[Jpn. J. Parasitol., Vol. 40, No. 1, 92–98, February 1991]

An Ultrastructural Study on Human Neutrophil Activity Against *Toxoplasma gondii*

MASAHARU NAKAO AND TAKEO MATSUMURA

(Accepted for publication; March 1, 1991)

Abstract

Human neutrophils were infected with tachyzoites of *Toxoplasma gondii in vitro* and were examined by an electron microscope (TEM) for changes in both host cells and parasites following infection. Two kinds of tachyzoites were observed with regard to their fates in phagocytic vacuoles (parasitophorous vacuoles) at 1 h after challenge; some were found to be degenerated showing vacuolization, and the others were intact. Phagocytic vacuoles containing the degenerated tachyzoite(s) at 6 h after infection, enlarged more than those at 1 h, while degenerated organisms became extremely diminished. The intact organisms grew and enlarged fully in the phagocytic vacuoles. The diminished bodies observed at 6 h were no more found in phagocytic vacuoles at 18 h, but 2–5 living organisms were nevertheless observed in one vacuole, suggesting that endodyogenies of tachyzoites occurred. These results indicated that human neutrophils have anti-*Toxoplasma* capacity in some degree in the absence of anti-*Toxoplasma* antibody, however, they would permit proliferation of *Toxoplasma* and would form foci of *Toxoplasma* infection if some conditions were prepared.

Key words: *Toxoplasma gondii,* Human neutrophil, Phagocytosis, Ultrastructure

Introduction

Various leukocytes are associated with immunity against *Toxoplasma gondii* infection in humans. Many detailed studies have been reported on cell-mediated immune responses, in which macrophages and lymphocytes act as effector cells. While this response is a slow defense mechanism, humans have other rapid systems. Phagocytic cells, such as polymorphonuclear leukocytes (PMNLs), monocytes, and macrophages act instantly after invasion of microorganisms. Neutrophils act most immediately and play major roles in initial defense mechanism.

Skillful mechanisms for avoiding phagocytosing and killing activity of macrophages have been reported in some bacteria, such as *Mycobacterium tuberculosis* (Flesch and Kaufman, 1987), *Legionella pneumonia* (Payne and Horwitz, 1987), which can be phagocytosed easily and proliferate in them. Similar mechanisms were also shown in *Toxoplasma* in some previous reports (Jones and Hirsch, 1972; Anderson and Remington, 1974).

Our infection experiments of neutrophils with tachyzoites *in vitro* indicated that tachyzoites proliferated in some neutrophils in the absence of antibody, suggesting that neutrophils seem to be responsible for dissemination of tachyzoites in the host during the initial phase of *Toxoplasma* infection until antibody production (Nakao and Konishi, 1991, in press).

The objective of our study was to clarify this proliferation of *Toxoplasma* in neutrophils using electron microscope and to discuss this phenomenon.

Materials and Methods

Toxoplasma. The virulent RH strain of *Toxoplasma* was harvested from the peritoneal cavities of 20–25g ICR mice on day 3 of infection. The exudates were filtered through a

Department of Medical Zoology, Kobe University School of Medicine, Kobe 650, Japan
中尾雅春　松村武男（神戸大学医学部医動物学教室）

polycarbonate membrane with a 3-μm pore size (Nuclepore Corp., Pleasanton, Calif.) and centrifuged at $300 \times g$ for 5 min to collect tachyzoites. The parasites were then washed twice with phosphate-buffered saline (PBS) and suspended Dulbecco's modified Eagles' minimum essential medium containing 10% heat-inactivated fetal calf serum (DMEM) at an appropriate concentration.

Preparation of human PMNLs. Blood was drawn from a healthy human volunteer in a sterile syringe containing 10 units of sodium heparin per ml of blood. The donor was negative for antibodies to *Toxoplasma* as determined by enzyme-linked immunosorbent assay (Konishi and Takahashi, 1983); the ELISA value of the serum was 0.058 for IgG antibody and 0.137 for IgM antibody. PMNLs were prepared from the blood by sedimentation of erythrocytes with 6% dextran followed by centrifugation at $400 \times g$ for 30 min of the leukocyte fraction over Ficoll-hypaque gradients (Mono-Poly Resolving Medium: Flow Laboratory, North Ryde, Australia). Residual erythrocytes were removed by lysis treatment with 0.2% NaCl for 30 second and adjusted to isotonic solution by equal volume of 1.6% NaCl. PMNLs were washed twice with PBS, and were suspended in DMEM at an appropriate concentration.

