[Jpn. J. Parasitol., Vol. 42, No. 1, 31–39, February, 1993]

Complement-dependent Encapsulation of the Third Stage Larvae of *Brugia pahangi* by Peritoneal Cells of DDY Mice

MUTSUO KOBAYASHI, KEIKO YAMADA AND HISASHI YAMAMOTO

(Accepted for publication; January 14, 1993)

Abstract

Male mice (DDY strain) inoculated with infective larvae of *Brugia pahangi* were dissected on 2 days post-infection and the cellular responses of mice against the larvae were investigated. A half of the larvae recovered from peritoneal cavity were encapsulated by peritoneal exudate cells (PEC) and one fourth of the larvae were completely encapsulated by many PEC. Electron microscopic observation of completely encapsulated larvae showed that macrophages and eosinophils were the major adhering cells to the cuticle. In some sections the pseudopodia of macrophages had invaded into the epicuticle of the larvae. The epicuticle where eosinophils attached also showed disintegration. The mid-layer of the capsule were the mixture of three types of cells including neutrophils. After incubation with normal mouse serum the larvae showed a positive reaction to FITC-conjugated anti-mouse C_3 antibody. When the larvae treated with normal or heat-inactivated serum were incubated with the peritoneal cells of naive mice *in vitro*, the former showed stronger encapsulation than the latter. The larvae recovered from peritoneal cavity of mice without any cell adhesion also showed a positive reaction to anti-mouse C_3 antibodies. From these data, the third stage larvae of *Brugia pahangi* were encapsulated by PEC through activation of complement alternative pathway *in vivo*. This strong cellular responses to filarial larvae seems to be related to low susceptibility of mice against filarial infection. The reason why all C_3-positive larvae were not encapsulated in the peritoneal cavity of mice is also discussed in terms of low responsiveness of macrophages.

Key words: *Brugia pahangi,* ddy mice, peritoneal macrophages, eosinophils, encapsulation, complement

Introduction

Since Ash and Riley (1970) reported the susceptibility of jirds to *Brugia pahangi* it become well known that susceptibility to filarial worms is variable among small laboratory animals. The underlying mechanisms controlling susceptibility of the host to filarial infection remains unknown. *In vitro* interrelation between filarial worms and effector cells of mammalian hosts have been reported repeatedly (Higashi and Chowdhury 1970, Ouassi *et al.* 1981, Haque *et al.* 1982, Brattig *et al.* 1991). In these *in vitro* works macrophages or eosinophils adhere to microfilariae (Mf), infective larvae, and adult worms under the presence of Ig E antibodies or another class of antibodies. Yates *et al.* (1985) showed human complement could mediate eosinophil adherence to the infective larvae of *Loa loa* and *B. malayi.* They also showed activation of complement alternative pathway in normal human serum by the infective larvae of *L. loa* and *B. malayi* and detected C_3 conversion products on larval cuticles by eosinophil adherence and by immunofluorescence with C_3c antiserum. Recently, Nakanishi *et al.* (1989) showed that macrophage blockade by injecting of carbon particles into peritoneal cavity of mice abolished sex differences in susceptibility of adult mice (BALB/c) to *B. pahangi.* These *in vitro* and *in vivo* experiments suggested that antibody and/or complement are involved in cellular defence responses of mammalian hosts to filarial larvae. In the present study we examined whether

Department of Medical Zoology, Dokkyo University School of Medicine, Mibu, Tochigi, 321-02 Japan.
小林睦生　山田圭子　山本　久（獨協医科大学医動物学教室）

primary defence responses of mice against third stage larvae of *B. pahangi* are also mediated by macrophages and/or eosinophils through the activation of alternative pathway of complement.

Materials and Methods

Animals and parasite

Mice used in this study were DDY strain raised in our laboratory under the conventional conditions. Infective larvae of *B. pahangi* were obtained from the mosquitoes, *Aedes aegypti* (Liverpool strain), that had been fed on infected jirds (*Meriones unguiculatus*) 11 days previously. Male mice aged 6–8 weeks were inoculated intraperitoneally with 100 infective larvae suspended in 0.6 ml of Hanks' balanced salt solution (HBSS). They were killed at 3, 24 and 48 hrs post-infection by an overdose of ether anesthesia. Worms were recovered from the peritoneal cavity by full washings with HBSS supplemented with 10 unit/ml of heparin and then all larvae were observed microscopically to grade the adhesion of peritoneal exudate cells (PEC).

