[Jap. J. Parasit., Vol. 32, No. 1, 43-54, Feb., 1983]

Detection of IgG Antibodies in Mongolian Jirds Infected with *Brugia pahangi* by Enzyme-Linked Immunosorbent Assay

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(Received for publication; December 27, 1982)

Key words: *Brugia pahangi*, microfilaria, ELISA, IgG

Introduction

Serological investigations on filariasis have been carried out for more than sixty years using various techniques for the diagnosis (Ambroise-Thomas, 1974; Beaver, 1970; Grove and Davis 1978; Kaliraj *et al.*, 1981; Kagan, 1963). In the most of serological tests for human filariasis a variety of related worms have been utilized as antigen (*e.g. Dirofilaria immitis* for the diagnosis of *Wuchereria bancrofti* infection) (Grove *et al.*, 1977; Weller *et al.*, 1980), because of the difficulty in obtaining sufficient amount of homologous antigens. None of the immunodiagnostic tests now available is satisfactory for defining accurately the presence of filarial infection in individuals, because of either lack of specificity or inability to discriminate between present and past infection (WHO, 1981). In order to develop a method to utilize the molecules with specific determinants, it is necessary to establish a technique with high sensitivity and stability using a small amount of antigenic materials.

In the present paper, we report on the IgG antibody responses of Mongolian jirds (*Meriones unguiculatus*) infected with *Brugia pahangi* and human cases infected with various parasite species measured by enzyme-linked immunosorbent assay (ELISA) using crude extracts of adult worms and microfilariae of *B. pahangi* as antigen.

Materials and Methods

Antigen preparation:

Adult worms (Ad) and microfilariae (Mf) of *B. pahangi* were washed out from the peritoneal cavity of jirds with saline 6 to 24 months after an intraperitoneal inoculation with 100 third- stage larvae.

To eliminate host cells from Ad or Mf preparation the suspension including Ad, Mf and host cells was transferred into a Petri dish with a Pasteur pipette. Ad were selectively transferred five times by a needle to another Petri dish after rinsing in saline. Then Ad were blotted with a filter paper, lyophilized and kept at −20 C until use. The suspension including Mf was poured into another Petri dish leaving adherent cells in the dish. The microfilaria suspension, 200,000 Mf/ml, was mixed an

The present study was partially supported by Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture, Japan.

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equal volume of 1% agarose dissolved in saline which was kept at 50 C in a water bath. The mixture was then transferred into a Petri dish and kept horizontally at room temperature. After the solidification of the mixture, 5 ml of Hanks' balanced salt solution (HBSS) was placed on the gel. The dish was then kept in an incubator at 37 C overnight. By this procedure intact and live Mf were collected effectively without host cell contamination (Nogami *et al.*, 1982). The Mf coming out of the gel into HBSS were recovered, washed with saline, lyophilized and kept at −20 C until use.

Crude extracts were obtained from Ad or Mf by the following procedure:

Worms were suspended in carbonate buffer (0.05 M, pH 9.5) and homogenized with a tefron homogenizer for 10 min and the homogenates were frozen and thawed in tubes 10 times. Then, the solution was sonicated for 10 min, stirred slowly for two days in a refrigerator, and centrifuged at 10,000 g for 1 hour at 4 C. The supernatant was filtered through a millipore filter (pore size: 0.45 μm) and lyophilized and kept at −20 C until use. For the sensitization of a microplate it was reconstituted to a concentration of 3 μg protein/ml in the carbonate buffer. Protein concentration was estimated by the Lowry-Folin method.

Serum samples:

Seventy eight jirds were inoculated intraperitoneally and 27 jirds subcutaneously with 100 third-stage larvae of *B. pahangi* when the jirds were 3–6 months old. They were bled at appropriate intervals after the inoculation. For studying the antibody titers of early course of infection, 5 jirds (6 months old) were inoculated intraperitoneally with 50 third-stage larvae of *B. pahangi*. Retro-orbital blood samples were collected at weekly intervals in capillary tubes. Sera were stored at −20 C until examination. A positive control serum was obtained by immunizing jirds with a crude extract of Ad emulsified with Freund's complete adjuvant followed by a booster injection without adjuvant.

