[Jpn. J. Parasitol., Vol. 33, No. 5, 385-391, October, 1984]

Interaction between *Toxoplasma gondii* and Mouse Peritoneal Macrophage: Entry of the Parasites and Host Responses to Its Development

KOEI SATO, YAYOI ISE, TAKASHI IIDA, TAKAKAZU SUZUKI AND KOHKICHI SHIMADA

(Received for publication; July 20, 1983)

Key words: *Toxoplasma gondii*, peritoneal macrophage, phagocytosis, β-glucronidase

Introduction

Toxoplasma gondii is a coccidian protozoan capable of infecting a wide range of vertebrates. This organism, which appears to reside in cytoplasmic vacuoles, can multiply even in phagocytic cells (Hohorst, 1962; Jones *et al.*, 1972; Nogueia and Cohn, 1978).

Large-sized foreigen bodies such as erythrocytes, polystyrene particles, or microorganisms, when ingested by mouse peritoneal macrophages, are enclosed within endocytic vacuoles or phagosomes (Armstrong and Hart, 1971). Foreign bodies within endocytic vacuoles or phagosomes are digested by lysosomes. Lysosomes of macrophages are capable of fusing and producing digestive vacuoles to make phagolysosomes within which the degradation of ingested materials takes place (Cohn and Fedorko, 1969; Murray and Cohn, 1979). In most intestances, there occur rapid death and destruction of ingested microorganisms. Certain protozoans and bacteria, however, can survive and multiply in phagocytic cells (Jones *et al.*, 1972; Robert, 1972). Many investigators have studied their survival within phagocytic cells, but the mechanism of the survival is not yet well known.

This paper describes interactions between active invasion of *Toxoplasma gondii* and phagocytosis of mouse peritoneal macrophages against to parasites.

Department of Biophylaxis, Tokyo Metropolitan Institute of Medical Sience. 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan.

Materials and Methods

Parasites: Three days after inoculation of *Toxoplasma gondii* (RH strain), 3 ml of physiological saline was injected into the peritoneal cavity of a Swiss albino mouse, and peritoneal fluid was harvested. To remove mouse peritoneal cells, the fluid was filtered through the cellulose powder CF-11 (Whatoman Ltd., England) according to the method of Tanabe *et al.* (1978). After the filtration, the parasites were washed twice with saline and suspended at a concentration of 1×10^7 parasites/ml in saline. The suspension was divided into four groups: 1) non-treated living toxoplasma (N); 2) living toxoplasma, coated with antibody for 30 min at 4°C (NA); 3) dead toxoplasma, fixed with 2% paraformaldehyde for 10 min at 4°C (F); 4) dead toxoplasma, coated with antibody for 30 min at 4°C (FA). Each preparation was suspended at a concentration of 1×10^7 parasites/ml in Ca^{++} free Krebs Ringer phosphate (KRP) buffer. The suspension was warmed to 37°C prior to use.

Rabbit anti-toxoplasma antibody: Rabbit anti-toxoplasma serum was obtained by repeated immunization of a New Zealand albino rabbit with a mixture of toxoplasma whole antigen and Freund complete adjuvant (Difco Laboratories, USA). IgG antitoxoplasma antibodies were prepared from the antiserum by salting out and cellulose column chromatography, and stored at −20°C until use. The IgG antibody showed a titer of 1:

8,000 by the dye test and 1 : 16,000 by the hemagglutination test. For sensitization of toxoplasma as described in 2) and 4) experiment the final concentration (1 : 8) of antibody equivalent to 1,000 dye test units was used.

Macrophage (Mϕ): Male mice weighing 20–30 g each were injected intraperitoneally with 3 ml of sterilized physiological saline containing 5 % casein sodium (Wako, Ltd.). The cells were centrifuged at 140 g for 5 min, followed by a brief osmotic treatment with 0.2 % NaCl for 30 sec to lyse red cells, then adjusted at 0.9 % NaCl solution by addition of 1.6 % NaCl. Furthermore, the Mϕ were separated from other peritoneal cells with Ficoll-Hypaque (Pharmacia Fine Chemicals). The separated cells were suspended at a concentration of 5×10^7 cells/ml in KRP buffer.

Reaction of the macrophages and toxoplasma parasites *in vitro*: One ml of toxoplasma suspension (1×10^7/ml) was incubated with 1 ml of mouse Mϕ suspension (1×10^5/ml) at 37°C. After incubation for 15, 30, and 60 min, the mixture was centrifuged at 200 G for 10 min, and the supernatant and the precipitate were separated.

