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Studies on Antibody Response in Rats Infected with *Paragonimus ohirai* by Immunofluorescent Staining Method

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Key words: *Paragonimus ohirai*, immunofluorescent staining method, IgG, IgM, antibody response

Introduction

It has been known that the infection of lung flukes causes parasite specific antibody responses, and various immunological examinations such as skin test (Yokogawa, 1956), complement fixation test (Yokogawa and Awano, 1956), Ouchterloney's gel diffusion test (Yogore *et al.*, 1965; Tada, 1967) and immunoelectrophoresis (Kruidenier and Katoh, 1959; Biguet *et al.*, 1965) have been applied to the diagnosis of paragonimiasis.

It is important to elucidate the courses of antibody formation against various sites of the fluke body, because it gives information on immunological relationships between hosts and parasites, thus making the immunological diagnosis more accurate. In experimental paragonimus, however, there are few detailed examinations undertaken using the indirect immunofluorescent (IIF) staining method for the above purpose, although some experimental studies have been reported on the chronological antibody formations in experimental paragonimiasis using various methods (So, 1959; Tada, 1967; Ikeda and Tada, 1977; Ikeda and Fujita, 1980). As for *Fasciola hepatica* the results of detailed examinations by the IIF method about the courses of antibody formation against the tegument, gut and excretory system were reported in relation to the morphological changes observed by electron microscope (Hanna, 1980a; Hanna and Trudgett, 1983).

In the present investigation, the courses of IgG and IgM antibody formations specific for adult juvenile flukes in *Paragonimus ohirai*-infected rats were examined by the IIF method. The chronological examinations of antibody formations in various sites of the fluke body were made after infection and the antibody titers to the tegument, gut and ovum were measured quantitatively.

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Materials and Methods

Animals: Female Wister rats weighing 150 to 180g were used to obtain serum samples and flukes.

Metacercariae: Metacercariae of *Paragonimus ohirai* were obtained from crabs (*Sesarma dehaani*), collected from the Maruyama River, Hyogo Prefecture, Japan.

Antisera: Each rat anesthetized with ethyl ether was inoculated intraperitoneally with 10 metacercariae by syringe (21G). This route of infection gave smaller deviation in the infection rate than the oral route. Three to six rats were bled from their femoral arteries weekly until the 15th week postinfection and then, at the 5th and 7th months postinfection. The sera separated from the collected blood were stored at −20°C until testing. Before experiments, pooled serum samples were pre-

pared by mixing sera of 3 to 5 rats of each experimental group.

Antigens: Sectioned adult flukes of *Paragonimus ohirai* aged over 12 weeks and 2-week juvenile flukes were used as antigens for IIF. Each rat was infected with 10 metacercariae in the same way as described above. The stage specific development and migration of *P. ohirai* has been previously reported (Okura, 1963; Tada, 1969). Based on these data, at 2 weeks postinfection, juvenile flukes were recovered from the host's liver parenchyma and adult flukes at 12 weeks postinfection from the cysts in the lungs. Each fluke was frozen immediately with dry ice-acetone, embedded in tissue tek II (Miles Leboratories, Co., Naperville, U. S. A.), and then sectioned longitudinally 4μm in thickness with cold microtome.

Indirect immunofluorescent (IIF) staining: Following the fixation with 95% ethanol, the sections of the fluke material were exposed to the pooled serum samples serially diluted with phosphate buffered saline solutions (PBS, pH 7.2) from 1/10 up to 1/1260 at 4°C overnight. The sections were washed with PBS and exposed for 60 min at 37°C to FITC-conjugated rabbit anti-rat IgG (Cappel Laboratories, Dawington, U. S. A.) diluted to 1/10 with PBS (I) or rabbit anti-rat IgM (Fujizoki, Co., Tokyo, Japan) diluted to 1/60 with PBS (II). After incubation, the sections were washed again with PBS for 15 min and the former (I) was mounted with buffered glycerine (9 parts glycerol+1 part PBS), while the latter (II) was labeled with FITC-conjugated sheep anti-rabbit IgG (Cappel Laboratories) followed by washing with PBS for 15 min and mounted with the buffered glycerine. The preparations were examined by microscope with substage illumination (Nikon, Fluophot, Japan). If necessary, sections were counterstained with hematoxylin-eosin to examine the morphological structures of the fluke in detail in relation to the finding of the IIF staining.