The human serum samples were collected in Japan and the Philippines. The patients were infected with *Wuchereria bancrofti, Anisakis* sp. (Type 1), *Loa loa, Gnathostoma* sp., *Strongyloides stercoralis, Schistosoma japonicum, Entamoeba histolytica* or *Echinococcus multilocularis.*

Anti-IgG antibodies conjugated with horseradish peroxidase:

Anti-human IgG goat IgG conjugated with horseradish peroxidase (HPO) was obtained from Miles Yeda Lab. Inc.

Anti-jird IgG rabbit IgG was conjugated with HPO (Sigma Chem. type VI) by the oxidation method (Nakane and Kawaoi, 1974) and stored at −20 C until use.

Anti-jird IgG rabbit IgG was prepared as follows: Jird IgG was purified from pooled jird serum by the affinity chromatography on Protein A Sepharose column. Rabbits were immunized with the IgG emulsified with Freund's complete adjuvant followed by a booster injection 4 weeks later. The rabbits were bled 2 weeks after the booster injection. The specificity of the serum was confirmed by immunoelectrophoresis. Rabbit IgG was purified from the serum by salting out in 40% saturated ammonium sulfate and on DEAE-cellulose column. The purity of the preparation was confirmed by immunoelectrophoresis using anti-rabbit whole serum (Miles Yeda Lab. Inc.).

ELISA microplate test procedure:

The optimal concentrations of antigens and conjugates were determined by a checker board titration using positive and negative sera. Disposable polystyrene 96 well microtiter plates (micro-ELISA plate,

Dynatech, M 129 A) were sensitized by adding to each well 0.1 ml of Ad or Mf antigen diluted in carbonate buffer (0.05 M, pH 9.5). The plates were incubated in a moisture box at 37 C for 2 hours and kept at 4 C overnight. The plates were washed with PBS including 0.05% Tween-20 (PBS /T). Then 0.2 ml of PBS containing 1% bovine serum albumin (PBS/BSA) was added to each well. The plates were incubated in a moisture box at 37 C for 1 hour and washed with PBS/T. After the addition of 0.1 ml to each well of test serum diluted appropriately with PBS/BSA the plates were incubated in a moisture box at 37 C for 40 min and washed three times with PBS/T. Then 0.05 ml of anti-IgG conjugated with HPO diluted in PBS/BSA was added to each well. Again the plates were incubated at 37 C for 30 min and washed three times. Subsequently, added to each well was 0.3 ml of working substrate which was prepared by adding 1 ml of substrate solution (containing 100 mg o-phenylenediamine in 10 ml methanol) and 0.1 ml of 3% hydrogen peroxide solution to 99 ml of distilled water. The plates were kept in dark at room temperature for 30 min. The reaction was stopped by adding 0.025 ml of 8 N H_2SO_4 to each well. The absorbances at 500 nm of the contents in each well were determined by a microplate photometer (Corona MTP-12, Nissei Co.).

Results

ELISA titration:

In the first experiments, sera from jirds heavily or lightly infected with *B. pahangi* were assayed for antibody against Ad or Mf antigen along with sera from uninfected jirds or from jirds immunized with Ad antigen with Freund's complete adjuvant. The results are shown in Fig. 1. Sera from both groups of infected jirds showed strong antibody activity in ELISA.