Assay of β-glucuronidase: β-glucuronidase in the supernatant and the precipitate was measured using *p*-nitrophenyl glucuronide (E. Merck Japan, Ltd.) as the substrate. Cleavage of this substrate to yield *p*-nitrophenol, which can be followed by extinction change at 405 nm, is proportional to the amount of β-glucuronidase (Rausch and Moore, 1975). After the incubation of Mϕ with parasites at 37°C, the mixture was centrifuged at 200 G for 10 min and separated into the surpernatant and precipitate fractions. The precipitate was sonicated at 100 kc for 5 sec. β-glucuronidase in the supernatant of the sonicated sample was measured.

Colchicine treatment: Colchcine (Sigma, Ltd.) was adjusted at 10^{-1} M in KRP buffer. Ten 1 of adjusted colchicine was added to 1 ml of the parasite and Mϕ solution, and each sample was pre-incubated at 37°C for 5 min. After preincubation, the mixture of the parasite and Mϕ solution was incubated for 3 different intervals (15, 30, and 60 min) at 37°C.

Electron microscope observation: The precipitate was fixed with glutaraldehyde solution (1 %) for 30 min at 4°C, and postosmicated (1 %) for 40 min at 4°C. The sample was dehydrated in a graded alcohol series, replaced in acetone-Epon 812 (Polyscience, Inc., USA), and embedded in Epon-812. Thin section were cut by an OMU-4-type (Reichert, Austria) ultratome and observed under a JEM-100 S electron microscope (Jeol, Ltd. Japan).

Results

The ratio of Mϕ containing *Toxoplasma gondii*: Mϕ were incubated for three different intervals (15, 30, and 60 min) at 37°C with living parasites (N), antibody coated living parasites (NA), dead parasites (F), and antibody coated dead parasites (FA). After the inoculation, the number of Mϕ containing parasites and the number of parasites in Mϕ were counted by an electron microsope. As shown in Fig. 1, 15 min after the inoculation, parasites were observed in many cells; in the NA and FA groups, parasites were observed in 80 % (number of parasites, 290) and 90 % (318) of the Mϕ. In contrast, in the N group, parasites were observed in 68% (148) of the Mϕ at 15 min and 83 % (205) at 30 min. In the F group, parasites were found in only 20% (39) of the Mϕ at 15 min, 32 % (62) at 30 min, and 64 % (113) at 60 min. Since the F and FA groups used parasites dead with paraformaldehyde, all parasites in Mϕ resulted from phagocytosis by Mϕ. On the other hand, the parasites in N and NA groups were living and some of them might have invaded into Mϕ by themselves. To prevent active invasion of parasites into Mϕ, colchcine was used at the final concentration of 10^{-3}M. The results are as shown in Fig. 2. There was little change in the ratio of Mϕ containing parasites in either F or FA group in the presence of colchicine: 19 % (43) for the former and 90 % (325) for

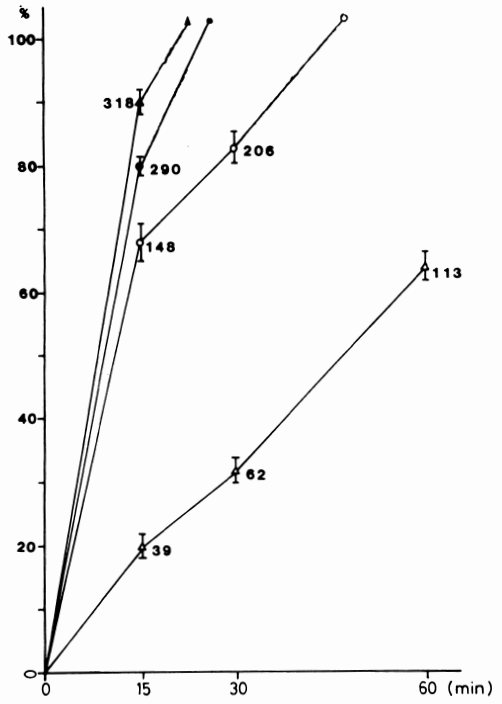

Fig. 1 Interaction between the mouse peritoneal macrophage and Toxoplasma (non-treated living parasites (○), antibody coated living parasites (●), dead parasites (△), and antibody coated dead parasites (▲)) in the absense of colchicine. The ordinate shows the ratio of macrophage containing parasites after inoculation and the abscissa incubation time. Figures beside the marks represent the numbers of parasites detected.