Infection of PMNLs with Toxoplasma. Infection experiment was performed using 3 tissue culture dishes (60×15 mm, Becton Dickinson & Co., Lincoln Park, N. J.). After 9.2×10^6 PMNLs in 8 ml of DMEM and 3.6×10^7 tachyzoite in 3 ml of DMEM were mixed and suspended in each dish; The PMNL and tachyzoite ratio was 1:4, they were incubated at 37°C in 5% CO_2 atmosphere. At 1-h incubation, one dish was fixed for electron microscopic observation, and the rest dishes were washed twice with fresh DMEM to remove extracellular tachyzoites. Then, the second dish was fixed at 6 h, and third one was fixed at 18 h after infection. As controls, dishes with PMNLs solution alone were served.

Fixation and electron microscopic observation. Materials were fixed only with 1% osmium tetraoxide (O_sO_4) in PBS (pH 7.4). One ml of 1% O_sO_4 was poured into each dish and cells were suspended and fixed for 3 min. After centrifugation at $700 \times g$ for 5 min, cells were re-fixed for 1 h with the same fixative. The fixed materials were dehydrated in graded series of ethanol and transferred to propylene oxide and embedded in Epon 812 (Oken Syoji Co. Ltd., Tokyo). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by Hitachi electron microscope (Hitachi H-300AT).

Results

Electron microscopic observation at 1 h after challenge with Toxoplasma.

Phagocytosing neutrophils with pseudopods and neutrophils which had already finished to phagocytose 1–2 tachyzoites were observed. Some organisms observed inside of phagocytic vacuoles began to degenerate and vacuolization could be found slightly in the bodies of parasites (Fig. 1). On the contrary, an intact organism could be found in such phagocytic vacuole, which size was almost as large as the size of a vacuole.

Electron microscopic observation at 6 h after challenge. Two kinds of phagocytic vacuoles, which involve a degenerated organism, or an intact organism, were observed in an infected neutrophil at 6 h. A few neutrophils involved both two kinds of vacuoles in one neutrophil.

Phagocytic vacuole at 6 h containing a degenerated organism, enlarged more than that at 1 h and became giantic. In addition, multiple vacuoles were observed in a body of organism itself, which became very small to be a diminished body with relative high density (Fig. 2). Vacuolization was also seen in the cytoplasm around a phagocytic vacuole (Fig. 2, Fig. 3).

Intact organisms grew and enlarged fully in each phagocytic vacuole, however, endodyogenies were not seen (Fig. 4).

Electron microscopic observation at 18 h after challenge. The diminished bodies observed at 6 h could not be found in phagocytic vacuoles at 18 h. All organisms in this stage had low electron density compared with the diminished bodies. No vacuolization was seen in the bodies of organisms. Two to five organisms were found in

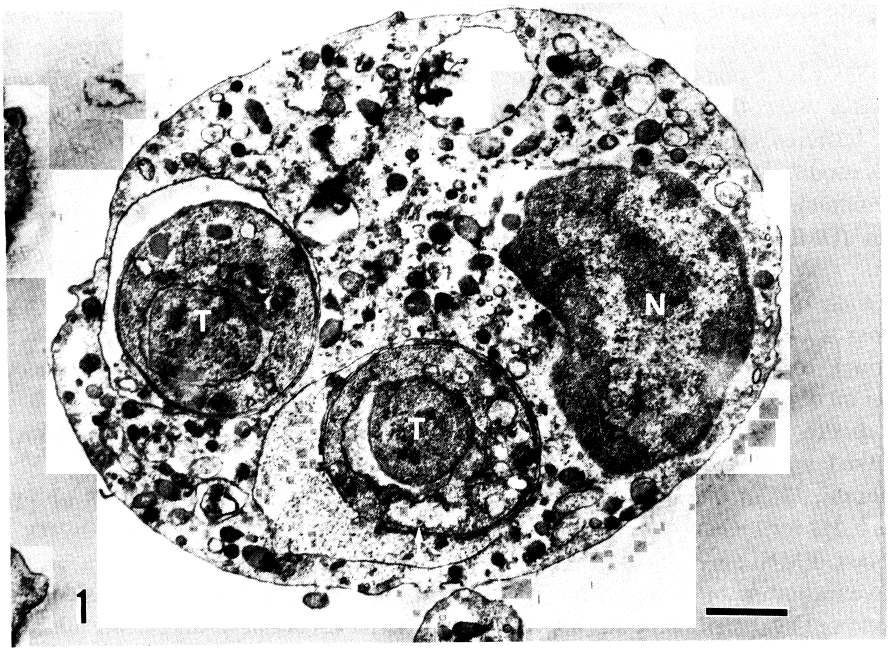

Fig. 1 Electron micrograph at 1 h after infection. Cross section of a neutrophil. Slight vacuolization (arrow) shows the beginning of degeneration. T: tachyzoite, N: nucleus of a neutrophil. Bar, 1 μm.