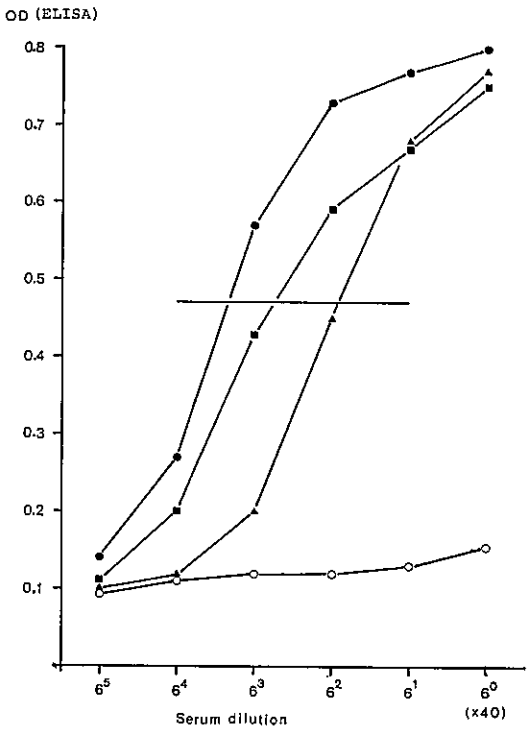

Fig. 1 ELISA of jird serum infected with *B. pahangi*. Six fold serial dilutions of sera initially diluted 1:40 in PBS/BSA were assayed by ELISA and the results were measured as optical density at 500 nm with Corona microplate photometer. Solid circles, positive control serum; open circles, negative control serum; solid triangles, serum of infected jird with large numbers of Ad (>11) and Mf ($>10^6$); solid squares, serum of infected jird with small numbers of Ad (1~5) and Mf (10^4~10^5).

On the other hand, optical density at 500 nm (OD 500) of uninfected jird serum did not exceed 0.18 at any dilution tested. In order to determine the background OD 500 of our assay system, serum samples from 40 uninfected jirds were examined at 1:40 dilution. Mean OD 500 of these samples and 99% confidence upper limit of OD 500 were 0.099 and 0.145, respectively. Antibody dose-response profiles (serum dilution in abscissa, OD 500 in ordinate) of infected sera and serum from jirds immunized with Ad with Freund's complete adjuvant showed sigmoid curve.

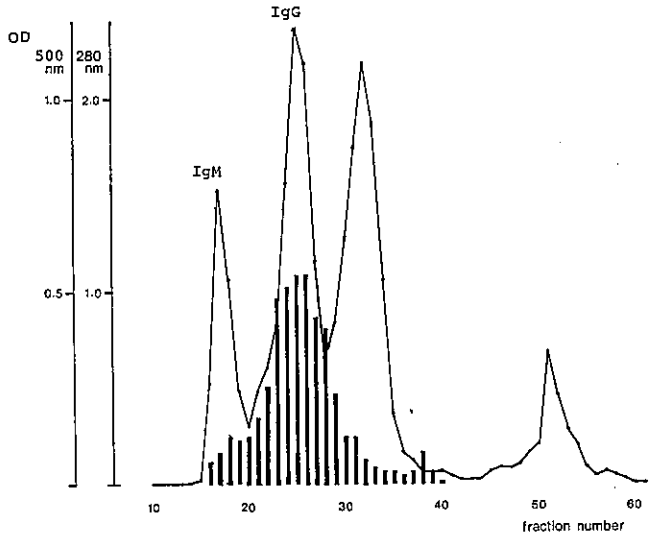

Fig. 2 Elution profile of jird serum infected with *B. pahangi* through Sephadex G-200 gel filtration. Protein concentration measured by OD 280 (solid circles connected by solid line) and antibody distribution in ELISA (OD 500, vertical bars). Note the values OD 500 in the first peak are less than 0.145.