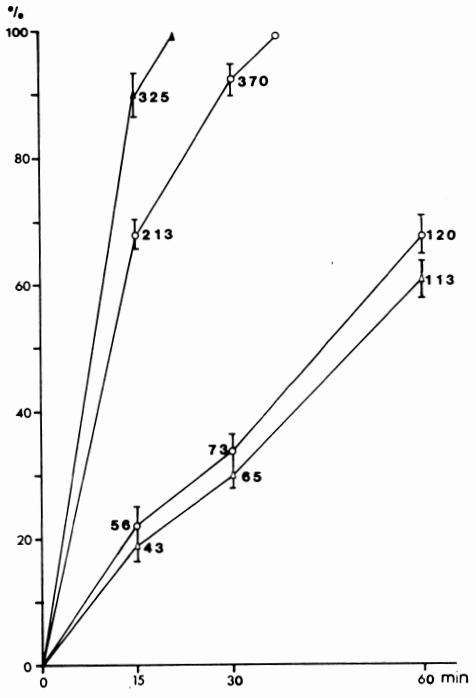

Fig. 2 Interaction between the mouse peritoneal macrophage and Toxoplasma (non-treated living parasites (○), antibody coated living parasites (●), dead parasites (△), and antibody coated dead parasites(▲)) in the presense of colchicine 10^{-3} M. The ordinate shows the ratio of macrophage containing parasites after inoculation and the abscissa incubation time. Figures beside the marks represent the numbers of parasites detected.

the latter at 15 min. The ratio, however, decreased markedly for both N and NA groups. This was especially true for the N group, which decreased from 68 % (148) to 22 % (56) at 15 min, almost as low as the ratio for the F group. In the NA group, the ratio decreased from 80 % (290) to 68 % (213) in the presence of colchicine.

Release of β-glucuronidase from phagocytic cells: For the measurement of digestion by the lysosomal enzyme, β-glucuronidase released from Mφ was measured by extinction change at 405 nm of *p*-nitrophenyl glucuronide (Fig. 3). The values indicate the released amount of β-glucuronidase per total amount of this enzyme in intact Mφ. The ratios of released β-glucuronidase from Mφ at 60 min incubation with parasites were 3.2 % in N, 5 % in F, 13 % in NA, and 16 % in the FA group. On the other hand, Mφ only was 2.1 %, and no β-glucuronidase was measured in parasites only.

Electron microscopic observation: In electron microscopy of native parasites (N) Fig. 4), the mitochondria and endoplasmic reticula of the host macrophage closely contact avacuole containing living parasites. Contrastingly, in electron microscopy of dead parasites (F) (Fig. 5), the mitochondria and endoplasmic reticulum of the host cell were not observed the vacuole containing dead parasites (F).

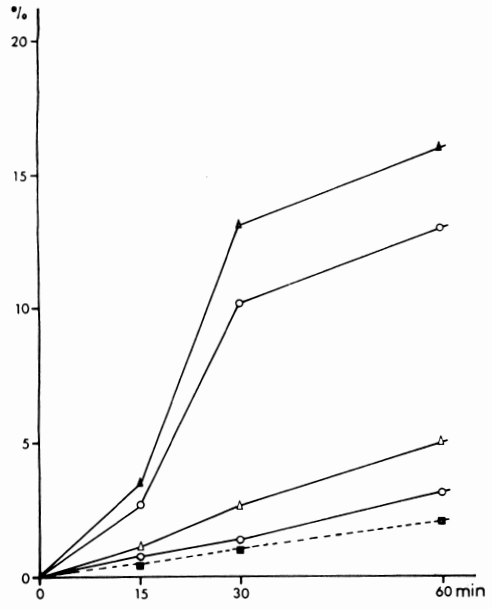

Fig. 3 Release of β-glucuronidase from phagocytic cells (macrophage) after incubation with non-treated living parasites (○), antibody coated living parasites (●), dead parasites (△), and antibody coated dead parasites (▲), and phagocytes only (■), the values indicate the released amount of β-glucuronidase per total amount of the enzyme within living macrophage.

Discussion

Toxoplasma gondii is infective for a great variety of phagocytic cells in various species and survives inside these cells. However, the course of the infection of this parasites and the responses of the phagocytic cells to this parasite are still not well understood.