Experimental method: The pooled serum samples were titrated against the fluke bodies, and the fluorescence of various tissues and organs of the flukes was observed. The IgG (I) and IgM (II) inducing antigens in adult flukes and the IgG inducing antigen (I) in juvenile flukes were examined by IIF method. The IgG and IgM antibody titers against the tegument, gut and ovum in adult flukes were measured quantitatively. As for juvenile flukes, only the IgG antibody titers against the tegument and gut were examined. Uninfected rat serum was used as a control throughout the present experiment. The titer of antibody in each pooled seurm sample was determined when the dilution of the serum reached to the level which gave just discernible labeling over the appropriate tissue sections in comparison with that of the control's. For the determination of the endopoint of antibody titer against the tegument, only the surface glycocalyx layer was noticed. Because this layer was always more fluorescent than any other sites of the tegument with any of the serum samples.

Results

IgG antibody response to adult fluke

The IgG antibody against the tegument was first detected by an IIF method in the serum at 2 weeks postinfection, although the titer was low (1/20). It increased markedly at 3 weeks postinfection with a serum titer of 1/60. The labeling occurred on the glycocalyx layer, throughout the surface syncytium, and over the cytoplasm of the tegumental cells. Above all, the glycocalyx layer showed the strongest fluorescence. The reaction appeared evenly on the surface, but the spines and tegumental cell nuclei were completely unlabeled (Photo. 1). With the serum obtained 5 weeks postinfection, fluorescence appeared also in the muscular layers beneath the syncytium layer (Photo. 2). The strongest labeling of glycocalyx layer was observed with 6-9-week-postinfection sera (1/640) (Photo. 3), and thereafter, there was a decreasing tendency in intensity of the labeling. The antibody titers in 5-and 7-month-postinfection serum samples were 1/40 and 1/20, respectively. The decrease of fluorescence was particularly remarkable in the region of the tegumental cells,

where the intensity decreased to less than 1/20 with post-12-week antiserum.

In the gut, a weak fluorescent labeling was first detected in the serum samples obtained at 2 weeks postinfection (1/10) and moderate fluorescence, at 4 weeks postinfection (1/40). However, the fluorescence of the guts was always much weaker than that of the teguments. The maximal antibody titer against the guts was observed in the 7-11-week-postinfection sera, although the titer was only 1/160 (Photo. 4). Thereafter, the intensity of fluorescence slightly declined and the antibody titer in both 5-and 7-month-postinfection sera was 1/40. The reaction was induced mainly over the cytoplasm of the epithelial cells, the lamella inside the lumens, and villi.

The antibody against the ovum was first detected in 6-week-postinfection sera, and the maximal titer, at 9-11 weeks postinfection (1/80) (Photo. 5). However, there was a gradual dercease in titer to ovum after 12 weeks, remaining around 1/40 for a long time. Specific fluorescence was recognized in the contents of ovum in the shape of granules, while the shells showed yellowish non-specific autofluorescence.

The fluorescent labeling of the excretory system first appeared in the sera obtained 3 weeks postinfection (Photo. 6), but there was only weak fluorescence at 5 and 7 months postinfection. With the lapse of time, the sites bound with IgG expanded. In 6-week-postinfection sera, fluorescence was recognized in the oral and ventral suckers (Photo. 7) and testis, too. In 8-week-postinfection sera, the interstitial tissue also showed weak fluorescence. Generally speaking, there was a tendency that in contrast to the gradual decrease of fluorescent labeling in the tegument, gut and ovum, the fluorescence in other organs increased, although not strong. However, after 10 weeks there was a gradual decrease in fluorescence in these sites. No significant fluorescence was observed with the control serum (Fig. 1).