Electron microscopy

Strongly encapsulated larvae were fixed in 0.3% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.3 for 3 hrs. After washing with several changes of 0.2 M cacodylate buffer, they were post-fixed in 1% OsO_4 in phosphate buffer (pH 7.0) for 1.5 hr, dehydrated, and then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL-100 B electron microscope at 80 KV.

Immunofluorescence assay

Normal serum was obtained from mice by venepuncture of external jugular vein and after incubation for 2 hrs at room temperature serum was collected by centrifugation. The serum was stored in ice until used. An aliquot of normal sera were inactivated at 56°C for 1 hr. Infective larvae of *B. pahangi* collected from the mosquitoes were incubated with either sera at 37°C for 2 hrs and washed three times with PBS and then incubated with 50 μl of a 1:5 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse C_3 (Cappel Lab. Inc.) at 37°C for 30 min. The larvae were then washed three times with PBS, mounted in glycerol solution (50% in PBS) and examined by a fluorescence microscope (Nikon Microphot-FXA). The third stage larvae collected from mosquitoes were also directly incubated with 50 μl of a 1:5 dilution of FITC-conjugated goat anti-mouse C_3 at 37°C for 30 min as a control.

In vitro encapsulation of serum-treated third stage larvae by peritoneal cells of mouse

The larvae treated with normal or heat-inactivated serum were incubated with peritoneal cells in the V-shaped microplate (Greiner). Briefly, 10 μl of HBSS containing 10 to 15 larvae was put into the wells of microplate. The peritoneal cells were harvested by peritoneal lavage with ice-cold HBSS and, after centrifugation, they were resuspended in HBSS to make 10^6 cell/ml. Fifty μl of cell suspension was gently poured into the well and incubated for 90 min at 37°C in 5% CO_2. After fixing with 10% formaldehyde in HBSS the larvae were observed microscopically to grade the cell adhesion.

Results

Encapsulation of filarial larvae in the peritoneal cavity of mice

Cellular responses against filarial larvae inoculated into the peritoneal cavity of mice 2 days post-infection are shown in Table 1. Recovery rate of the larvae from peritoneal cavity was 46.7 ± 4.2%. About fifty percent of the larvae showed no sign of cell adhesion, while other fifty percent of larvae were partially or completely encapsulated. Moreover, 53.9% of all encapsulated larvae were completely encapsulated by numerous cells (Fig. 1a). Some larvae showed a weak cellular adhesion (Fig. 1b). Completely or partially encapsulated larvae were also observed in the larvae recovered 3 and 24 hrs post-infection in the preliminary experiments (data not shown).