We showed active invasion of this parasite and response of Mϕ against infection. None of fixed parasites were alive ; they were not capable of invading actively by themselves. Thus, dead (F) and antibody coated dead (FA) parasites in Mϕ were due to phagocytosis by Mϕ. As shown in Fig. 1, when parasites dead with paraformaldehyde were coated with antibody (FA), the ratio of phagocytes increased up to 90 % as compared with dead but not coated parasites (F). Release of β-glucuronidase also increased in parallel. These results suggest that normal mouse peritoneal Mϕ are capable of phagocytosis against F and FA groups and that antibody enhances the phagocytosis of the cells as already shown by many investigators. A correlation between the ratio of Mϕ containing (F and FA groups) and the ratio of release of the digestive enzyme for parasites (F and FA groups) in Mϕ was also demonstrated. In contrast, there was no correlation between the ratio of Mϕ containing parasites (living

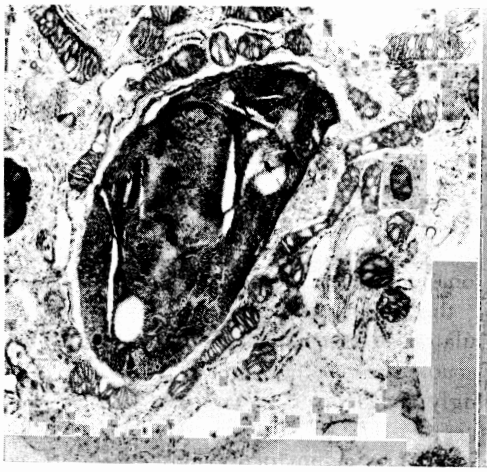

Fig. 4 Electron microscopy of non-treated living parasites. (20,000 X)

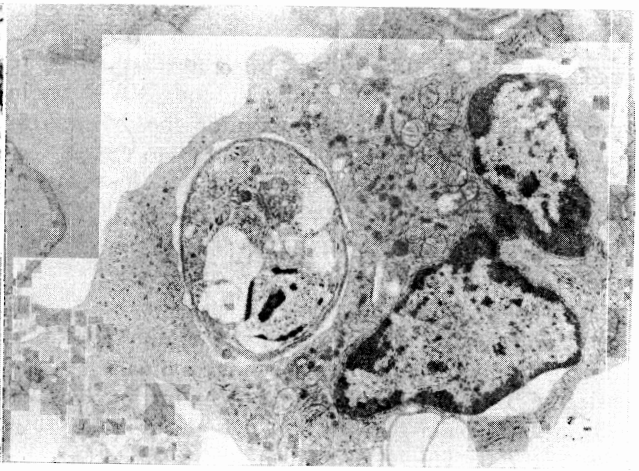

Fig. 5 Electron microscopy of dead parasites. (10,000 X)

parasites and antibody coated living parasites) and the ratio of release of the digestive enzyme from Mϕ. Antibody coated parasites (NA) increased to 80 % from 68 % of the ratio of Mϕ containing living parasites (N) but the difference between antibody coated living parasites and non-antibody coated living parasites is much smaller as compared with the difference between antibody coated dead parasites and non-antibody coated dead parasites (Figs. 1, 2). In the release of the digestive enzyme, a significant difference was observed between N and NA groups (Fig. 3). This fact suggests that the digestion of parasites hardly took place in the Mϕ incubated with living parasites. Living parasites may inhibit the fusion of lysosome to phagosome as is the case with tuberculosis bacilli (because acid lipid containing sulfatide alters the phagosome membrane, the fusing of the lysosome is inhibited) or may actively invade insuch a manner as not to induce the phagocytosis of Mϕ. Some authors (Anderson and Remington, 1974 ; Klainer *et al.*, 1973 ; Pulvertaft *et al.*, 1954 ; Thomas and Hirsch, 1972 ; Visher and Suter, 1954) have suggested that the parasites are phagocytized and that this is how they enter the host cells. Other researchers (Garnhum *et al.*, 1962 ; Ludvik, 1963 ; Lycke *et al.*, 1975 ; Norrby, 1970) have proposed active invasion as the made of entry of parasites. However, it is not certain whether infection of parasites is phagocytosis or active invasion.