IgM antibody response to adult flukes

The IgM antibody was detected against the tegument and gut, but no any other tissues or

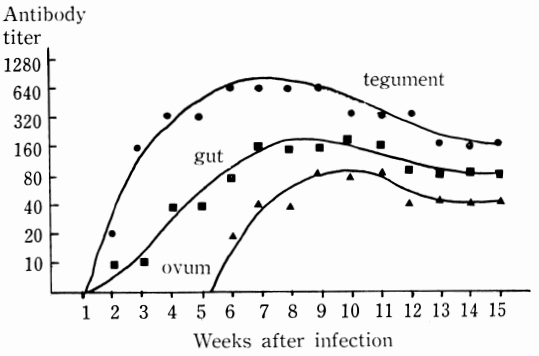

Fig. 1 Kinetics of IgG antibody response to adult *P. ohirai* in rats.

organs. With the sera obtained 4-5 weeks postinfection, the fluorescence at both tegument and gut reached their maximum, which decreased thereafter.

On the tegument, the fluorescence labaling occurred in the tegumental cells and syncytium layer, which first appeared with 2-week-postinfection serum (1/20) and was the strongest with the 4-5-week-postinfection sera (1/320). In IgM, unlike IgG, the fluorescence of the surface glycocalyx layer was not so strong as that in the syncytium layer. No fluorescence was observed in either muscle layer or spine (Photo. 8).

In the gut, week fluorescence was first recognized with the 2-week-postinfection sera (1/10) over the cytoplasm of the epithelial cells and villi, which reached maximum at 4-6 weeks postinfection (titer, 1/40) (Photo. 8). No significant fluorescence was observed with the control serum (Fig. 2).

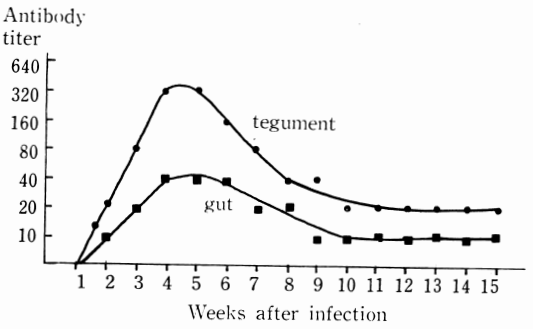

Fig. 2 Kinetics of IgM antibody response to adult *P. ohirai* in rats.

IgG antibody response to juvenile flukes The IgG antibody against the tegument of juvenile flukes was first detected in 2-week postinfection sera revealing a little higher titer than that of adult flukes. The titer increased with time reaching a maximum (1/160) at 4-6 weeks postinfection and declined subsequently during the course of the infection. Except the sera obtained until 4 weeks postinfection, all the IgG antibody titers to juvenile flukes were lower than those of adult flukes. The fluorescence appeared over the apical surface, syncytium and tegumental cells. However, the muscle layers and the spines were not stained (Photo. 9, 10). No specific fluorescence was recognized in any other sites than the tegument and gut throughout the course of experimental infection (Fig. 3).

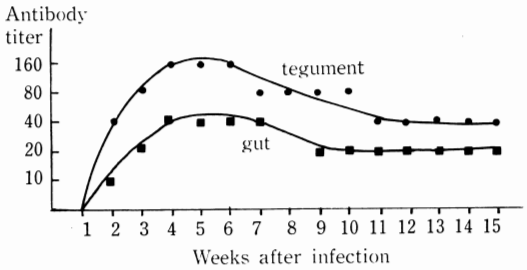

Fig. 3 Kinetics of IgG antibody response to juvenile *P. ohirai* in rats.

Discussion

The range of IgG binding sites expanded with the stages of infection. At 10 weeks postinfection, a great variety of sites such as ovum, oral and ventral suckers, testis and connective tissue showed antigenicity. This finding coincides with the results of the previous expriment by using Ouchterloney's gel diffusion method (Ikeda and Tada, 1977), where the number of precipitin arcs increased together with the stages of infection, showing 10-15 arcs at 10 weeks postinfection between serum and worm extract.

In the present study, the tegument and gut found specific IgG antibody at an early stage of infection and the antibody level in the serum reached its peak at 8 weeks postinfection. A correlation was recognized in kinetics of the antibody titers between the IHA method (Ikeda and Tada, 1977 ; Ikeda and Fujita, 1980) and the present IIF method until 10th week postinfection. However, after the 10th week, a difference was recognized between them ; the IIF titer revealed a decrease thereafter, while those by the IHA method remained almost unchanged. But it is assumed that the results obtained by these two methods should not be necessarily in accord, because as the stage of the infection progresses, antigens other than tegument and gut also may begin to participate in the reaction as revealed by the IIF.