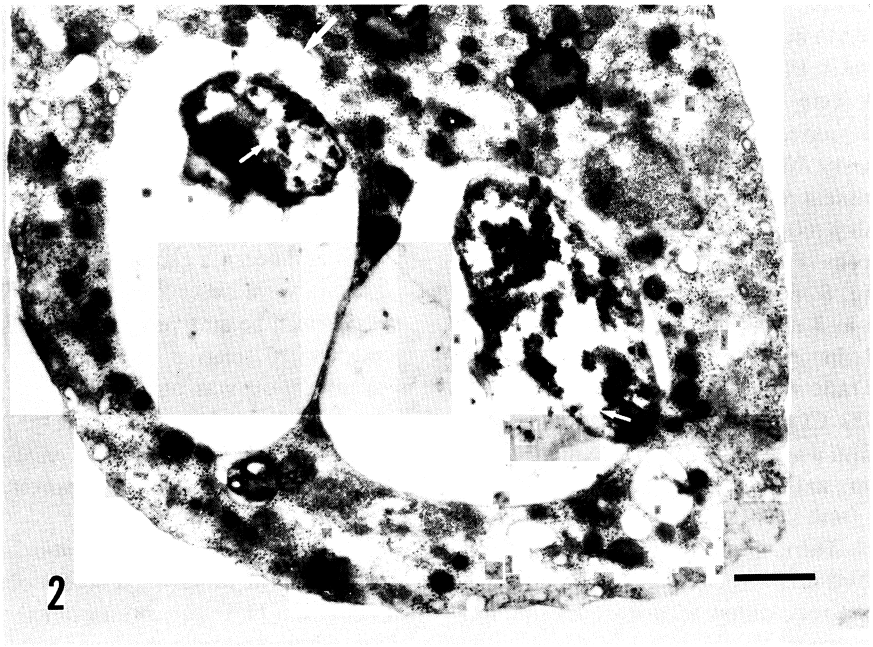

Fig. 2 Phagocytic vacuoles in a neutrophil at 6 h after infection. Multiple vacuoles (small arrows) are observed in each body of parasite. In addition, phagocytic vacuoles are enlarged and giantic. Vacuolization (large arrow) is also seen around vacuoles. Bar, 1 μm.

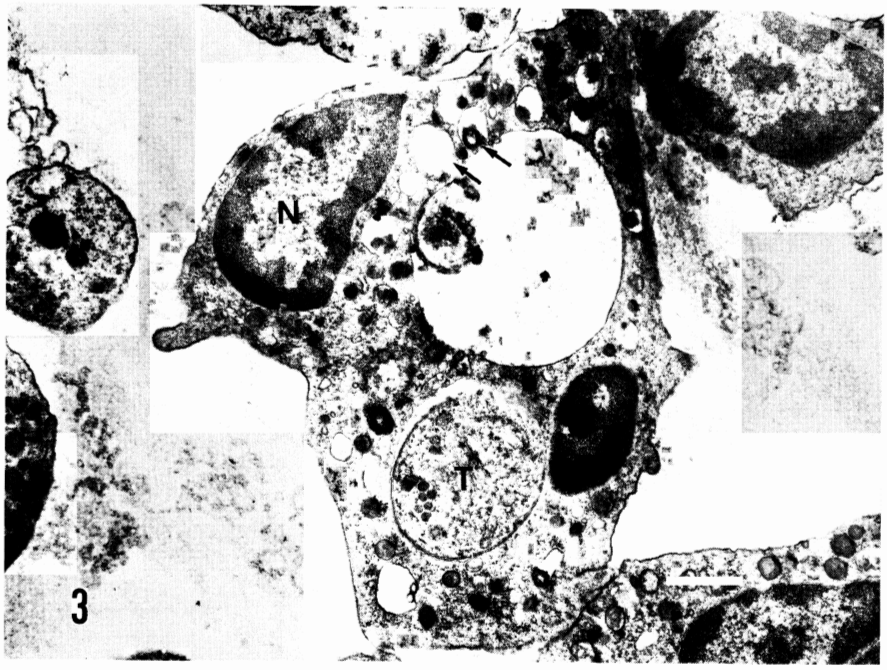

Fig. 3 Electron micrograph at 6 h after infection. Vacuolization (arrows) is also seen in cytoplasm near a phagocytic vacuole, which contains a diminished body. T: an intact tachyzoite, N: nucleus of a neutrophil. Bar, 1 μm.