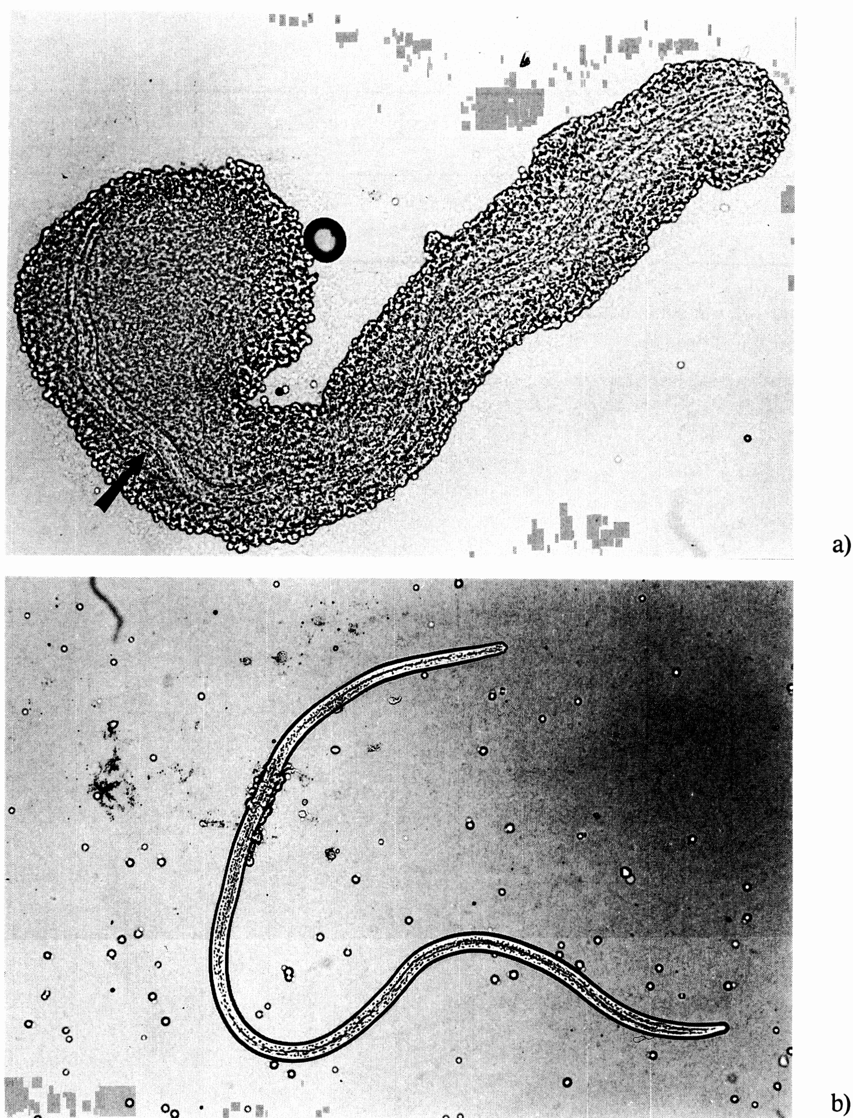

Fig. 1 Encapsulated third stage larvae of *Brugia pahangi* recovered from the peritoneal cavity of mice 2 days after inoculation.
a: Completely encapsulated larva (arrow shows the larva in the capsule)
b: Partially encapsulated larva

Electron microscopic observation of the cells adhered to filarial larvae

Macrophages attached tightly to the cuticle by many pseudopodia. In some specimens the pseudopodia invaded into the epicuticle (Fig. 2). Small number of eosinophils with typical eosinophilic granules were also observed on the cuticle (Fig. 3). The some areas of epicuticle adhered by eosinophils also seemed to be disintegrated. In the mid-layer of capsule, eosinophils which had only 1 or 2 typical granules in their cytoplasms were observed around larvae (data not shown). This means degranulation of

Table 1 Cellular reaction against filarial larvae of *Brugia pahangi* in the peritoneal cavity of mice 2 days post-infection

No. of mouse	Intact larvae	Encapsulated larvae		Average no. of larvae recovered
		Partially	Completely	
6	23.2 ± 6.6 (49.7%)	10.8 ± 5.0 (23.1%)	12.7 ± 6.0 (27.2%)	46.7 ± 4.2 (100%)