Hanna (1980a) reported that the antigenicity of the tegument of *F. hepatica* decreased in post-10-week antiserum. This author presumed that once the flukes entered the bile ducts, their various antigenic products were no longer available for stimulating the immune system of the host and consequently the concentration of circulating antibodies decreased. This explanation may also be applicable to the case of *P. ohirai*. Once flukes were surrounded by cyst wall in the lung, it might be difficult to recognize them by the host's immunological mechanism.

IgM antibody against the tegument and gut were detected in early stage of infection. Thereafter, the IgM antibody titer reached its maximal level 4-5 weeks postinfection and then decreased rapidly. This transient response was well consistent with the general pattern of IgM antibody response at primary time of infectious diseases. It was assumed that at an early stage of infection, the IgM antibodies were produced mainly against tegument and also partially against the gut. The tegument and gut seemed to be the only effective antigens for inducing the IgM antibodies throughout the course of *P. ohirai*-infection.

In the sera obtained after 4 weeks postinfection, the tegument and gut of juvenile flukes were less intensely labeled by IIF method than those of adult flukes. This finding suggests that there are some differences in antigenicity between juvenile and adult flukes. Rajasekariah and Howell (1978) reported that

adult *F. hepatica* subcutaneously implanted in rats could not protect the rats from a challenge with metacercariae of this fluke, whereas juvenile flukes protected the animals. Hanna (1980a) reported that the fluorescence of adult *F. hepatica* was less intense than that of juvenile when examined using the same infected serum. Similar result was reported by Thorpe (1965) using *F. hepatica*. These findings suggested difference in antigenicity between adult and juvenile flukes. In *F. hepatica*, Hanna (1980a) indicated that change of tegument antigenicity occurred with development of distinct type of the tegument cell and the author (1980b, c) observed the active secretion process of the tegument antigens. It is quite possible that similar changes may occur also in the tegument of *P. ohirai*, which will be effective for evading the immunological attack by the host. Further study is necessary to elucidate the relation between changes of tegument antigenicity and the morphological changes of tegument cell.

It was clarified that the IIF method was very sensitive to detect antibody against *P. ohirai* and, therefore, effective to distinguish the intensity of fluorescence at various sites of the fluke body among various periods after infection.

Summary

Kinetics of IgG and IgM antibodies responses in rats experimentally infected with *P. ohirai* was studied by the IIF method, and the following findings were obtained.

1) The IIF method was sensitive for the detection of antibody against *P. ohirai* as well as other immunological methods.

2) Only tegument and gut were found binding specific IgM and IgG antibodies in the early stage of infection, while in the latter stage, various other tissues such as ovum, excretory system, suckers, testis and interstitial tissues bound IgG antibody.

3) Some antigenic differences were seen between adult and juvenile, especially in their tegument.

4) It seems that flukes surrounded by cyst wall in the lung could not stimulate effectively to the immune system of rats.

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大平肺吸虫感染ラットにおける抗体産生の螢光抗体法による研究

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寄生蠕虫感染宿主において寄生体各部に対する抗体産生を明らかにすることは，宿主と寄生体との相互関係を検討するために重要であり，ひいてはより正確で精度の高い免疫診断に有用であると思われる．本研究では，大平肺吸虫（*Paragonimus ohirai*）感染ラットにおける成虫および幼若虫の虫体各部に対する IgG および IgM 抗体産生の変化を凍結切片を用いた間接螢光抗体法により観察した．血清は，*P. ohirai* のメタセルカリアをウイスター系ラットに感染させ，1週毎に15週目まで採取したものを用いた．表皮，腸管，虫卵に対する抗体では，抗体価の測定を行なった．

