J. Biosci., Vol. 6, Number 2, June 1984, pp. 165–171.

Evaluation of fractionated *Wuchereria bancrofti* microfilarial excretory-secretory antigens for diagnosis of bancroftian filariasis by enzyme linked immunosorbent assay

M. V. R. REDDY, ASHOK MALHOTRA, G. B. K. S. PRASAD and B. C. HARINATH*

Department of Biochemistry, M. G. Institute of Medical Sciences, Sevagram, Wardha 442 102, India

MS received 14 October 1983

Abstract. The *Wuchereria bancrofti* microfilarial excretory-secretory antigens were fractionated into ES1, ES2, ES3 and ES4 by ultra-membrane filtration and evaluated for their diagnostic utility by enzyme linked immunosorbent assay. Three of the four fractions showed antigenic activity (ES2, ES3 and ES4). The antigen fractions ES2 and ES4 were highly active in the detection of filarial IgM antibody in clinical filariasis and microfilaraemia respectively. The chemical characterization of the ES2 and ES4 antigen fractions showed that they were glycoproteins.

Keywords. Microfilarial excretory-secretory antigen; ultramembrane filtration; enzyme linked immunosorbent assay.

Introduction

Excretory-secretory (ES) antigens were highly sensitive and specific tools for the diagnosis of parasite diseases such as Chagas disease (Tarrant *et al.*, 1965), toxocoriasis (de Savigny *et al.*, 1977) and onchocerciasis (Schiller *et al.*, 1980). Studies from this laboratory have shown that the *Wuchereria bancrofti* microfilarial (mf) ES antigens are highly sensitive in detecting antibody in filarial sera as well as in filter paper blood samples (Kharat *et al*., 1982; Malhotra *et al*., 1982). However mf ES antigen from culture fluid could not distinguish active infection from clinical filariasis in enzyme linked immunosorbent assay, using either mixed or specific anti-immunoglobulin-enzyme conjugates. Separation and characterization of different antigen fractions from the whole antigen has proved to be a meaningful approach in obtaining an active fraction with the required specificities (Sawada *et al*., 1969; Marcoullis *et al*., 1978; Kaliraj *et al*., 1982). This communication presents the fractionation and characterize-ation of mf ES antigens and studies on their diagnostic utility for detecting human filariasis.

* Address for correspondence and reprint requests.

Abbreviations used: ES, Excretory-secretory; ELISA, enzyme-linked immunosorbent assay; mf, microfilarial; PBS/T, 0·01 M Sodium phosphate buffer saline pH 7·2, containing 0·05% Tween 20; IgG+M+A, antihuman immunoglobulins; GMRT, geometric mean of the reciprocal of antibody titre.

Materials and methods

Microfilarial excretory-secretory antigens

The mf ES antigens were prepared as described by Kharat *et al*. (1982). *W. bancrofti* mf were separated from microfilaraemia blood samples by nucleopore membrane filtration and maintained for 15 days in medium 199 (3–4 thousand mf/ml medium) supplemented with organic acids and sugars of *Grace's medium* (Paul, 1975). The medium was changed after every 24 h and the culture fluid was centrifuged at 13,000 *g* for 15 min. The supernatant was collected and the protein was estimated by Lowry's method (Lowry *et al*., 1951). Then it was stored at –20°C until used, after addition of sodium azide to a final concentration of 0·1% as preservative.

Sera

Human sera (32 samples), belonging to different groups *viz.,* normal subjects from endemic and non endemic regions (endemic and non endemic), filarial patients (microfilaraemia and clinical filariasis) were screened in this study. Filarial blood samples were collected from Sevagram and surrounding villages, which is an endemic area for nocturnally periodic form of *W. bancrofti.* Endemic normal samples were from healthy individuals living in Sevagram and its surrounding villages and having neither mf in their blood nor any clinical symptoms. The presence or absence of mf was confirmed by night blood (wet smear) examination. Non endemic normal blood samples were collected from students of this Institute coming from places like Chandigarh, Kashmir etc. where there is no filariasis immediately after their admission. Sera were separated from blood samples and stored at –20°C after addition of sodium azide as preservative.

