ANIMAL FILARIAL ANTIGENS IN SEROLOGICAL TEST FOR HUMAN FILARIASIS

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INTRODUCTION

Filariasis, a parasitic disease found in many countries of Southeast Asia, is caused by either *Brugia malayi* or *Wuchereria bancrofti* nematodes. The presence of microfilaria in the blood has been used routinely for the diagnosis of filariasis and immunological techniques have been carried out by many investigators such as the skin test (Sawada *et al.*, 1965; Grove *et al.*, 1977), complement fixation (Danaraj *et al.*, 1959), haemagglutination (Tanaka *et al.*, 1968), precipitation (Desowitz *et al.*, 1976), and fluorescent-antibody (Yong, 1973; Ambroise - Thomas, 1974). The antigens employed in the investigations mentioned, were mostly prepared from the filaria of heterologous host animals which were not specific and gave cross-reaction with sera from patients infected with many other parasites. The present study is to prepare the antigen for hemagglutination test from adult *Dirofilaria immitis* in order to use for the serological diagnosis of human filariasis caused by *Brugia malayi.*

MATERIALS AND METHODS

D.immitis worms were collected alive from the heart of the infected dogs at autopsies. The worms were separated from blood clots and washed in several changes of cold physiological saline solution (PSS) to remove as much as possible the host contaminants. Finally, these were soaked in distilled water and dried by lyophilization (lyophilized worms) or blot with filter paper under slight finger press (dehydrated worms). The crude extract antigens (CrAg) of the *D.immitis* were prepared as previously described for rat filariasis by Tanaka, *et al.*, (1968) with slight modifications. Sixty milligrams of dehydrated worms or ten milligrams of lyophilized worms were suspended in one ml of phosphate buffered saline pH 7.2 (PBS pH 7.2) and the worms were ground using tissue grinder. The grinding was performed 4-10°C.When the preparation looked homogeneous, it was subjected to MSE ultrasonicator for 20 minutes. The preparation was kept at 4°C overnight, then it was centrifuged at 10,000 g at 4°C for 15 minutes. The collected supernatant was kept at 4°C for two days. Recentrifugation at 13,000 g at 4°C for 30 minutes. The supernatant fluid was collected and dialysed against excess volume of cold distilled water overnight. The protein content of the preparation was determined and the volume was measured. The extract was concentrated to the desired protein concentration by lyophilization and was kept at-20°C for use as crude antigen (CrAg). The protein concentration of the antigen was determined using micromodification of Folin-Ciocalteu method (Kabat and Mayer 1961).The bovine serum albumin was employed for preparing standard curve.

Ninety-four filariasis sera (F) were collected from persons infected with *B.malayi*, as examined by the presence of the microfilaria in the peripheral blood. These persons lived in Hulu Sungai Utara, South Kalimantan which is one of the filariasis endemic areas in Indonesia. The microfilaria were examined from thick smear slides of 20 c.mm finger -

prick blood stained with Giemsa. Forty eight of these persons are males. The age of persons ranged from 17 to 70 years old. The microfilarial density ranged from 1 to 158 per thick smear. Some of these infected persons showed clinical manifestations, i.e. lymphangitis in 5 persons and elephantiasis in 3 persons.

Sera from thirty patients, who were admitted to the Hospital for Tropical Diseases, Bangkok, Thailand, were used as a comparative control. Twenty-one of them are females and their age ranged from 13 to 66 years old. All of them had hookworm egg positive in stools with the egg count of 400 to 34,000 eggs per gram of faeces. Some of these patients had other helminthic infections such as opisthorchiasis (16 persons) and fasciolopsiasis(1 person). This group of sera was regarded as O.

Forty-nine sera collected from normal, healthy individuals (N) whose stools were negative for parasitic egg were used as negative control group.

