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No Antibody Production Observed against Cuticle Layer of *Brugia pahangi* in Naturally Infected Rat – Implication of an Evasive Mechanism against Host Immunity –

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Abstract

This investigation was conducted to assess the course of antibody formation against various parts of the worm bodies of adult *Brugia pahangi* filariae by way of an immunofluorescent antibody method using the IgG-, IgM-, and IgE-monoclonal antibodies and sera from infected rats. The results were compared with previous results of a study using the lung fluke (Ohara *et al*., 1985b), in which differences between nematoda and trematoda in terms of immune response and the mechanism of evading the immune response of the host were investigated.

Two weeks after infection, antigenic sites against IgM and IgE antibodies were identified on the outer surfaces of the inner organ, in the body cavity fluid, in muscle layers, on the surfaces of microfilariae in the uterine and excretory canals of the worms. At the same time, we observed a small number of antigenic sites against the IgG antibody around the outer surfaces of the inner organs and in the body cavity fluid of the worms. Thereafter, serum levels of the antibodies against those sites increased until 12 weeks infection.

Antigenic sites against IgG, IgM, and IgE were almost identical; strong antigenicity was found at the sites which contained large amounts of excretory and secretory products, such as in body cavity fluid, inside the excretory canal. On the contrary, no antibody was observed against the cuticle layer throughout the experiment.

The absence of antigenicity on the surface of worm body (cuticle layer) in *B. pahangi* suggests a strong evasion of the immune response of host and difference in evasive from trematoda.

Key words: *Brugia pahangi*; filaria; nematoda; antibody; immunofluorescent method; antigenicity.

Introduction

To understand relationship between hosts and parasites in terms of immunology, it is informable to clarify the localization of sites of antigenicity in the worm and the courses of antibody formation against various sites within the worm body. Some studied on the nematode *filaria* have investigated IgG- and IgE-inducing antigens in the worm body from an immunohistologic point of view (Ishii *et al*., 1969; Fujita and Tsukidate, 1982; Ohara *et al*., 1985a). In one of these studies, Ohara *et al*. (1985b) reported that IgG class antibodies strongly reacted with the site of musculature inside the cuticle and the body cavity fluid, while IgE class antibodies reacted with body cavity fluid and excretory canal.

Similar studies have been conducted using the trematodes *Fasciola hepatica* (Hanna, 1980a) and *Paragonimus ohirai* (Ohara *et al*., 1985a; Ikeda and Oikawa, 1990; Ikeda *et al*., 1991). All of these studies showed that the tegument had strong antigenicity to IgG antibody. In *F.hepatica*, it has been shown that the antigenicity of the tegument changes

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frequently. This change is suspected to play an important role in helping the parasite evade the immune response of the host (Hanna, 1980b; Hanna, 1980c; Hanna and Trudgett, 1983). These findings suggest much difference in immune response by the host to parasite body exist between nematoda and trematoda.

In the present study, observation of antibody formation against various parts of the worm body of *B. pahangi* was conducted in detail using monoclonal antibodies and indirect immunofluorescent antibody (IIF) method. Comparisons were made between these results and the former results obtained on *P. ohirai* in terms of the difference in sites of antigenicity, and the possible difference in evading host immune response between nematoda and trematoda was discussed.

Materials and Methods

Animals

Adult male jirds weighing 50 to 60 g were used to obtain adult worm of *Brugia pahangi*, and adult male Wistar strain rats weighing 200 g were used to obtain serum samples.

Antigen

Sectioned adult worms were used as antigen for IIF. A jird was anesthetized with ethyl ether and was inoculated intraperitoneally with 200 third-stage larvae by 20G syringe. Six months after inoculation, the jird was killed, and adult worms were recovered from the peritoneal cavity, abdominal muscles, and inner organs. After washing with phosphate-buffered saline (PBS, pH 7.4), the worms were separated by sex. Furthermore, each male and female worm was separated into anterior, middle, and posterior portions.

Each group of 5 to 10 worms was frozen immediately with dry ice and acetone, embedded in Tissue Tek 2® (Miles Laboratories Co., Naperville, U.S.A.), and then sectioned longitudinally with a cold microtome to a thickness of 5 μm.

Antisera

Ten rats were infected with third-stage larvae of *B. pahangi* in the manner described above. Three to four rats were bled from the orbital plexus every 2 weeks starting 2 weeks postinfection until 30 weeks postinfection, and then at 10 months postinfection. The existence of microfilariae in the blood samples was confirmed by microscopic examination, and the sera of only microfilaria-positive rats were used throughout this experiment. The sera separated from the collected blood were stored at –20°C until testing. Before the experiments, pooled serum samples were prepared by mixing sera of each experimental group.