Colchicine was used to analyse the mode of infection of parasites, because it can suppress the assembly of the microtubules and toxoplasma parasites have microtubules under the membrane. Malawista and Bodel (1967) reported that in the presence of 10^{-3}M colchicine, Mϕ can normally phagocyte bacteria, although digestive vacuoles are not formed within them (Malawista, 1971 ; Curnutle and Babior, 1975). When 10^{-3}M colchicine was used, dead parasites with or without antibody coating were phagocytized by Mϕ as well as in the absence of colchicine (Figs. 1, 2). Phagocytosis of Mϕ against NA group is not much affected by addition of colchicine. On the other hand, by addition of colchicine, the ratio of Mϕ containing living parasite (N) at 15 min markedly decreased to only 22 % from 68 % of the ratio in the absence of colchicine. It is a very interesting finding that invasion of parasites was inhibited by colchicine. Since colchicine did not inhibit the phagocytosis of Mϕ, we thought that many toxoplasma parasites are capable of invading actively into Mϕ and invading parasites can survive within phagocytic cells. Ryning and Remington (1978) reported that cytochalacin D did not prevent attachment but did prevent invasion of toxoplasma into Mϕ. Jenson and Edgar (1976) reported that motility of *Eimeria magna* was inhibites in the presence of cytochalacin. Cytochalacin induces a phagocytosis-like metabolic pattern in Mϕ and polymorphonuclear cells (Nakagawa *et al.*, 1974) but phagocytosis was inhibited. The motility of toxoplasma may be inhibited in the presence of cytochalacin as repored by Jensen and Edgar (1976). It is very interesting that invasion of parasites was also inhibited by addition of cytochalacin D as reported by Ryning and Remington (1978), because cytochalacin is an inhibition reagent of microfilaments (F-actin). Colchicine did not prevent either attachment of parasite to Mϕ (data not shown) or phagocytosis of parasite by Mϕ. Parasitic infection is considered to follow two different cources ; one is attachment and the other is invasion as reported by Ryning and Remington (1978).

The results of our study that living parasites can actively invade in such a manner as not to induce the phagocytosis of Mϕ and the phagocytic cells hardly respond to their invasion by releasing digestive enzymes. Therefore, after invasion, parasites seem to reside and multiply inside Mϕ without any action of digestive enzyme such as β-glucuronidase of Mϕ except that mitochondria and endoplasmic reticula of host cell close by contact vacuoles containing surviving parasites as reported by Jones and Hirsh (1972), Jones *et al.* (1972), and Thomas *et al.* (1972).

Summary

When mouse peritoneal macrophages were incubated with antibody coated native parasites or antibody coated dead parasites, incorporation of the parasites into macrophages was enhanced significantly through phagocytic activity of macrophage. A parallel increase in release of digestive enzyme β-glucuronidase in macrophages was also observed. Phagocytosis of dead parasites was moderate and β-glucuronidase release by macrophages was also moderate. The incorporation of antibody coated parasites or dead parasites was not inhibited by 10^{-3}M colchicine. When living parsites were mixed with mouse peritoneal macrophages, incorporation of parasites was observed more effectively than the dead parasites. In this case, however, release of β-glucuronidase from infected macrophages was of a low grade and intake of parasites was strongly inhibited by 10^{-3}M colchicine. From these observations, it may be concluded that intake of dead or antibody coated parasites is due to phagocytic process of macrophages while intake of living parasites is due to active invasion of parasites into macrophage. After living parasites invade into the cells, they seem to reside in macrophages escaping the effect of digestive enzymes of macrophages.