成虫では，表皮および腸管に対する IgG 抗体は感染後2週目に認められ，7～12週目で最高値に達し，その後漸減した．表皮のうち，glycocalyx 層および tegumental cell では感染早期より強い螢光が認められ，5週目からは筋層にも螢光が認められた．感染経過とともに表皮，腸管のほか，虫卵，排泄管，精巣および結合組織などにも螢光が認められた．IgM 抗体は，表皮と腸管のみに認められ，4～5週目に最高値に達し，その後漸減した．また，対照として正常ラット血清を用いた時には殆ど螢光が認められず，宿主体内においてすでに虫体付着している IgG および IgM 抗体は少ないものと考えられた．幼若虫に対する IgG 抗体は，感染5週目以後は常に成虫に対するより低く，成虫と幼若虫との間の抗原性の差異が示唆された．

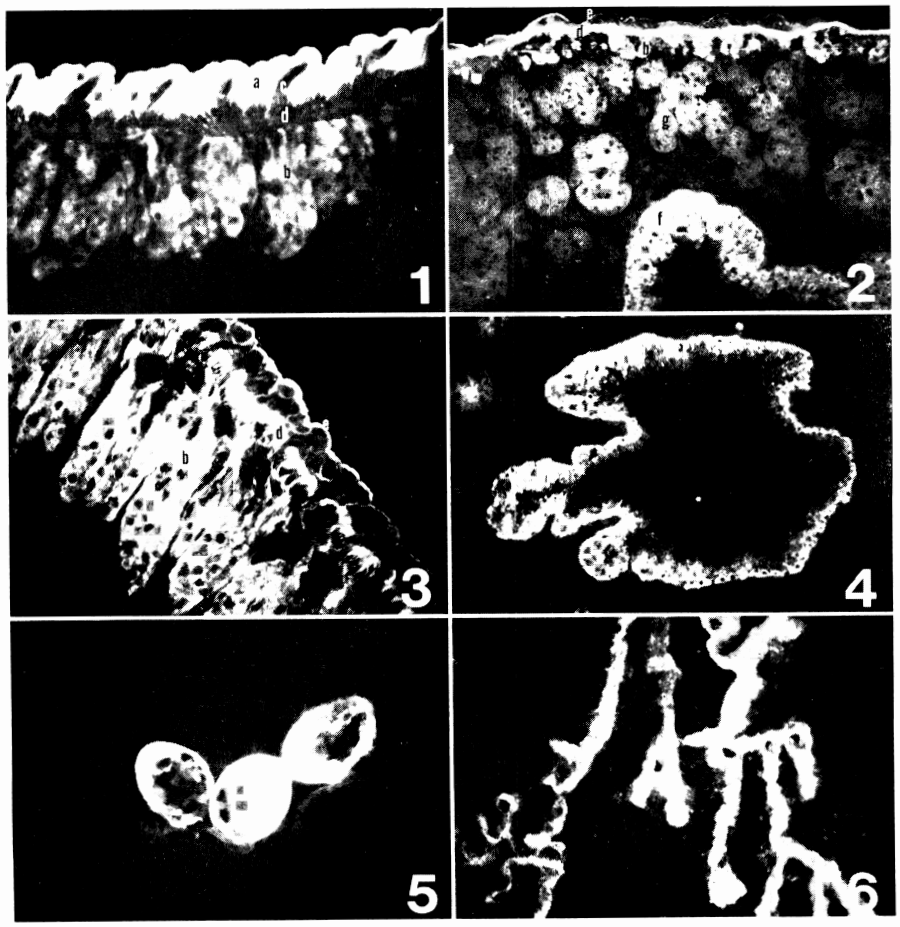


Photo. 1 *IgG antibody response to adult fluke.* The tissue was treated with 3-week-postinfection serum (1/40) prior to fluorescent staining. The surface syncytium (a) is strongly labeled, while the tegumental cells (b) are weakly labeled. The spines (c), muscular layer (d) and tegumental cell nuclei are negative. ×294.