Indirect immunofluorescent antibody method

After pretreatment with a 95% solution of ethanol, the sections of worm material were exposed to the pooled serum samples (serially diluted with PBS, pH 7.4) from 1:4 to 1:1,280 at 4°C overnight. The sections were washed with PBS and exposed for 60 min. at 37°C to an FITC-conjugated serum of monoclonal antibodies. Three kinds of monoclonal antibodies were used: FITC-conjugated mouse anti-rat IgE (Sera Lab Co., Tokyo, Japan) diluted at a ratio of 1:4 with PBS, FITC-conjugated mouse anti-rat IgM (Sera Lab Co., Tokyo, Japan) diluted at a ratio of 1:10 with PBS, and FITC-conjugated mouse anti-rat IgG(ab')$_2$ (Sera Lab Co., Tokyo, Japan) diluted at a ratio of 1:10 with PBS. After incubation, the sections were washed again with PBS for 15 min. and mounted with buffered glycerin (9 parts glycerol to 1 part PBS). The preparations were examined by microscope with substage illumination (Nikon, Fluophot, Tokyo, Japan). When necessary, sections were counter stained with hematoxylin and eosin to examine the morphologic structures of the worm in detail in relation to the findings of the IIF method.

Observation of antigenic sites

The pooled serum samples were titrated against the worm bodies, and the fluorescence of various tissues and organs of the worm were observed. The IgG-, IgM-, and IgE-inducing antigen sites of the worm bodies were examined, and progressive changes in antigenicity were observed. IgG-, IgM-, and IgE-antibody titers at the outer margins of the female intestine were measured because this site showed continuous, strong fluorescence when exposed to any of the serum samples. Uninfected rat serum was used as a control throughout the present experiment. The titer of antibody in each pooled

serum sample was determined when the dilution of the serum reached the level of discernible labeling in the appropriate tissue sections in relation to the antibody titer of the control group.

Comparison of antigenic sites with fluke

Comparison of IgG-, IgM, and IgE-inducing antigen sites was conducted using the present results and the previous results from experiments on *Paragonimus ohirai* (Ohara *et al.*, 1985b; Ikeda *et al.*, 1991).

Results

Antibody response to worm body

Figure 1 shows the results of antibody titers against the outer surface of the intestine of the female after infection. Antigenic sites against IgM were first recognized around the inner organ (intestine), in the muscle layers, in the body cavity fluid, and on the surface of the microfilariae in the uterine and excretory canals 2 weeks after infection. Antibody titer against these sites reached the maximum level at 3 to 4 weeks after infection and declined thereafter. No fluorescence was present after 8 weeks. As shown in Figure 2, antigenicity was recognized in the cuticle layer or in the intestine wall.

Antigenic sites against IgG began to appear 2 weeks after infection around the inner organs and in the body cavity fluid. The antibody titer reached the maximum level at 12 weeks (Fig. 1), when strong fluorescence was recognized in the muscle layer, around the inner organs, in the body cavity fluid and on the surface of the microfilariae in the uterine and excretory canals. Antibody titer and antigenic sites remained similar thereafter. No antigenicity was recognized in the cuticle layer nor in the intestinal wall (Figs. 3 and 4).

Antigenic sites against IgE were recognized first around the inner organs, in the body cavity fluid, in the muscle layer, and on the surface of the microfilariae in the uterine and excretory canals after 2 weeks. Antibody titer against those sites increased gradually and reached the maximum level 8 weeks after infection (Fig. 1). The antibody titers remained at the same level and the location of the antigenic sites remained the same thereafter. No antigenicity was recognized in the cuticle layer nor in the intestinal wall. The IgE-antibody titer was lower than that of IgM or IgG, however, the location of the antigenic sites of the immunoglobulins were nearly identical (Figs. 5 and 6).