References

1) Anderson, S. E. and Remington, J. S. (1974): Effect of normal and activated human macrophages on *Toxoplasma gondii*. J. Exp. Med., 139, 1154-1174.
2) Armstrong, J. A. and Hart, P. D. (1971): Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J. Exp. Med., 134, 713-740.
3) Cohn, Z. A. and Fedorko, M. E. (1969): The formation and fate of lysosomes. In Lysosomes in Biology and Pathology (Dingle, J. T. and Fell, M. E., eds). North. Holland Publishing Co., London. 1, 43.
4) Curnutle, J. T. and Babior, B. M. (1975): Effects of anaerobiosis and inhibitors on O_2^- production by human granulocytes. Blold., 45, 851-861.
5) Garhuam, P. C., Baker, J. R. and Bird, R. G. (1962): Fine structure of cystic form of *Toxoplasma gondii*. Brit. Med. J., 1, 83-84.
6) Hohorst, H. J. (1962): Methoden der enzymatischen Analyse., ed. by Berbmeyer, H. V. Verlag Chemine, Weinheim, 1st. ed., pp. 266.
7) Jensen, J. B. and Edger, S. A. (1976): Effect of anti-phagocytic agents on penetration of Eimeria Magna sporozoites into cultured cells. J. Parasitol., 62, 203-206.
8) Jones, T. C. and Hirsch, J. G. (1972): The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. Exp. Med., 136, 1173-1194.
9) Jones, T. C., Yeh, S. and Hirsch, J. G. (1972): The interaction between *Toxoplasma gondii* and mammalian cells. I. Mechanism of entry and intercellular fate of the parasites. J. Exp. Med., 136, 1157-1172.
10) Klainer, A. S., Krahen Buhl, J. L. and Remington, J. S. (1973): Scanning electron microscopy of *Toxoplasma gondii*. J. Gene. Micro., 75, 111-118.
11) Ludvik, J. (1963): Electron microscopy of some parasites protozoa, In Proc. Ist. Internat. Congre. Protozool., ed. by Ludvik, J., Blom, J. and Vawra, I. Publishing House of the Czecho slovak Academy of Science, Preque. pp. 387-399.
12) Lycke, E., Carlberg, K. and Norrby, R. (1975): Interaction between *Toxoplasma gondii* and its host cells: Function of the penetration-enhancing factor of Toxoplasma. Infect. Immun., 11, 853-861.
13) Malawista, S. E. and Bodel, P. D. (1967): The dissociation by colchicine of phagocytosis from increased oxygen consumption in human leukocytes. J. Clin. Invest., 46, 786-796.
14) Malawista, S. E. (1974): Vinblastine: Colchicine-like effects on human blood leukocytes during phagocytosis. Blood., 37, 519-529.
15) Murray, H. M. and Cohn, Z. A. (1979): Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. J. Exp. Med., 150, 938-949.
16) Nakagawa, K., Takeshi, G.E. and Minakami, S. (1974): Induction of a phagocytosis-like

metabolic pattern in polymorphonuclear leucocytes by cytochalasin E. Exp. Cell Res., 87, 392-394.
17) Norrby, R. (1970): Host cell penetration of *Toxoplasma gondii*. Infect. Immun., 2, 250-255.
18) Nogueia, N. and Cohn, Z. A. (1978): Trypanasoma cruzi: In vitro induction of macrophage microbicidal activity. J. Exp. Med., 148, 288-300.
19) Pulvertaft, R. J. V., Valentine, J. C. and Lane, W. F. (1954): The behaviour of *Toxoplasma gondii* on serum-agar-culture. Parasitol., 44, 478-484.
20) Rausch, P. G. and Moore, T. G. (1975): Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. Blood, 46, 913-919.
21) Robert, R. F (1972): Interaction of L cells and Chlamydia psittaci: Entry of the parasites and host responses to its development. J. Bact., 110, 706-721.
22) Ryning, F. W. and Remington, J. S. (1978): Effect of cytochalasin D on *Toxoplasma gondii* cell entry. Infect. Immun., 20, 739-743.
23) Tanabe, K., Kimata, I., Tabuse, Y., Furusawa, M. and Tanaka, S. (1978): *Toxoplasma gondii*: Penetration into differentiating Friend erythroleukemia cells. Exp. Parasitol., 46, 72-82.
24) Thomas, C. J. Shirley, Y. and James, G. H. (1972): The interaction between *Toxoplasma gondii* and mammalian cells. J. Exp. Med., 136, 1157-1172.
25) Visher, W. A. and Suter, E. (1954): Intracellular multiplication of *Toxoplasma gondii* in adult mammalian macrophages cultivated *in vitro*. Pro. S. Exp. Bio. Med., 86, 413.

Toxoplasma とマウスマクロファージとの相互関係：Toxoplasma の進入とマクロファージの応答

佐藤功栄　伊勢やよい　飯田　孝　鈴木貴和　嶋田孝吉
((財)東京都臨床医学総合研究所免疫室)

マウス腹腔マクロファージを抗体感作した生虫体あるいは死虫体と反応させた場合，これらの虫体のマクロファージへのとり込みは著明に増加し，この食作用と平行してマクロファージのリソゾーム酸素（β-グルクロニダーゼ）活性も上昇した．なお，これらのマクロファージの食作用は，リソゾームと食胞の融合を阻止するコルヒチンの存在下でも変化はなかった．

一方，抗体を感作しない生虫体とマクロファージを反応させた場合，マクロファージ内に多数の虫体が観察されたが，β-グルクロニダーゼの遊離は低く，コルヒチンの存在下ではマクロファージ内の虫体数は減少した．

以上のことから，抗体感作虫体，死虫体の場合はマクロファージによる食作用が行なわれ，一方，生虫体は自らマクロファージへ侵入し，マクロファージの消化酵素の作用をのがれるものと考えられた．