Gel-filtration column chromatography : Preswollen Sephadex G_{200} (in distilled water for 72 hours) was packed into a 2cm × 90 cm glass column, then the column was equilibrated with the 0.05 Tris-HCl buffer pH 8.6. Five ml of antigens containing 20 mg protein per ml was fractionated. Before applying to the column, the antigen was dialysed against the tris buffer at 4°C for at least 2 hours then centrifuged at 13,000 g at 4°C for 15 minutes. The supernatant collected was applied to the column. Elution was done using the same buffer at a flow rate of 9.6 ml per hour. The eluate was collected at 3 ml per fraction. The protein content of each fraction was determined by measuring absorbency at 280 mμ using a spectrophotometer.

Ion-exchange column chromatography : DE_{52} cellulose microgranules (preswollen) was equilibrated completely with 0.05 M phosphate buffer pH 7.0 and packed into 1.5 cm × 25 cm glass column. Sample containing 20 mg protein per ml (volume 2 ml) was applied to the column. The gradient was set up from 0.05 M phosphate buffer pH 7.0 to 1 N sodium chloride solution at a flow rate of 36 ml per hour. The antigen to be applied to the column was dialysed extensively against the phosphate buffer and centrifuged as for Sephadex column. Collection of eluate and determination of protein were performed in the same manner as the Sephadex.

The passive haemagglutination test (HA) was carried out as described by Tanaka *et al.*, (1968) with slight modification. Human group O red blood cells were prepared at 2.5% concentration in phosphate buffer saline (PBS) pH 7.2. The red blood cells were tanned by adding equal volume of 1 :120,000 tannic acid solution to the 2.5% red blood cells. The mixture was kept in ice bath with occasional shaking for 15 minutes. The cells, then, were washed once with cold PBS pH 7.2 and resuspended to the original concentration. The tanned cell suspension was mixed with an equal volume of the required antigens diluted in PBS pH 6.4 to the final protein concentration of 2 mg per ml. The mixture was incubated at 37°C for 15 minutes with rotation. The cells were washed twice with cold normal saline solution containing 1% heat inactivated normal rabbit serum. Finally the cells were restored to 2.5% concentration in the same diluent.

The microtitration was carried out in a U glass microhaemagglutination tray. Each serum to be tested was heated at 56°C for 30 minutes before the titration. It was diluted in serial two fold manner with the 1% normal rabbit serum in normal saline solution. To each well of the serum dilution 1 drop (0.025 ml) of the antigen sensitized tanned cells was added and mixed well. The tray was incubated at 37°C for 30 minutes and at 4°C overnight.

The haemagglutinating titre was determined by Jacob and Lunde (1957) classification.

RESULTS

The ranges and geometric mean HA titres (GMT) of sera from group F, group O and group N are shown (Table 1). Table 2 revealed the number of sera from each group at different HA titres. For example, 2 sera in group F had HA titre 1 : 32; 5 sera at 1 : 64; 25 sera at 1 : 128 etc.

The HA test using CrAg sensitized tanned cells could differentiate only 9.5% of group F sera (9 from 94 sera showing HA titre 1 : 1024 or higher) from group O sera. GMT of both groups showed no significant difference ($p > 0.01$) although they are different from the GMT of the N group ($p < 0.01$). The CrAg was, therefore, fractionated by Sephadex G_{200} column chromatography in order to obtain the more specific antigen for group F sera.

Table 1

The geometric mean HA titres (GMT) and titre ranges from patients with filariasis, with other parasites and normal persons.

Sera	No. tested	GMT	Titre range
Filariasis	94	1:256	1:32-1:2048
Other	30	1:181	1:64-1:512
Normal	49	1:56	1:16-1:256

Four protein profiles, P_1S, P_2S, P_3S and P_4S, were obtained from the Sephadex fractionation of CrAg (Fig. 1). Each profile, after dialyses against excess volumes of distilled water and dried by lyophilization, was used to sensitize tanned red blood cells. The cells were tested against 26 randomly selected group F sera and 9 sera of group O which yielded high HA titres (> 1 : 128) against the CrAg sensitized tanned cells.