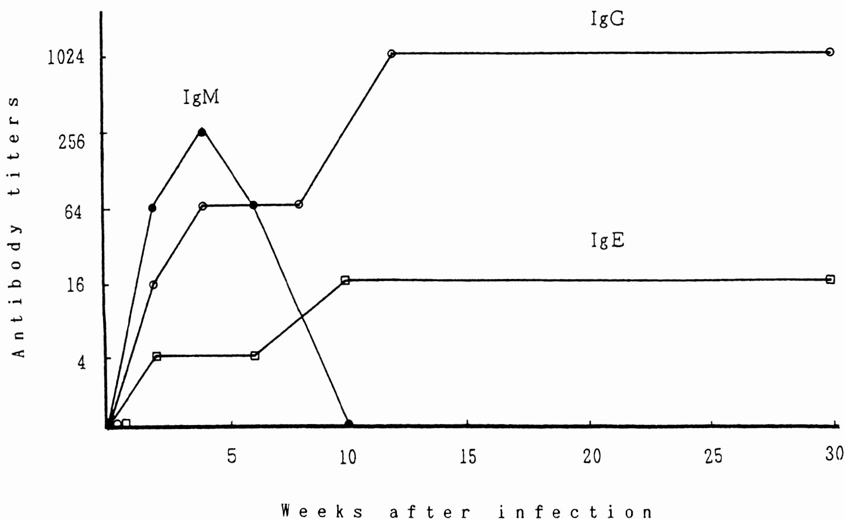

Fig. 1 Kinetics of antibody response against the outer surface of the intestine to adult female *B. pahangi*.

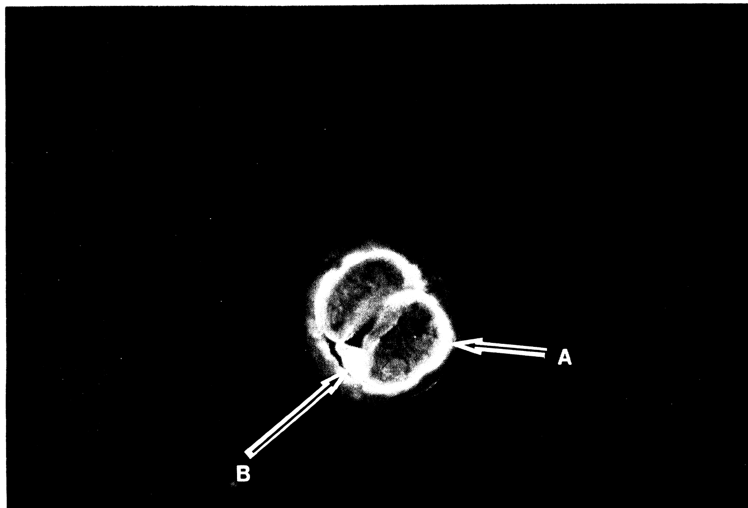

Fig. 2 IgM-antibody response to male worm of *B. pahangi*. The tissue was treated with serum that had been infected for 4 weeks (1:16) before fluorescent staining. Fluorescence is observed around the intestine and in the body cavity fluid (A), and in the excretory canal in the lateral lines (B) (magnification, 200×).

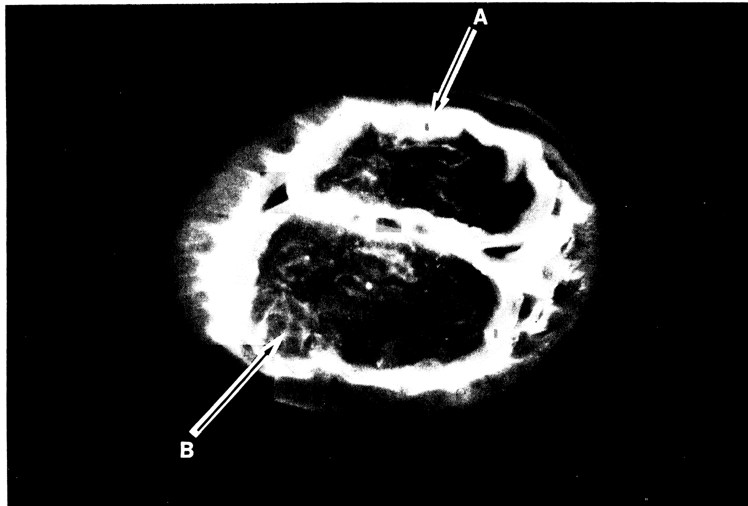

Fig. 3 IgG-antibody response to female worm of *B. pahangi*. The tissue was treated with serum that had been infected for 12 weeks (1:64) before fluorescent staining. Fluorescence is observed around the inner organ (uterus) and in the body cavity fluid in the lateral lines, and at the sites where the body cavity fluid is solidified inside the muscle layer (A). The surface of microfilariae in the uterine canal also shows fluorescence (B). Cuticle layer is negative (magnification, 400×).