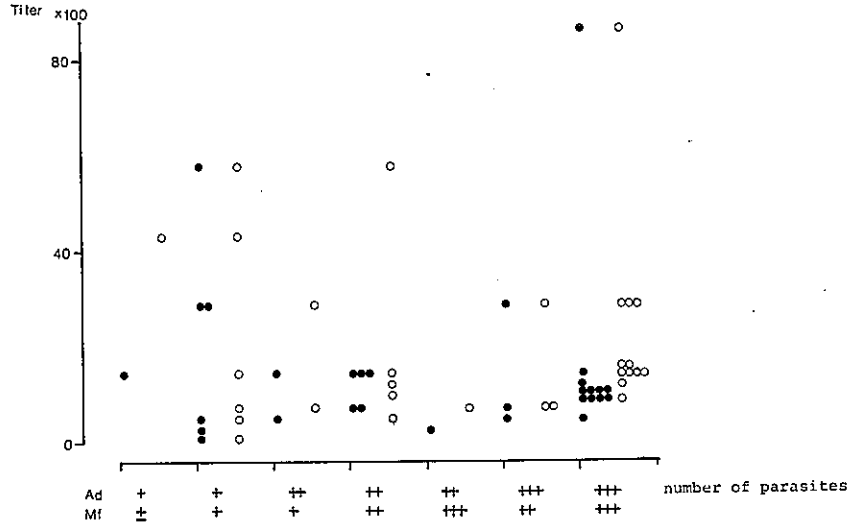

Fig. 3 ELISA titers of sera from infected jirds and numbers of Ad and Mf recovered from the peritoneal cavity. Solid circles, anti-Ad titers; open circles, anti-Mf titers. Ad: +, 1–5 worms/jird; ++, 6–10 worms/jird; +++, 11 worms/jird and over. Mf: ±, less than 10^4 worms/jird; +, 10^4 worms/jird up to 10^5 worms/jird; ++, 10^5 worms/jird up to 10^6 worms/jird; +++, 10^6 worms/jird and over.

The antibody titers of the serum samples, therefore, were determined thereafter by the serum dilution which showed equal OD 500 to 50% transmission of the dose-response curve of positive control serum assayed on each plate.

Reacting antibody in ELISA:

In order to see if the antibody reacting in the ELISA was IgG, serum from infected jirds was applied to a Sephadex G-200 column equilibrated in PBS. The fractions were assayed for antibody activity against Mf in terms of ELISA. The results are shown in Fig. 2 along with the optical density at 280 nm of each fraction. The antibody activities of the serum were distributed mainly in the second peak of the elution profile and did not show distinct peak in IgM rich fractions. The results strongly indicated that antibody reacting in our system to Mf antigen in infected jird serum were almost exclusively IgG.

Worm numbers and titers:

Figure 3 shows the correlation between the ELISA titers of serum from the jirds inoculated intraperitoneally with 100 third-stage larvae and the numbers of adult worms or microfilariae recovered from the peritoneal cavity after 180 days or more of infection. As shown in the figure there seemed to be no correlation between the number of worms recovered and antibody titers against Ad or Mf antigen. Despite the presence of live Ad and Mf in the peritoneal cavity, five sera of infected jirds (older than 30 months) were negative in ELISA. The antibody titers in jirds seemed to decrease with age (Fig. 4).

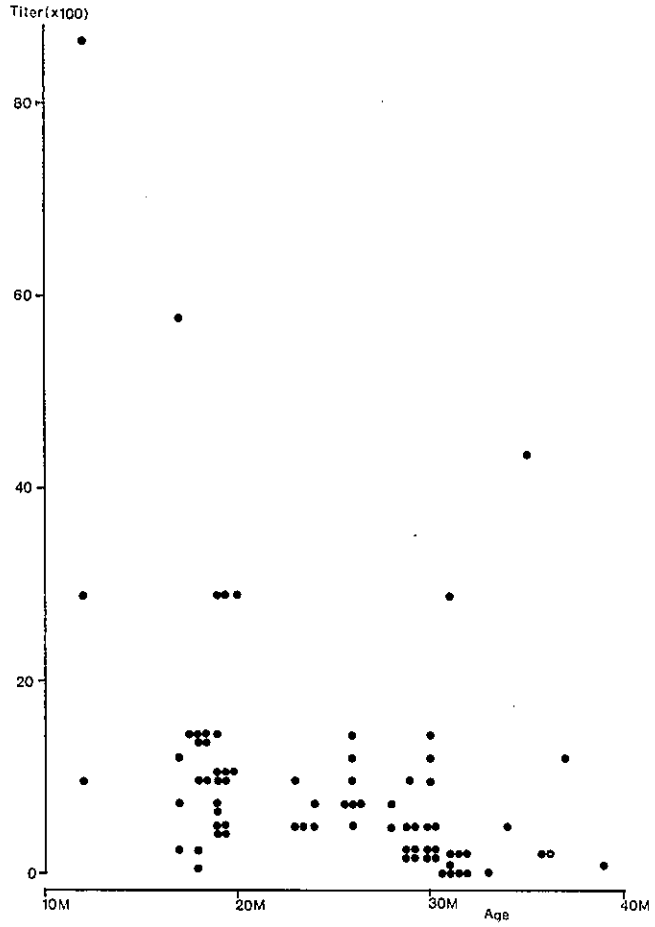

Fig. 4 Age dependency of ELISA titers of infected jirds. Note that the five jirds over 30 months of age are negative and the titers tend to decrease with age.