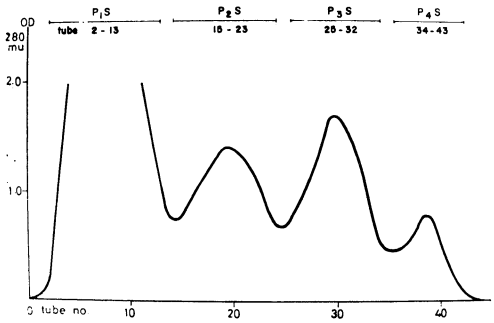

Fig. 1—Sephadex G_{200} column chromatography of the CrAg.

It was found that all of the selected group F and group O sera gave negative result when tested against the P_3S and P_4S sensitized cells. Therefore the P_3S and the P_4S are not suitable antigenic fractions in our system. The HA titres of 26 sera of group F and 9 sera of group O against the P_1S and P_2S coated cells are shown in Table 3. The P_1S coated cells yielded higher GMT to group F than the GMT of group O. However, opposite

Table 2

The comparative number of F,O and N sera at various HA titres against the CrAg, sensitized tanned cells.

Sera	HA titres							
	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2045
F	0	2	5	25	35	18	5	4
O	0	0	2	15	9	4	0	0
N	3	15	23	5	3	0	0	0

results were observed when the P_2S coated cells were used in the HA.

Table 4 shows the numbers of sera which gave different HA titres against P_1S and P_2S coated cells. The P_1S coated cells could differentiate about 81% of the group F sera from group O sera at the cut off titre 1:128.

From these results the P_1S seemed to be the most reactive antigen obtained from *D.immitis* against the filariasis sera in our study. The P_1S was, therefore, fractionated further by ion-exchange chromatography. The P_1S at 20 mg protein per ml was applied to DE_{52} column equilibrated with 0.05 M phosphate buffer pH 7.0 to 1 N NaCl. Four groups of proteins namely P_1D, P_2D, P_3D and P_4D were obtained from the fractionation of the P_1S (Fig. 2).

HA test was performed on 14 sera, out of the 26 randomly selected group F using

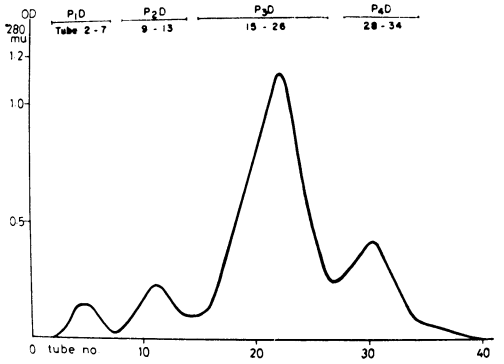

Fig. 2—DE_{52} cellulose column chromatography of the P_1S.

tanned cells sensitized with either P_1D, P_2D P_3D, or P_4D. The results are shown in Table 5. The P_3D coated tanned cells gave the highest GMT against the 14 sera. When these sensitized cells were tested against the same 9 sera from group O as for those tested with P_1S and P_2S sensitized cells the GMT was remarkably lower than of group F.

Table 3

The geometric mean HA titres and titre ranges of F and O sera against P_1S and P_2S sensitized tanned cells.

Sera	Antigens	No.tested	GMT	Titre range
F	P_1S	26	1:154	1:64 - 1:512
	P_2S	26	1: 28	1:16 - 1:128
O	P_1S	9	1: 47	1:32 - 1: 64
	P_2S	9	1:128	1:64 - 1:256

Table 4

The comparative number of F and O sera at various HA titres as tested against the P_1S and P_2S sensitized tanned cells respectively.

Sera	Antigens	HA titres					
		1:16	1:32	1:64	1:128	1:256	1:512
F	P_1S	0	0	5	13	4	4
	P_2S	12	9	3	2	0	0
O	P_1S	0	4	5	0	0	0
	P_2S	0	0	2	5	2	0

Table 5

The geometric mean titres (GMT) and titre ranges of F and O sera obtained from HA test using either P_1D, P_2D, P_3D, P_4D sensitized tanned cells.