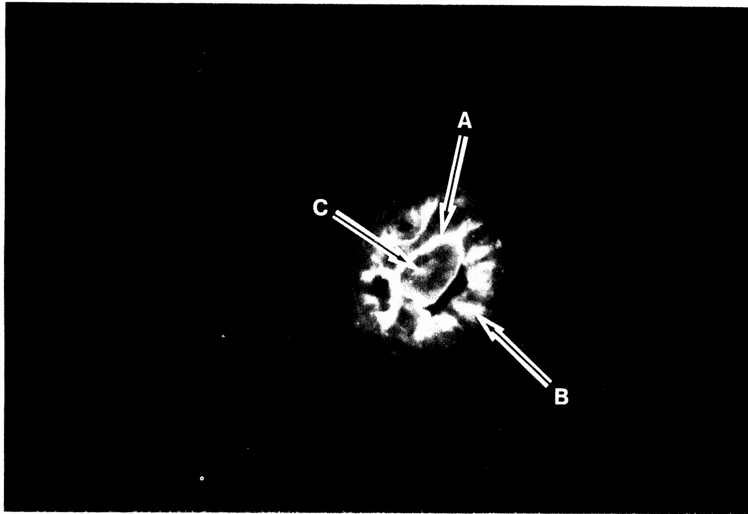

Fig. 4 IgG-antibody response to male worm of *B. pahangi*. The tissue was treated with serum that had been infected for 12 weeks (1:64) before fluorescent staining. Fluorescence is observed around the intestine and at the site where the body cavity fluid (A) is solidified inside the muscle (B) layer. Content of intestine (C) also shows fluorescence (magnification, 200×).

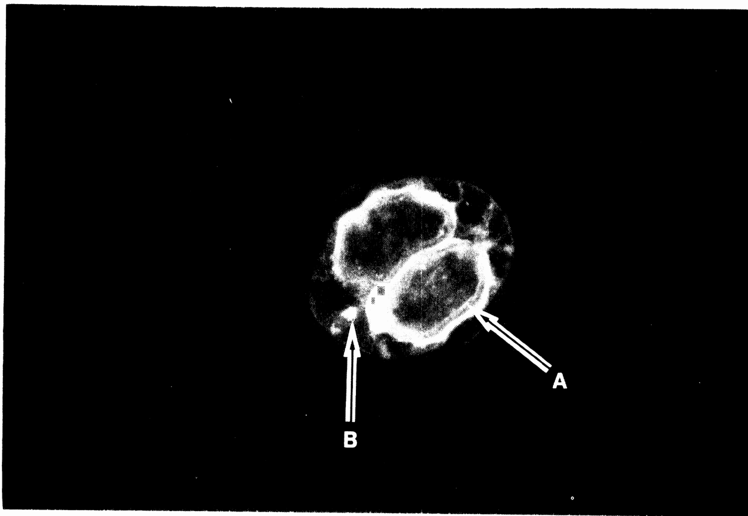

Fig. 5 IgE-antibody response to male worm of *B. pahangi*. The tissue was treated with serum that had been infected for 6-weeks (1:4) before fluorescent staining. Fluorescence is observed around the intestine and in the body cavity fluid (A), and in the excretory canal in the lateral lines (B) (magnification, 200×).

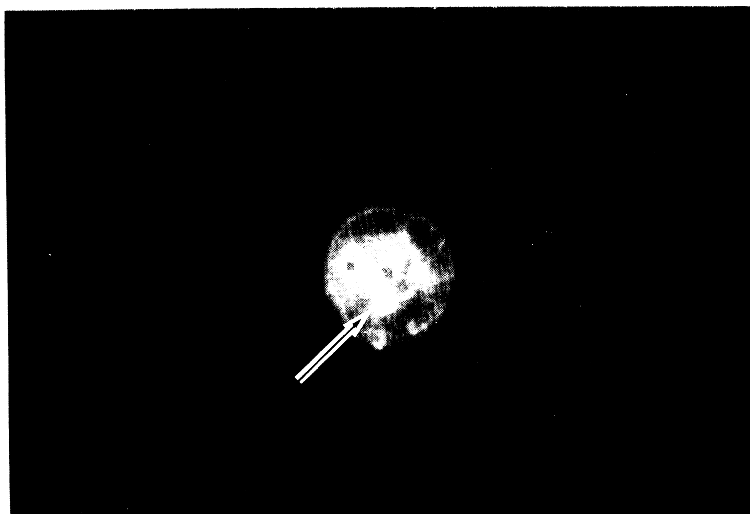

Fig. 6 IgE-antibody response to female worm of *B. pahangi*. The tissue was treated with serum that had been infected for 6 weeks (1:4) before fluorescent staining. Body cavity fluid shows strong fluorescence (magnification, 200×).