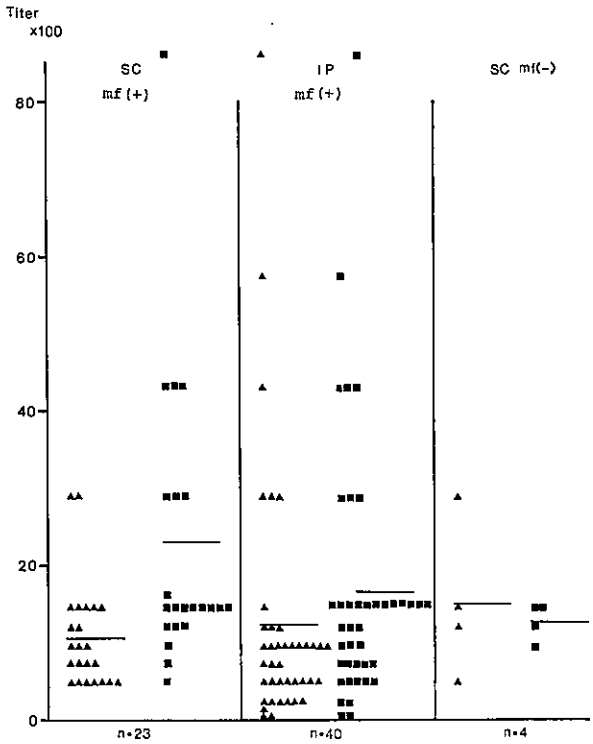

Fig. 5 ELISA titers of anti-Ad and anti-Mf in jirds inoculated with larvae subcutaneously (SC) with microfilaremia, SC mf (+), or without microfilaremia, SC mf (—), and jirds inoculated intraperitoneally (IP) and have Mf *in situ*, IP mf (+). Solid triangles, anti-Ad titers; solid squares, anti-Mf titers.

Table 1 ELISA titers in jirds after intraperitoneal inoculation of 50 *B. pahangi* larvae

Jird number	Anti-Ad titers at weeks after inoculation										
	0	1	2	3	4	5	6	7	8	9	10
1	0	0	0	8	40	240	240	960	1,440	960	720
2	0	0	4	0	4	4	8	14	8	28	720
3	0	0	0	0	0	*	4	80	20	40	20
4	0	0	*	0	0	4	20	40	120	120	120
5	0	0	0	*	28	8	28	40	40	480	960
Jird number	Anti-Mf titers at weeks after inoculation										
	0	1	2	3	4	5	6	7	8	9	10
1	0	0	0	4	8	40	80	240	480	240	240
2	0	0	0	0	0	0	4	4	30	30	160
3	0	0	0	0	0	0	0	*	*	*	9
4	0	0	16	*	24	8	16	24	32	80	120
5	0	0	0	0	4	20	80	20	20	4	20

*: less than 4 of ELISA titer, but absorbances of more than 0.2 were measured at 500 nm.
'0 week' denotes preinfection.