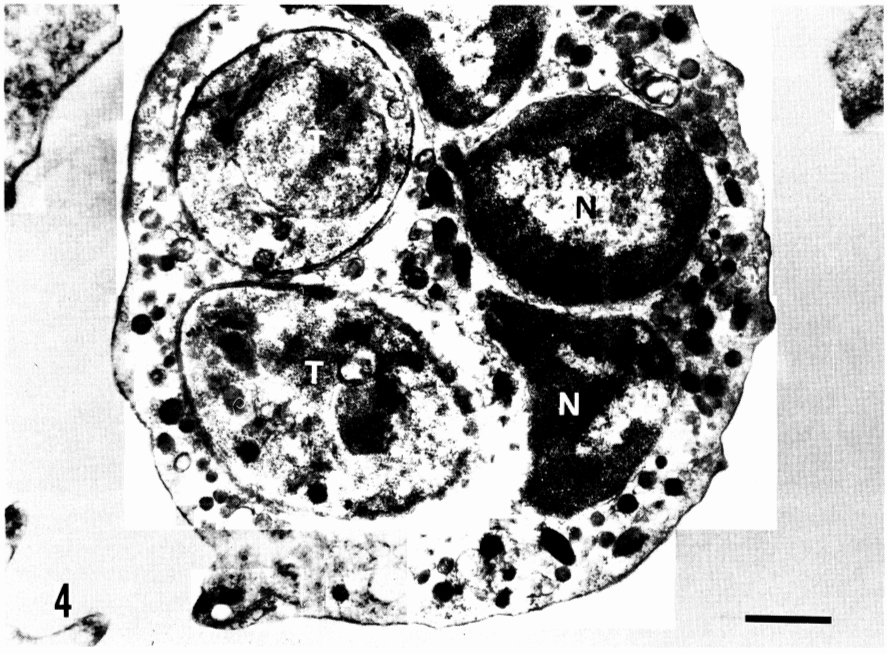

Fig. 4 Cross section of a neutrophil containing intact organisms at 6 h after infection. Each tachyzoite expands fully in each phagocytic vacuole. T: tachyzoite, N: nucleus of a neutrophil. Bar, 1 μm.

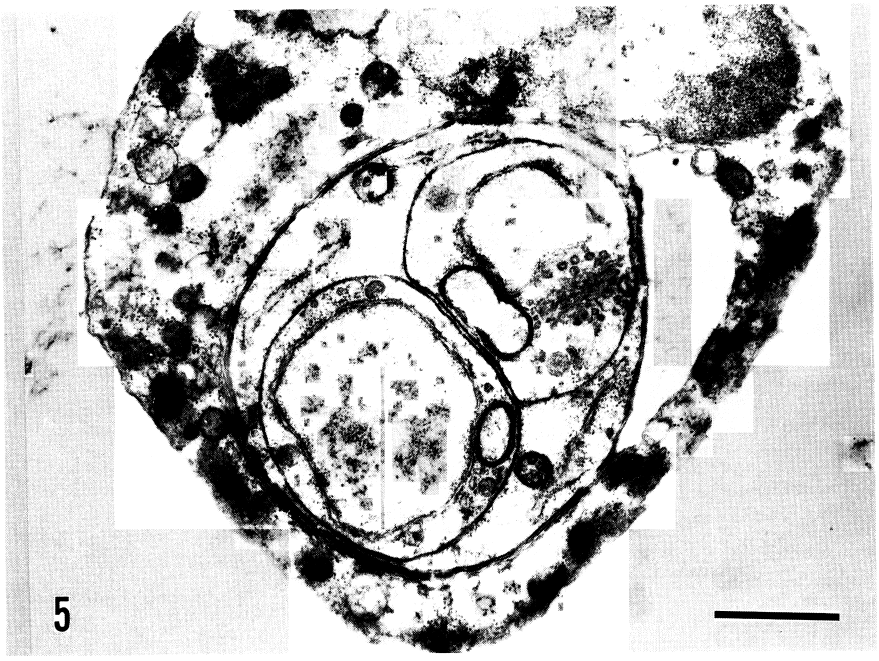

Fig. 5 Early stage of endodyogeny observed in one phagocytic vacuole at 18 h. Immature daughter tachyzoites appear inside the mother tachyzoite. Bar, 1 μm.