All mice were inoculated with 100 infective larvae

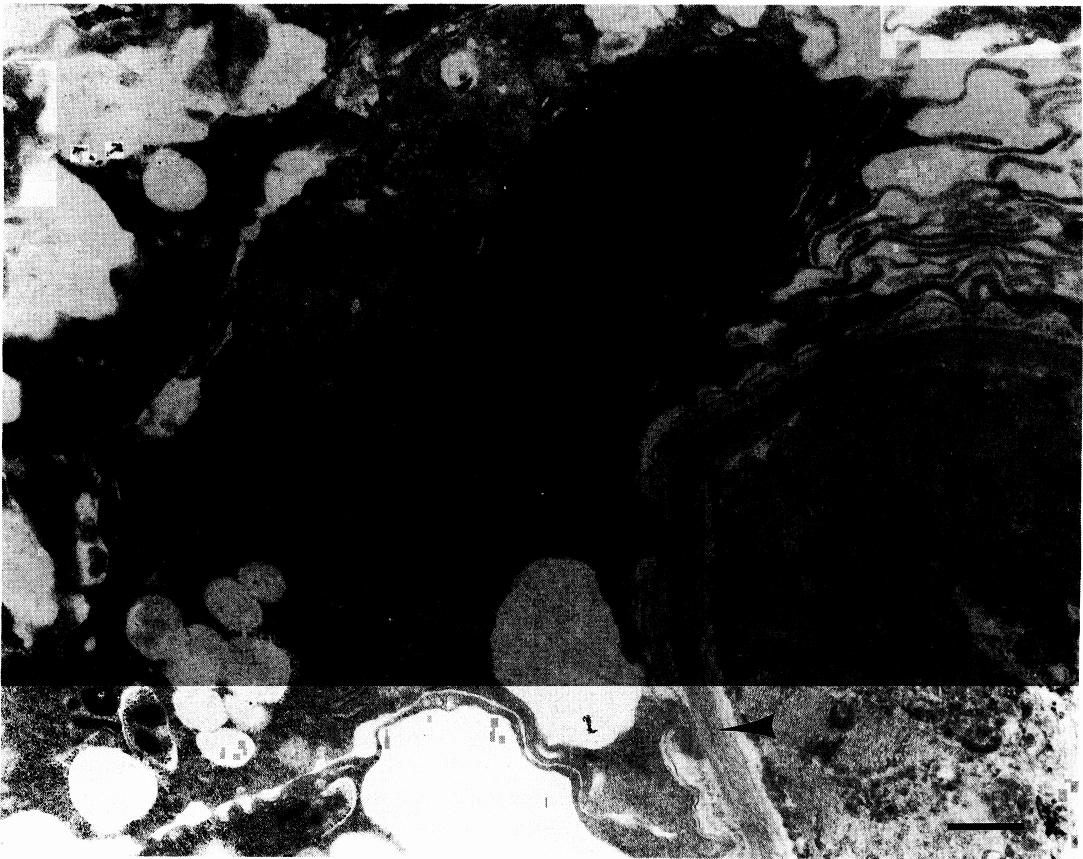

Fig. 2 Macrophage attached to the surface of the third stage larva of *Brugia pahangi* recovered from the peritoneal cavity of mice 2 days after inoculation. (arrowheads show disintegration of larval cuticle which contacts with pseudopodia of macrophage) Bar = 1 μm