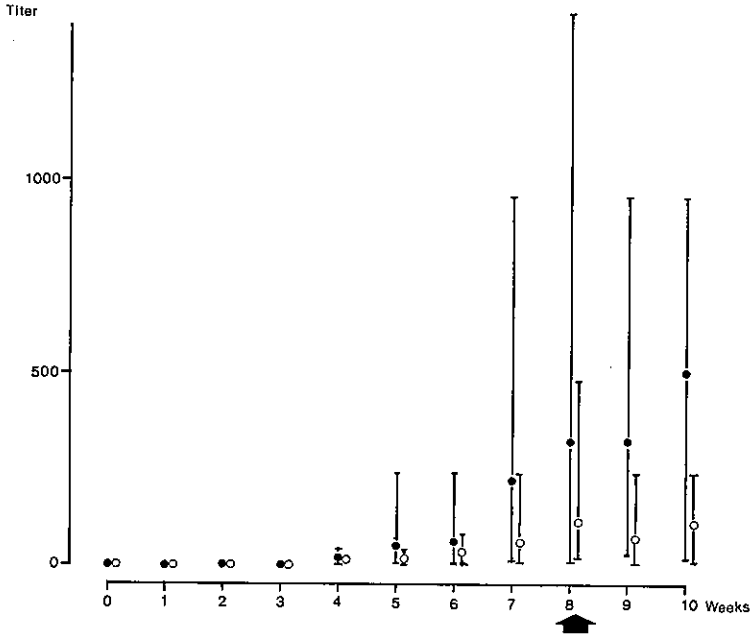

Fig. 6 ELISA titers in early course of infection in jirds inoculated with 50 larvae intraperitoneally. "0 week" denotes preinfecion. Solid circles, anti-Ad titers; open circles anti-Mf titers; an arrow, intraperitoneal appearance of Mf. Note the decrease of titers at 9 weeks after the inoculation.

Table 2 ELISA titers in human cases with parasitosis

Parasite	*Wuchereria bancrofti*											*Loa loa*
Area	Okinawa					the Philippines						Africa
Case number	1	2	3	4	5	1	2	3	4	5	6	1
Sex	M	M	M	M	M	M	M	M	M	M	M	M
Age	85	80	78	85	84	37	39	40	70	47	38	youth
Mf density/ml	0	0	0	0	0	8	6	2	47	3	720	?
Titer of anti-Ad	30	20	10	24	0	36	40	40	10	36	400	1,280
anti-Mf	12	10	0	10	0	20	20	40	10	20	400	640

Parasite	*Gnathostoma* sp.	*Strongyloides stercoralis*		*Schistosoma japonicum*		*Anisakis* sp.		*Entamoeba histolytica*	*Echinococcus multilocularis*
Case number	1	1	2	1*	2	1	2	1	1
Sex	M	M	M	M&F	M	M	M	M	M
Age	22	youth	56	—	40	youth	youth	50	45
Titer of anti-Ad	320	0	0	40	16	0	0	0	0
anti-Mf	140	0	0	4	10	0	0	0	0

*: pooled sera of 30 cases with schistosomiasis in the Philippines.

Routes of inoculation and titers:

Significant differences of antibody titers were not found between jirds receiving intraperitoneal inoculation and subcutaneous one in terms of both anti-Ad and Mf antigens. When jirds were inoculated

with larvae subcutaneously, most animals with microfilaremia showed similar antibody titers against both Mf and Ad antigens to animals without microfilaremia, although a few sera from the jirds with patent microfilaremia revealed a little higher titers against Mf antigen than against Ad (Fig. 5).

Titers in early course of infection:

Table 1 shows the changes of antibody titers in the jirds after the intraperitoneal inoculation of 50 third-stage larvae. As early as 2 to 4 weeks after the inoculation, the antibody was detected against Ad or Mf antigen and the titers were increased thereafter. As shown in Figure 6 a transient decrease of titers seemed to occur at 9 weeks coincidentally to the first appearance of the Mf.

Anti-B. pahangi *antibodies in patients with parasitosis*:

The sera of human cases infected with various species of parasites were examined for cross-reacting antibodies against *B. pahangi* antigens in ELISA (Table 2). In most of the patient sera antibody titers against Ad were higher than those against Mf except in the cases of *W. bancrofti* with microfilaremia, in whom the titers of anti-Ad and anti-Mf were same.

1) *Wuchereria bancrofti*: Antibodies were detected in the sera of 4 patients out of 5 who had been diagnosed as being infected with *W. bancrofti* and untreated with any parasiticidal drugs more than ten years in Okinawa, and had symptoms of chyluria, elephantiasis, or hydrocele without microfilaremia. They were older than 70 years of age. All patients with microfilaremia in the Philippines except one had higher titers than the patients without microfilaremia in Okinawa.

2) *Loa loa*: A Japanese young patient infected in Africa had antibody at the highest titer in human cases examined.

3) *Gnathostoma* sp.: A Malaysian student with positive skin test for gnathostomiasis had creeping eruption a few weeks after eating a slice of raw catfish in Thailand and had antibody titer against both Ad and Mf.