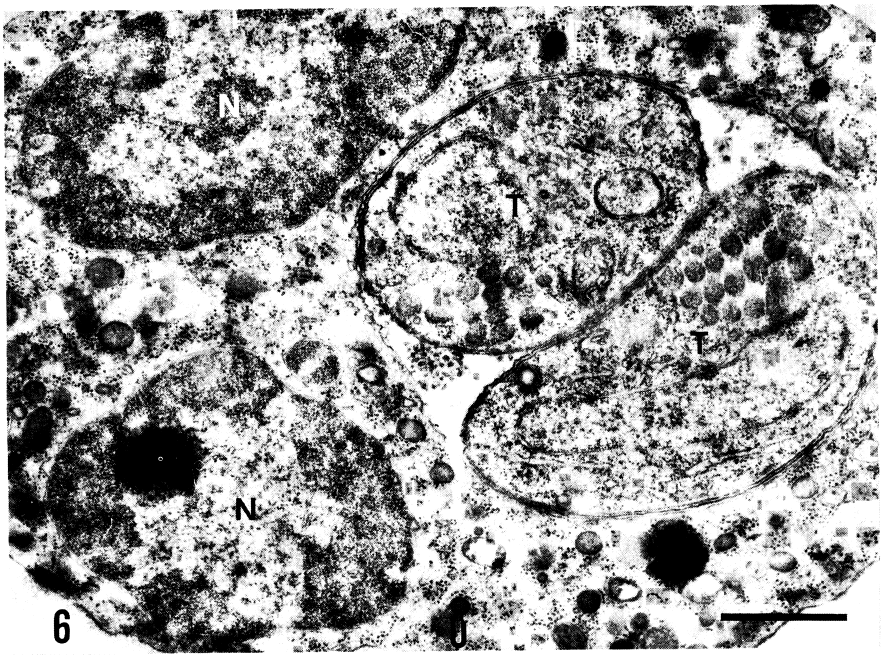

Fig. 6 Last stage of endodyogeny observed at 18 h. Two mature tachyzoites in one phagocytic vacuole. T: tachyzoite, N: nucleus of a neutrophil, Bar, 1 μm.

one vacuole (Fig. 5). Although endodyogenies of tachyzoites were observed, their mitochondria aggregation was not seen (Fig. 5, Fig. 6).

Discussion

The interaction between *Toxoplasma* and macrophages in mammalian cells was studied in detail and the proliferation mechanisms of this parasite in them are well known (Jones and Hirsch, 1972). As for human leukocytes, the roles of human macrophages against this parasite have been investigated. Human alveolar macrophages and peripheral blood monocytes are resistant to *Toxoplasma,* whereas monocyte-derived macrophages are susceptible to this parasite (Murray et al., 1985).

Human neutrophils act instantly in bacterial infection as phagocytic cells as well as human macrophages. Nevertheless, only a few studies have been reported about human neutrophils in protozoan infections. Holland & Sleamaker (1970) and Wilson & Remington (1979) reported that neutrophils phagocytosed *Toxoplasma* easily and destroyed immediately in their experiments *in vitro*. However, our previous experiment revealed that tachyzoites proliferated in 25.0–35.0% of neutrophils that phagocytosed tachyzoites within 1 h of incubation in the absence of antibody (anti-*Toxoplasma* IgG), whereas they did not proliferate in human monocytes in the absence of antibody (Nakao and Konishi, 1991, in press). This is the first report describing the proliferation of *Toxoplasma gondii* in human neutrophils.

From our light microscopic observation, tachyzoites in neutrophils by Giemsa stain retained their color at an initial phase after infection, but they turned to be reddish in the presence of antibody after 2 h of incubation. This phenomenon was considered that oxygen-dependent killing mechanism against *Toxoplasma* was working in human neutrophils that phagocytosed tachyzoites. Especially, 99.5% of tachyzoites in neutrophils that phagocytosed tachyzoites turned to be reddish and all tachyzoites disappeared at 18 h in the presence of antibody. This phenomenon corresponded with massive superoxide production within 1 h after infection in the presence of antibody (Nakao and Konishi, 1991, in press). On the contrary, the color of tachyzoites in some of neutrophils that phagocytosed tachyzoites retained their color in the absence of antibody.