Sera	Antigens	No. tested	GMT	Titre range
F	P_1D	14	1:12	1: 8 - 1: 32
	P_2D	14	1: 4	1: 2 - 1: 8
	P_3D	14	1:52	1:32 - 1:128
	P_4D	14	1: 9	1: 4 - 1: 32
O	P_3D	9	1: 9	1: 4 - 1: 16

When the P_3D was used to sensitize the tanned red cells, the cells could differentiate 100% of group F sera from group O sera on the basis of cut off HA titre 1 : 32.

DISCUSSION

Although filariasis is usually diagnosed by the presence of microfilaria in the blood circulation, serological diagnosis is sometimes required. The antigens used in most immunological tests for the diagnosis of human filariasis are prepared from heterologous filarial species for example *D.immitis* (Sawada *et al.*, 1965). These antigens have been used extensively because of the easy availability and supplies of large quantities. The ideal antigen which could be used for the diagnosis of human filariasis should give either specific reaction only to human filariasis sera or stronger reaction (higher antibody titre) with the filariasis sera than with sera of other parasitic infections.

To prepare such antigen, adult *D.immitis* were extracted with phosphate buffer pH 7.2. This extract (CrAg) when used to sensitized the tanned red cells gave the geometric mean haemagglutinating titre (GMT) at 1 : 256 to the filariasis (F) sera and 1 : 181 to sera of patients infected with other parasites (O). These GMT were significantly different from the GMT of normal (N) sera (1 : 56). However, the GMT of the F and O sera were not statistically different. Nevertheless, the CrAg sensitized tanned cells could differentiate about 10% of the F sera from the O sera at the cut off titre 1 : 1024.

Fractionation of the CrAg through Sephadex G_{200} column yielded four protein groups of different molecular sizes namely P_1S, P_2S, P_3S and P_4S respectively. The P_3S and P_4S were found to be non-reactive in our experiments. The P_1S coated red cells gave high titre with the F sera (GMT 1 : 154) which was significantly different ($p < 0.01$) from the GMT of the O sera (1 : 47). The opposite results were found with the P_2S coated cells. The results indicate, perhaps, that the P_1S contains the most reactive protein antigens against filariasis sera. Besides, about 80% of the F sera could be differentiated from group O sera on the basis of HA titre 1 : 128 against the P_1S coated cells.

Attempts were made to prepare antigen from the P_1S which would be able to differentiate 100% of the F sera from the O sera. The concentrated P_1S yielded four proteins profiles after fractionation by DE_{52} column. Among these four proteins, the P_3D was the most reactive one in the HA test against the F sera. The P_3D coated red cells could differentiate 100% of the group F from the group O sera at the cut off titre at 1 : 32.

It was concluded that the P_3D was the best antigen obtained from *D.immitis* in the passive haemagglutination test towards human filariasis. Unfortunately, sera of patients infected with *B.malayi* without circulating microfilaria were not available. The P_3D sensitized cells, therefore, have not been tested against the sera of the occult filariasis.

SUMMARY

The results of passive haemagglutination tests showed that geometric mean titres (GMT) of filariasis sera (F) and the sera from patients with other helminthic infections (O) were significantly different from the GMT of the sera from normal individuals (N) when tested against tanned cells coated with crude extract of adult *D.immitis*. The GMT of the F and the O group sera were not significantly different from each other when the same antigen were used. About 10% of the F group sera could be differentiated from the sera of the O group by the crude antigen sensitized cells. Four protein peaks were obtained from the fractionation of the crude antigen through Sephadex G_{200} column chromatography. The results of haemagglutination test showed that the first peak (P_1S) gave significantly higher titres with the F sera than with the O sera. About 80 % of the F sera could be differentiated from the sera of the O group using the P_1S coated red blood cells. The second peak (P_2S) coated cells yielded significantly higher titres against the O sera than the F sera. The P_1S was further fractionated by DE_{52} column chromatography. Four protein profiles were obtained. They were termed P_1D, P_2D, P_3D and P_4D respectively. The results of the haemagglutination test showed that the P_3D was the most reactive fraction against the F sera among the four DEAE peaks. The P_3D coated cells differentiated 100% of the F sera from the O sera. There was significant difference between the GMT of the F and the GMT of the O sera.

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