4) *Schistosoma japonicum*: The pooled serum of thirty patients in the Philippines complicated with *Ascaris, Trichuris* and/or hookworm infections showed the low antibody titer against *B. pahangi* antigen, although it showed strong antibody activity against *S. japonicum* egg antigens in ELISA. An another serum with an "old" *Schistosoma* infection without current infection of any nematoda revealed low titers of cross-reacting antibody to *B. pahangi*.

5) *Strongyloides, Entamoeba, Echinococcus* or *Anisakis* sp.: Antibody cross-reacting with *B. pahangi* antigens in ELISA was not detected in the sera from patients infected with these parasites.

Discussion

Immunological investigations of *B. pahangi* and *B. malayi* infection in experimental animals or human cases have been reported. In these reports various immunological techniques have been used: complement fixation test, indirect fluorescent antibody techniques (IFAT), ELISA, etc. (Benjamin and Soulsby, 1976; Desowitz *et al.*, 1978; Grove, 1981; Ridley and Hedge, 1977; Spencer *et al.*, 1981). The correlation between microfilaremia and antibody responses has been complicated, so far. During the microfilaremic phase of the infection, serum antibody against microfilaria surface antigen was reported not to be detected (Ponnudurai *et al.*, 1974). Some investigation have reported that there was a significant correlation between the amicrofilaremia and the anti-sheath antibody detected by the IFAT using an intact microfilaria as antigen (McGreevy

et al., 1980; Piessens *et al.*, 1980b). Sonicated microfilariae have been reported to be suitable antigen for the detection of antibodies against microfilariae in jirds (Singh *et al.*, 1980) and even for the diagnosis of human filariasis (Hedge and Ridley, 1977) irrespective of the presence of microfilaremia. Cytoplasmic antigen would be exposed by the sonication.

In the present study, IgG anti-Mf antibodies could be detected in ELISA irrespective of the presence of microfilariae in blood or peritoneal cavity in jirds using water-soluble microfilarial antigen. The anti-Mf antibody detected in our system could be considered to react with cytoplasmic antigens of microfilariae. In our results titers of anti-Mf antibody were almost parallel to the titers of anti-Ad antibody in infected jirds. A lack of specificity had been indicated by Hedge and Ridley (1977) with regard to cytoplasmic antigens of microfilariae.

In order to investigate the differences in antigenicity between adult worm and microfilarial antigen of *B. pahangi*, absorption experiments were carried out. The serum from infected jirds was mixed with the homogenate of male adult worms and incubated for 30 minutes at 37 C. The absorbed serum showed anti-Ad antibody titer less than one sixteenth of the original serum and anti-Mf antibody titer, one third. Thus, there seems to be difference between Ad and Mf in their antigenicity.

The antibody titers were independent of the numbers of worms recovered six months after infection. These results suggest that a small number of worms (approximately less than 10,000 microfilariae or 5 adult worms in a jird) can be enough to elicit the antibody production against *B. pahangi*.

It is generally accepted that worms inoculated in the peritoneal cavity of the jirds produce Mf in the cavity and the Mf thus produced and localized in the cavity follow an aberrant mode of life different from the lymph-dwelling worms which were inoculated subcutaneously, although the Mf from jird peritoneal cavity was proved to reach the infective stage in mosquitoes (Chuang *et al.*, 1979). However, the findings that antibody titers did not depend on inoculating routes and localization of worms revealed that the 'aberrant worms' could cause antibody production in jirds as the lymphatic worms.

In our experiments no antibody could be detected in some old jirds despite the patent infection of Ad and Mf. An additional experiment was carried out to see if these findings could be due to the age-related immunodeficiency. Various ages of jirds (6–32 months) were immunized with sheep blood cells and antibody titers were examined by the hemagglutination.