This change of the color of tachyzoites was considered to correspond to that of electron density of tachyzoite bodies discussed in this study, indicating that blue tachyzoites immediately observed after challenge corresponded with low dense ones, and that red tachyzoites at 6 h corresponded with high dense ones. Although detailed structures of a phagocytic vacuole and a tachyzoite in an infected neutrophil could not be observed by light microscope, electron microscope enabled to investigate their morphologic changes and their positional relations. As we described in the results, two kinds of tachyzoites (intact organisms and degenerated organisms) were observed in neutrophils in the course of infection. The former tachyzoites enlarged fully in phagocytic vacuoles at 6 h and finally divided into two daughter tachyzoites at 18 h of incubation. These divisions of tachyzoites were recognized as endodyogenies. Immature daughter tachyzoites appeared inside of the mother tachyzoite in the early stage of endodyogeny (Fig. 5) and finally divided into two mature tachyzoites in one phagocytic vacuole (Fig. 6). On the other hand, the latter ones reduced in size and finally became diminished bodies in phagocytic vacuoles at 6 h. In this stage, numerous vacuoles could be observed within tachyzoites. These indicates destructions of tachyzoites. When we paid more attention, many small vacuoles also could be found around the phagocytic vacuoles. These suggested to be scars of garnules, indicating a possibility that some enzymes might have been released from lysosomes in the neutrophils. Chinchilla et al. (1986) described that vacuolization was observed at 1 h as initial destructions of parasite bodies in rat peritoneal macrophages which were resistant to *Toxoplasma* with electron microscope. Their observations were in concord with ours. Jones and Hirsch (1972) clarified that 50% of tachyzoites were proliferated in macrophages and mitochondria aggregation was

observed around phagocytic vacuoles containing proliferating tachyzoites. These observations suggested that tachyzoites needed high-energy intermediate such as ATP produced in mitochondria in the host cell for their rapid proliferation. As for human neutrophils, these mitochondria aggregation was not observed, so that they might not be as profitable as mouse peritoneal macrophages for proliferation of *Toxoplasma*.

However, as neutrophils permitted tachyzoite proliferation in some degrees *in vitro*, they might be foci of these organisms *in vivo* if some conditions such as hypofunction of host neutrophils were prepared.

Acknowledgements

The authors wish to thank Mr. Yoshio Kawamoto for his technical assistance of the electron microscope throughout this study and Mr. Eiji Konishi for his helpful suggestions.

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) Chinchilla, M., Portilla, E. and Guerrero, O. M. (1986): Rat macrophage activity against *Toxoplasma gondii* studied by electron microscopy. Rev. Biol. Trop., 34, 83–88.
3) Flesch, I. and Kaufman, S. H. E. (1987): Mycobacterial growth inhibition by interferon-γ activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. J. Immunol., 138, 4408–4413.
4) Holland, P. and Sleamaker, K. (1970): Motile phagocytic defence against protozoa and fungi. J. Reticuloendothel. Soc. 7, 635.
5) Jones, T. C. and Hirsch, J. (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.
6) Konishi, E. and Takahashi, J. (1983): Reproducible enzyme-linked immunosorbent assay with a magnetic processing system for diagnosis of Toxoplasmosis. J. Clin. Microbiol., 17, 225–231.
7) Murray, H. W., Rubin, B. Y., Carriero, S. M., Harris, A. M. and Jaffee, E. A. (1985): Human mononuclear phagocyte antiprotozoal mechanisms: oxygen-dependent vs oxygen-independent activity against intracellular *Toxoplasma gondii*. J. Immunol., 134, 1982–1988.
8) Nakao, M. and Konishi, E. (1991): Proliferation of *Toxoplasma gondii* in human neutrophils *in vitro*. Parasitol. (in press)
9) Payne, N. R. and Horwitz, M. A. (1987): Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J. Exp. Med., 166, 1377–1389.
10) Wilson, C. B. and Remington, J. S. (1979): Activity of human blood leukocytes against *Toxoplasma gondii*. J. Infect. Dis., 140, 890–895.