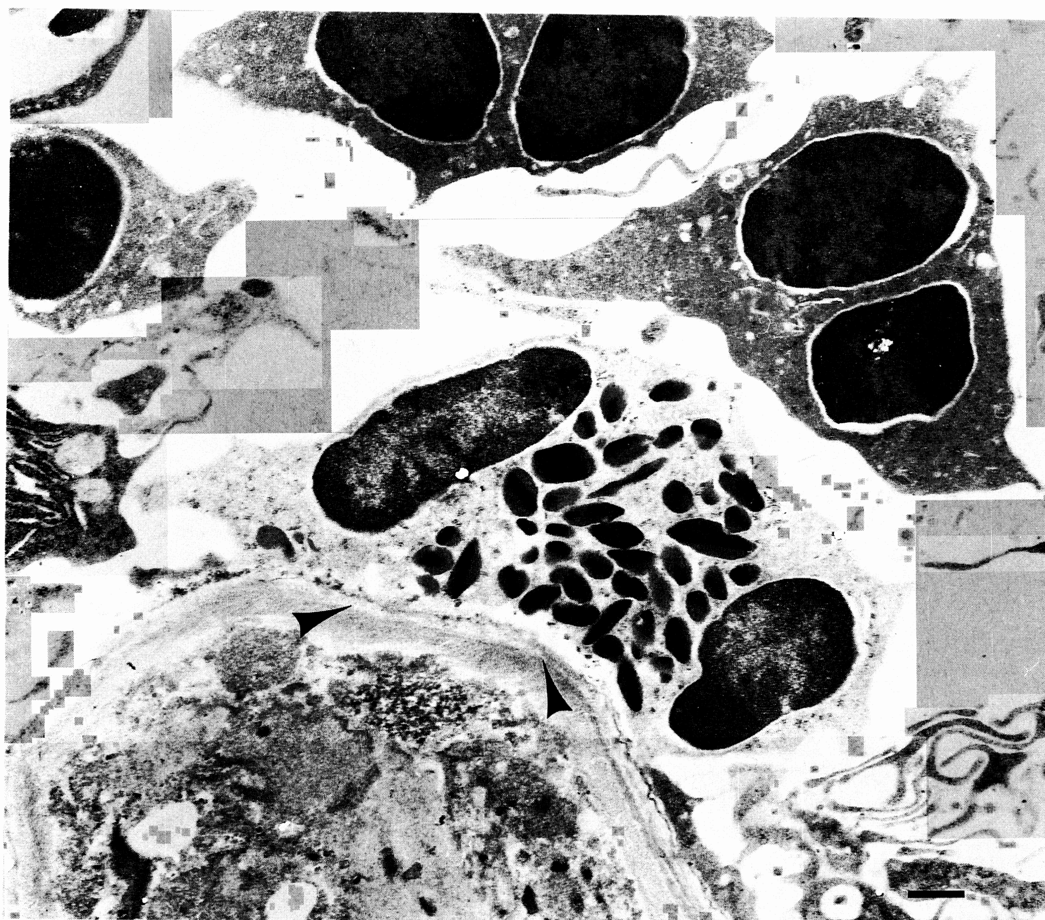

Fig. 3 Eosinophil attached to the surface of the third stage larva of *Brugia pahangi* recovered from the peritoneal cavity of mice 2 days after inoculation. (arrowheads show disintegration of larval cuticle which contacts with eosinophil) Bar = 1 μm

Table 2 Population of adhered cells to the cuticle of *Brugia pahangi* larvae recovered from peritoneal cavity of male mouse 2 days post-infection

Total number of cells counted	Macrophages	Eosinophils	Others
169* (100%)	120 (71.0%)	8 (4.7%)	41 (24.3%)

*Analysis of attached cells was carried out using 60 photographs from 4 strongly encapsulated larvae.

Fig. 4 Degranulated eosinophil attached to the larval cuticle of *Brugia pahangi* in the peritoneal cavity of mice 2 days after inoculation. (arrowheads show disintegration of larval cuticle which contacts with pseudopodia of the cell) Bar = 1 μm

eosinophils frequently occurs in the capsule. An exocytosed eosinophil attached to the cuticle and the line of epicuticle was clearly disintegrated by invasion of pseudopodia-like extension of the cell (Fig. 4). Population of adhered cells to the cuticle was analyzed (Table 2). Seventy one percent of total observed cells (169 cells) was macrophages, only 4.7 percent was eosinophils with specific granules and remaining cells were not fully identified at the present. But, the majority of these cells seems to be degranulated eosinophils morphologically.

In the mid-layer of capsule the cells were not tightly packed with each other so that the cells might be able to migrate towards inner side easily from the outer side of the capsule.

Detection of C_3 component on the surface of filarial larvae by FITC-conjugated anti-mouse C_3 antibody

Infective larvae incubated with normal mouse serum showed a positive reaction to FITC-conjugated anti-mouse C_3 antibody (100%, 23/23). On the other hand, the larvae incubated either with inactivated serum or with phosphate buffered saline did not show fluorescence (0%, 0/21; 0%, 0/19 respectively). In positive larvae the striation of larval cuticle was seen (Fig. 5). These results indicate that the infective larvae of *B. pahangi* activate the alternative pathway of complement system. Almost all larvae recovered from the peritoneal cavity of infected mice without any cell adhesion also reacted with FITC-conjugated anti-mouse C_3 antibody (Table 3),

Table 3 Binding of anti-mouse C_3 antibody to *Brugia pahangi* larvae recovered from peritoneal cavity of mouse (ddy strain)

Infective larvae	Positive rate*	Degree of fluorescence†
3 hrs post-infection	94.4% (34/36)	+ ~ + + +
24 hrs post-infection	89.3% (25/28)	+ ~ + + +
48 hrs post-infection	100.0% (20/20)	+ + ~ + + +

* Experiments were duplicated.
† +: weak fluorescence, ++: moderately strong fluorescence, +++: strong fluorescence

