for careful reviewing of the manuscript. Grateful acknowledgement is also made to Associate Professor Yoshihiro Ito, Department of Parasitology, School of Medicine, The University of Tokushima for continuous guidance and electron microscopic observation.

References

1) Araujo, F. C. and Remington, J. S. (1974): Protection against *Toxoplasma gondii* in mice immunized with *Toxoplasma* cell fractions, RNA and synthetic polyribonucleotides. Immunology, 27, 711–721.
2) Cerottini, J. C. and Brunner, K. T. (1974): Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. Adv. Immunol., 18, 67–132.
3) Frenkel, J. K. (1967): Adoptive immunity to intracellular infection. J. Immunol., 98, 1309–1319.
4) Gardner, I. D. and Remington, J. S. (1978): Aging and the immune responses II. Lymphocyte responsiveness and macrophage activation in *Toxoplasma gondii*-infected mice. J. Immunol., 120, 944–949.
5) Greaves, M. F., Owen, J. T. and Raff, M. C. (1974): T and B lymphocytes: Origins, properties and role in immune responses. Excerpta med., Am. Elsevier Publ. Co., New York, 316 p.
6) Ito, Y., Furuya, M., Doi, M., Hayashi, H., Yagyu, M., Oka, Y. and Osaki, H. (1975): Protective role of immune lymphoid cells and phagocytes in experimental trichomoniasis in mice. Jap. J. Parasitol., 24, 333–339.
7) Jones, T. C., Len, L. and Hirsch, J. G. (1975): Assessment in vitro of immunity against *Toxoplasma gondii*. J. Exp. Med., 141, 466–482.
8) Julius, M. H., Simpson, E. and Herzenberg, L. A. (1973): A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol., 3, 645–649.
9) Kaufman, H. E. and Maloney, E. D. (1962): Multiplication of three strains of *Toxoplasma gondii* in tissue culture. J. Parasitol., 48, 358–361.
10) Krahenbuhl, J. L., Ruskin, J. and Remington, J. S. (1972): The use of killed vaccines in immunization against an intracellular parasite: *Toxoplasma gondii*. J. Immunol., 108, 425–431.
11) Mackaness, G. B., Lagrange, P. H. and Ishibashi, T. (1974): The modifying effect of BCG on the immunological induction of T cells. J. Exp. Med., 139, 1540–1552.
12) Mitchell, G. F. (1979): Effector cells, molecules and mechanisms in host-protective immunity to parasites. Immunology, 38, 209–223.
13) Nakano, K. (1973): Cell mediated immunity (1). In Ikaken Seminar of Immunology, 1971, ed. by T. Matuhasi, Bunkodo, Tokyo, 78–92. (in Japanese).
14) Ruskin, J. and Remington, J. S. (1971): Resistance to intracellular infection in mice immunized with *Toxoplasma* vaccine and adjuvant. J. Reticuloendothel. Soc., 9, 465–479.
15) Sekino, S. (1973): Invasion of *Toxoplasma gondii* into cultured cells. Niigata Med. J., 87, 564–575. (in Japanese)
16) Shirahata, T., Shimizu, K. and Suzuki, N. (1975): An in vitro study on lymphocyte-mediated immunity in mice experimentally infected with *Toxoplasma gondii*. Jap. J. Vet. Sci., 37, 235–243.
17) Shirahata, T., Shimizu, K. and Suzuki, N. (1976): Effects of immune lymphocyte products and serum antibody on the multiplication of *Toxoplasma* in murine peritoneal macrophages. Z. Parasitenk., 49, 11–23.
18) Tsuchimoto, M. (1978): Protective antigenicity of cell homogenate and centrifugal cell fractions of *Toxoplasma gondii* in mice. Jap. J. Parasitol., 27, 433–443. (in Japanese with English Summary)
19) Zweerink, H. J., Courtneidge, S. A., Skehel, J. J., Crumpton, M. J. and Askonas, B. A. (1977): Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. Nature (London), 267, 354–356.

Toxoplasma gondii tachyzoite の培養 L 細胞侵入に対する免疫脾リンパ細胞の抑制作用

篠原信之
（徳島大学医学部寄生虫学教室）

Toxoplasma gondii の感染に対する免疫防御機構解析の一助として，免疫マウスからリンパ細胞を分離し，原虫の細胞侵入に対する抑制効果を *in vitro* で検討した．

T. gondii tachyzoite のホモジネート（10^8 原虫相当）に等容量の Freund's complete adjuvant (FCA) を添加の上マウス腹腔内に接種し，30日後に分離した脾リンパ細胞を，あらかじめ培養したL細胞（L-929）に原虫と共に加え，原虫のL細胞侵入に及ぼす作用を調べた．また，培養液にそれぞれ抗トキソプラズマ血清，抗マウス IgG 血清及び抗マウス全血清血清並びに抗原と免疫脾リンパ細胞との培養上清を加え，原虫侵入に対する免疫リンパ細胞の抑制効果への影響を調べた．更に，免疫リンパ細胞をウサギ抗マウス胸腺細胞血清で処理し，その由来（TかBか）を検討した．別に，免疫リンパ細胞と原虫との相互作用を走査型電子顕微鏡で観察した．

その結果，FCA 添加の有無に関係なく，免疫された脾リンパ細胞は原虫の細胞侵入を抑え，この抑制は抗トキソプラズマ血清の影響も受けず，抗マウス IgG 及び抗マウス全血清によっても阻害されなかった．また，免疫リンパ細胞の代わりに，抗原と免疫リンパ細胞との培養上清をL細胞培養液に加えても，原虫の細胞侵入は抑制できなかった．一方，ウサギ抗マウス胸腺リンパ細胞血清で処理した免疫リンパ細胞は，その抑制効果が明らかに減弱していた．また，免疫リンパ細胞と原虫との特異的な接着現象を走査電子顕微鏡下で観察した．

以上から *T. gondii* の宿主細胞への侵入に対する免疫宿主の直接障害性T細胞による抑制が示唆された．