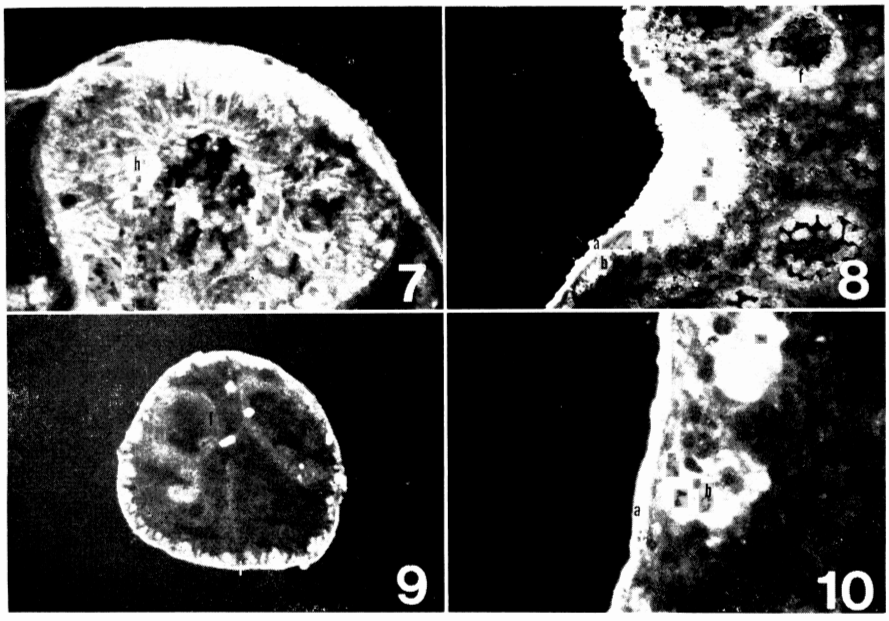
Photo. 2 *IgG antibody response to adult fluke.* The tissue was treated with 5-week-postinfection serum (1/20) prior to fluorescent staining. Glycocalyx layer (e), muscle layer (d) and tegumental cells (b) are strongly labeled, while moderate fluorescence is seen in the gut (f). The vitelline glands (g) beneath the syncytium layer are showing yellowish autofluorescence. ×247.

Photo. 3 *IgG antibody response to adult fluke.* The tissue was treated with 7-week-postinfection serum (1/160) prior to fluorescent staining. Glycocalyx layer (e), muscle layer (d) and tegumental cells (b) are strongly labeled. ×294.

Photo. 4 *IgG antibody response to adult fluke.* The tissue was treated with 8-week-postinfection serum prior to fluorescent staining. In gut, the fluorescence is observed mainly over the cytoplasm of the epithelial cells, the lamella inside the lumens, and villi. Weak fluorescence is observed in lumen itself, too. ×294.

Photo. 5 *IgG antibody response to adult fluke.* The ova were treated with 9-week-postinfection sera (1/20) prior to fluorescent staining. The contents of ova are stained, while the shell showing yellowish autofluorescence. ×294.

Photo. 6 *IgG antibody response to adult fluke.* The tissue was treated with 4-week-postinfection sera (1/40) prior to fluorescent staining. Fluorescence is observed along the excretory canals. Interstitial tissues surrounding the canals are completely unlabeled



with this stage of serum. ×294.

Photo. 7 *IgG antibody response to adult fluke.* The tissue was treated with 8-week postinfection sera (1/40) prior to fluorescent staining. Both oral (h) and ventral suckers are showing moderate fluorescence. ×247.

Photo. 8 *IgM antibody respoonse to adult fluke.* The tissue was treated with 4-week-postinfection sera (1/40) prior to fluorescent staining. Strong fluorescence is observed throughout the deapth of the syncytium layer (a) and tegumental cells (b). Moderate fluorescence is observed over the epithelial cells of the guts (f). ×147.

Photo. 9 *IgG antibody response to juvenile fluke.* The tissue was treated with 4-week-postinfection sera (1/40) prior to fluorescent staining. The fluorescence is observed only in the tegument (i) and gut (f), although the fluorescence of the gut was very weak. The fluorescent three points in the upper half are regarded as artifacts. ×59.

Photo. 10 *IgG antibody response to juvenile fluke.* The tissue was treated with 4-week-postinfection sera (1/40) prior to fluorescent staining. In tegument the fluorescence is observed over the apical surface, syncytium (a) and tegumental cells (b). The spines (c) and muscular layers are unlabelled. ×294.