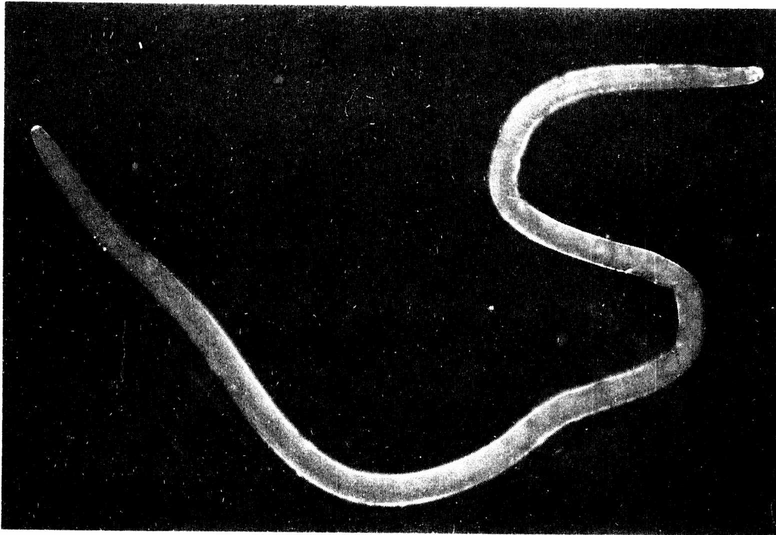

Fig. 5 Third stage larva of *Brugia pahangi* which had been incubated with normal mouse serum showing a positive to FITC-conjugated anti-mouse C_3 antibody.

suggesting that the larvae could activate the complement alternative pathway *in vivo.*

In vitro encapsulation of the third stage larvae treated with normal or heat-inactivated serum

The larvae treated with normal serum were encapsulated by peritoneal cells from naive mice *in vitro.* In this method 56.0% of the total number of larvae treated with normal serum was partially or completely encapsulated, although the reaction is not so strong as compared with *in vivo* reaction. On the other hand, heat-inactivated serum-treated larvae did not show any strong cell adhesion (Table 4).

Discussion

In parasite infection of mammalian hosts,

Table 4 *In vitro* encapsulation of the third stage larvae of *Brugia pahangi* by peritoneal cells of mouse

Treatment	Intact larvae (%)	Partially encapsulated larvae (%)	Completely encapsulated larvae (%)	Total number of larvae observed
Inactivated serum	39 (88.6)	5 (11.4)	0	44 (100.0)
Normal serum	22 (44.0)	27 (54.0)	1 (2.0)	50 (100.0)

*Experiments were duplicated.

killing of helminth larvae by eosinophils has been reported over the last two decades (Butterworth, 1984). In filarial infection eosinophils are known to adhere to both infective larvae and Mf of *B. malayi* and *B. pahangi* (Chandrashekar *et al.*, 1985 a and b; Yates and Higashi, 1986). In these reports antibody-dependent cellular cytotoxicity was considered as a major effective system.

Infective larvae of *L. loa* and *B. pahangi* activate the complement system of humans (Yates *et al.*, 1985) and the larvae of *B. malayi* also activate alternative pathway of complement system of rats (Chandrashekar *et al.*, 1986). From these and present results it is suggested that alternative pathway of the complement system is activated by the infective larvae and C_3 component attaches to the surface of the larvae. Complement-dependent encapsulation of the larvae by macrophages and eosinophils in the peritoneal cavity of mice seems to be important and essential defence responses in the early stage of intra-peritoneal infection and this strong reaction may lead to killing of the larvae. In the present experiment intact larvae without any cell adhesion were recovered from peritoneal cavity of mice 3, 24 and 48 hrs post-inoculation. The reasons why these larvae could escape from the cellular defence responses of mice are not clear at the present. Recently, we reported that the treatment of β-1,3 glucan to mice caused clear mitigation of cellular defence responses and simultaneously cause the drastic alternation in the distribution of the surface receptor for C_3b on peritoneal macrophages (Kobayashi *et al.*, 1992). It is plausible that the responsiveness of peritoneal macrophages of infected mice against C_3-positive larvae coexisted in the peritoneal cavity lower by the same alternation in the receptors of macrophages.

It is reported that the recovery rate of *B. pahangi* larvae were different among 5 strains of mice at 2 weeks after peritoneal inoculation (Sakamoto *et al.*, 1989). In their report ICR strain showed a slightly higher recovery rate (17.0%) as compared with that of DDY strain (2.3%). The difference might be related to the variance of complement activation system and/or responsiveness of macrophages and eosinophils, such as expression of C_3 receptor of the cells, among these strains of mice.