Table 1 Comparison of the sites of antigenicity to IgM, IgG, and IgE antibodies between *B. pahangi* and *P. ohirai* by immunofluorescent antibody method

	B. pahangi	*P. ohirai*[†]
IgM	Body cavity fluid (+++)* Outer surface of (+++) inner organs Excretory canal (+++) Muscle layer (+) Microfilariae in uterus (+)	Tegument (+++) Intestine (+++) (epithelium, contents)
IgG	Body cavity fluid (+++) Outer surface of (+++) inner organs Muscle layer (++) Site of the musculature (+++) inside of cuticle Microfilariae in uterus (+)	Tegument (+++) Intestine (+++) (epithelium, contents) Excretory Canal (+++) Eggs (+) Testis (+)
IgE	Body cavity fluid (+++) Excretory canal (+++) Outer surface of (+++) inner organs Muscle layer (++) Microfilariae in uterus (+)	Intestine (+++)[‡] (epithelium, contents) Tegument (+++)[‡]

*Intensity of fluorescence: (+++), strong; (++), intermediate; (+), weak.
[†]Ohara *et al.*, 1985a
[‡]Ikeda *et al.*, 1991

Comparison of antigenic sites of nematoda with trematoda

A comparison of antigenic sites of *B. pahangi* and *P. ohirai* is shown in Table 1. Similar results were obtained concerning the course of formation of IgM and IgG antibodies, however, a difference in antigenic sites was recognized between the two worms. In *P. ohirai*, strong fluorescence was observed on the surface of the worm body, whereas no fluorescence was recognized on the surface of *B. pahangi*. In both worms, strong fluorescence was observed in the excretory vesicle.

Discussion

In the present study, body cavity fluid and the outer surfaces of the intestine and excretory canal showed strong antigenicity of IgM, IgG, and IgE antibodies, and no remarkable differences were found in the location of the antigenic sites among these antibodies. These results suggest that the antigenic sites to IgM, IgG, and IgE antibodies are almost identical, and antigenic substances are contained in large amounts in the excretory products and body cavity fluid and at the sites of their attachment.

The findings that no antigenicity to IgM, IgG, and IgE antibodies was recognized on the surface of the *B. pahangi* worm body throughout the course of infection suggest that antibodies that are formed during filarial infection are mainly against the excretory and secretory (ES) products and the body cavity fluid. These results coincide with the results of our previous study, which examined IgG-, and IgE-antibody-inducing sites using *Dirofilaria immitis* (Ohara *et al.*, 1985b) and with the results of Ikeda *et al.* (1991), in which IgE-inducing sites were detected most strongly in intestinal epithelium and luminal contents.

In an experiment using *Litomosoides carinii*, Ishii *et al.* (1969) also reported that IgG-inducing antigen was contained mainly in body cavity fluid, and many other reports have confirmed that large amounts of IgE-inducing antigens are contained in ES products (Fujita *et al.*, 1979; Fujita and Tsukidate, 1981; Fujita and Tsukidate, 1984). Those previous reports and the present study suggest that ES products and body cavity fluid are major antigenic stimulants not only to IgE but also to IgM and IgG.

The IgM, IgG, and IgE antibodies are known to form against the ES products of filaria because in the course of natural infection, only the ES products (except for the surface cuticle layer) have contact with the immune mechanism of the host. The fact that the cuticle layer does not show antigenicity suggests that it is not recognized as a foreign object and does not lead to the formation of antibodies. In addition, ES products and body cavity fluids are known to have close antigenicity.

In trematoda such as *P. ohirai*, however, strong antigenicity is recognized in the tegument (Ohara *et al.*, 1985; Ikeda and Oikawa, 1990). In *F. hepatica*, Hanna (1980a) indicated that a change in antigenicity of the tegument occurred with the development of a distinct type of tegumental cell and observed the process of active secretion of the tegumental antigens (Hanna, 1980b; Hanna, 1980c; Hanna and Trudgett, 1983). This process is effective for evading the immunologic attack of the host.

Cuticle layer is suspected to have characteristic which is difficult to be recognized as foreign object. This characteristic is suspected to be acting as one of the effective mechanisms to evade responses of the hosts in nematoda. However, cell-mediated immunity, cooperating closely with antibody production also played a vital role in the immune response of the host to filaria and other parasitic infections (Horii *et al.*, 1988; Nakanishi *et al.*, 1988; Nakanishi *et al.*, 1989). The present study investigated antibodies only, however, and further study is needed to examine the evasive mechanism in terms of the antibody-cell-mediated immunity interaction.

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