No significant difference was observed in antibody responses among various age groups tested, indicating that the absence of antibody responses in old infected jirds can not be caused by aging. More over, abilities of antibody responses against sheep red blood cells were not suppressed after the inoculation of live microfilariae into the peritoneal cavity (500,000 Mf/jird) (data not shown). McGreevy *et al.* (1980) suggested that anti-sheath antibodies might play a role in regulating peripheral microfilaremia. Further, the correlation between regulation of patent microfilarial infection and cellular immunity has been reported (Piessens *et al.*, 1980a,c; Subrahmanyam) *et al.*, 1978; Suswillo *et al.*, 1980, 1981; Thompson *et al.*, 1981). The present findings, however, revealed that the antibodies detected by our system can not directly be related with the regulation of peripheral microfilaremia nor that of intraperitoneal Mf. The results presented in this paper suggest the possibility that the ELISA system can be applied for screening test of human filariasis patients taking advantage

of cross-reaction between *B. pahangi* and *W. bancrofti*. For this purpose the adult worm antigen seems to be better than the microfilarial antigen at the same protein concentration. The test is so sensitive as to reveal past infection of bancroftian filariasis, although some aged patients give low titers or negative results.

Summary

IgG antibodies in the sera from the jirds infected with *Brugia pahangi* and from human with parasitosis were detected by ELISA using crude extracts of adult worms or microfilariae of *B. pahangi* recovered from the peritoneal cavity of jirds as antigens.

1) In infected jirds the antibodies were detected as early as 2 to 4 weeks after inoculation. The titers were almost equal to each other against both antigens from 6 to 24 months of infection. The titers seemed to decrease with age. Antibodies could not be detected in some old jirds.

2) There was no significant difference in titers among the groups of jirds divided by the worm burden. Marked difference in titers was found neither between microfilaremic and amicrofilaremic jirds, nor between jirds inoculated subcutaneously and intraperitoneally.

3) By the absorption of infected jird serum with homogenized male adult worms, the titer was decreased to one sixteenth or less against adult antigen and one third against microfilarial antigen.

4) Antibodies cross-reacting against adult and microfilarial antigens of *B. pahangi* were found in the sera from human cases infected with *Wuchereria bancrofti, Loa loa* or *Gnathostoma* sp. The sera of patients infected with *Schistosoma japonicum* were weakly positive against both antigens mentioned above.

Acknowledgement

Grateful acknowledgment is made to Prof. R. Kano, Tokyo Medical and Dental University for his constant interest and guidance in this investigation. Thanks are tendered to Prof. H. Tanaka, Institute of Medical Science, The University of Tokyo for helpful discussion. Thanks are also due to Dr. M. Sakamoto, Department of Parasitology, Institute for Tropical Medicine, Nagasaki University for providing vector mosquitoes, to Dr. H. Matsuda, Institute of Medical Science, The University of Tokyo for providing pooled sera of schistosomiasis, and to Dr. K. Araki, Institute of Public Health for providing human sera of anisakiasis and strongyloidiasis.

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Brugia pahangi 感染スナネズミにおける IgG 抗体産生の ELISA による検出

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Brugia pahangi 感染スナネズミならびにヒト寄生虫症例の血清中の IgG 抗体を，スナネズミ腹腔より得た *B. pahangi* の成虫と仔虫の抽出物を抗原として ELISA で検出した．

1) 感染スナネズミでは，接種後 2～4 週で抗体が検出された．感染 6 ケ月より24ケ月に於ては，成虫抗原と仔虫抗原に対し同様の抗体価を示すものが多かった．抗体価は月齢と共に低下の傾向を示し，老齢の数匹では抗体を検出できなかった．

2) 回収虫体数でスナネズミを分けても，抗体価に差を認めず，仔虫血症の有無，或は皮下または腹腔内という接種方法の違いによっても抗体価に差を見出せなかった．

3) 雄成虫体のホモジネートで感染スナネズミ血清を吸収すると，抗体価が成虫抗原に対し 1/16 以下，仔虫抗原に対しては 1/3 に低下した．

4) バンクロフト糸状虫，ロア糸状虫，顎口虫のヒト症例では，*B. pahangi* の成虫ならびに仔虫抗原と交叉反応を示す抗体を検出した．日本住血吸虫症では弱い交叉反応を認めた．

フィラリア症の感染成立を論ずる際，ミクロフィラリアに対する抗体の存在が宿主のミクロフィラリア血症を調節する重要な要因であるか否か問題となるが，本実験においてミクロフィラリア血症の有無にかかわらず感染スナネズミがミクロフィラリアに対する抗体を有していることが判明した．