Recently, Brattig *et al.* (1991) showed that the third stage larvae, but not in the fourth larvae of *Onchocerca volvulus* were encapsulated by eosinophils *in vitro*. These data might be important to understand eliminating process of filarial larvae in the early stage of infection. Further studies on relationships between the amount of complement components or the activation system of complement and responsiveness of the effector cells will be helpful to understand the cellular defence responses of the hosts to parasites in the early stage of infection.

Acknowledgements

The authors are grateful to Dr. Keiichirou Yamaguchi for kindly performing observation by electron microscopy and taking microphotos. The authors thank to Misses Mayumi Ooshita and Junko Taya for excellent technical

assistance. This work was partially supported by a grant provided by Japan-U.S. Cooperative Medical Science Program.

References

1) Ash, L. R. and Riley, J. M. (1970): Development of *Brugia pahangi* in the jird, *Meriones unguiculatus,* with notes on infections in other rodents. J. Parasitol., 56, 967–973.
2) Brattig, N. A., Tischendorf, F. W., Strote, G. and Garza, C. E. M. (1991): Eosinophil-larval-interaction in onchocerciasis: heterogeneity of *in vitro* adhesion of eosinophils to infective and fourth stage larvae and microfilariae of *Onchocerca volvulus.* Parasite Immunol., 13, 13–22.
3) Butterworth, A. E. (1984): Cell-mediated damage to helminths. Adv. in Parasit., 23, 144–282.
4) Chandrashekar, R., Rao, U. R. and Subrahmanyam, D. (1985a): Serum dependent cell-mediated immune reactions to *Brugia pahangi* infective larvae. Parasite Immunol., 7, 633–642.
5) Chandrashekar, R., Rao, U. R., Parab, P. B. and Subrahmanyam, D. (1985b): *Brugia malayi*: Serum dependent cell-mediated reactions to microfilariae. South East Asian J. Trop. Med. and Pub. Heal., 16, 15–21.
6) Chandrashekar, R., Rao, U. R., Parab, P. B. and Subrahmanyam, D. (1986): *Brugia malayi*: rat cell-interactions with infective larvae mediated by complement. Exp. Parasit., 62, 362–369.
7) Haque, A., Ouaissi, A., Joseph, M., Capron, M. and Capron, A. (1981): Immunoglobulin E antibody in eosinophil and macrophage mediated *in vitro* killing of *Dipetalonema vitae.* J. Immunol., 127, 716–725.
8) Higashi, G. I. and Chowdhury, A. B. (1970): *In vitro* adhesion of eosinophils to infective larvae of *Wuchereria bancrofti.* Immunol., 19, 65–83.
9) Kobayashi, M., Yamada, K. and Yamamoto, H. (1992): Effect of β-1,3 glucan on the cellular defence responses in the peritoneal cavity of mice and jirds to the third stage larvae. *Brugia pahangi.* Jpn. J. Parasit., 41, 378–383.
10) Nakanishi, H., Horii, Y., Terashima, K. and Fujita, K. (1989): Effect of macrophage blockage on the resistance to a primary *Brugia pahangi* infection of female BALB/c mice. Trop. Med. Parasit., 40, 75–76.
11) Ouassi, M. A., Haque, A. and Capron, A. (1981): *Dipetalonema viteae*: ultrastructural study on the *in vitro* interaction between rat macrophages and microfilariae in the presence of Ig E antibody. Parasitol., 82, 55–62.
12) Sakamoto, M., Shigeno, S., Fujimaki, Y., Miura, M., Tachibana, Y. and Aoki, Y. (1989): Difference in the reaction of peritoneal cells to *Brugia pahangi* in several strains of mice. Trop. Med., 31, 125–129.
13) Yates, J. A., Higashi, G. I., Lowchick, A., Orihel, T. C., Lowrie, R. C. and Eberhard, M. L. (1985): Activation of alternative pathway of complement in normal human serum by *Loa loa* and *Brugia malayi* infective larvae. Acta Trop., 42, 157–163.
14) Yates, J. A. and Higashi, G. I. (1986): Ultrastructural observations on the fate of *Brugia malayi* in jirds previously vaccinated with irradiated infective stage larvae. Am. J. Trop. Med. Hyg., 35, 982–987.