Fractionation of mf ES antigen

The fractionation of mf ES antigen into different fractions by ultra membrane filtration was essentially as described by Nash *et al*. (1974) with *S. mansoni* homogenate and by Mok *et al.* (1977) with *Histoplasma capsulatum* antigen, except that Millipore ultrafiltration system (Millipore Corporation, Bedford, USA) was used in this study. One ml of culture fluid containing mf ES antigen was diluted to 20 ml with 0·05 M sodium phosphate buffer pH 7·2 and sequentially passed through CX-10 (NMWL 10,000), CX-30 (NMWL 30,000) immersible ultrafilters and PTHK 02510 (NMWL 100,000) pellicone ultra-filter membrane. At each stage of filtration, the filtrate was collected and the concentrate (0·1 ml) was diluted to 20 ml with the same buffer and refiltered. The process of dilution and refiltration was repeated twice and these filtrates were discarded. The concentrate was diluted to 20 ml with 0·05 M sodium phosphate buffer pH 7·2 for passing through the next filter in sequence. The filtrates collected first from CX-10, CX-30 and PTHK-02510 ultra filters were labelled as ES1, ES2 and ES3 fractions respectively and the concentrate on PTHK-02510 was diluted to 20 ml with 0·05 M sodium phosphate buffer pH 7·2 and labelled as ES4. All the four fractions were diluted 500 times with sodium carbonate buffer 0·06 M, pH 9·6 and used in indirect enzyme linked immunosorbent assay (ELISA) to assess their diagnostic utility.

*Characterization of ES*2 *and ES*4 *antigen fractions*

Treatment with enzymes: Three ml each of diluted (50 times) mf ES antigen fractions ES2 and ES4 in 0·05 M sodium phosphate buffer, pH 7·2 were treated separately with an equal volume of the following enzymes (at final concentration, 50μg/ml); chymotrypsin (Council of Scientific and Industrial Research Centre for Biochemicals, Delhi) in 0·05 M Tris-HCl buffer pH 7·4, α-amylase (Fluka, Switzerland), in 0·05 M Sodium phosphate buffer, pH 7·2 and lipase (Scientific and Industrial Supplies Corporation, Bombay) in 0·05 M citrate buffer pH 6·0. The incubation of the antigen-enzyme mixture was at 37°C for 24 h as described by Rolfe and Finegold (1979), followed by centrifugation at 13000 *g* for 20 min at 4°C. The supernatant was collected and diluted (5 times) to the optimum concentration of each antigen fraction with 0·06 M sodium carbonate buffer, pH 9·6 and used in ELISA to see the effect of different enzymes on the antigen fraction.

Treatment with sodium periodate: The antigen fractions ES2 and ES4 were diluted 50 times with 0·05 M sodium phosphate buffer pH 7·2 and treated separately with an equal volume of 0·1 M sodium periodate and kept at 37°C for 24 h (Mok *et al.,* 1977). The mixtures were centrifuged and the supernatants were used in ELISA as described above.

Heat inactivation: The tubes containing diluted (50 times) ES2 and ES4 antigen fractions were kept in boiling water bath for 45 min, the fractions were then centrifuged and the supernatants were used in ELISA after diluting 10 times with 0·06 M sodium carbonate buffer pH 9·6.

In one control experiment, the antigen fractions (ES2 and ES4) were mixed with an equal volume of 0·05 M Tris-HCl buffer pH 7·4, kept at 37°C for 24 h and used in ELISA. In another control experiment each antigen fraction was treated separately with heat inactivated enzymes and after centrifugation (13000*g* for 20 min), the supernatants were collected and used in ELISA.

ELISA

Penicillinase Type I (5 mg) Sigma Chemical Co., Saint Louis, Missouri, USA with specific activity of 2100 units/mg protein was conjugated to 40 mg of antihuman immunoglobulins (anti IgG+M+A) or individual anti-IgG or anti IgM (Immunodiagnostics, New Delhi) by the single step method of Avrameas using glutaraldehyde (Avrameas, 1969). The substrate consisted of soluble starch (150mg) in 27·5 ml of 0·2 M sodium phosphate buffer pH 7·0 containing 10·64 mg penicillin 'V' (Sigma Chemical Co., USA) and 65 μl of 0·08 M iodine in 3·2 M potassium iodide solution. The substrate was prepared fresh before use.

Micro ELISA was carried out as described by Kharat *et al.* (1982) in Cooke polyvinyl chloride microtitre U plates (No. 1-220-29). The optimally diluted mf ES, ES2 and ES4 antigen fractions in 0·06 M sodium carbonate buffer pH 9·6 were added to the wells and incubated at 37°C for 3 h. The wells were drained and the plate was further incubated with 200 μl of 3 % bovine serum albumin in the same carbonate buffer at 37°C for 2 h. The plate was then washed 5 times with 0·01 M phosphate buffer saline pH 7·2 containing 0·05 % Tween 20 (PBS/T). Hundred μl of diluted test sera (1:300) or serially

diluted (2 fold) test sera in PBS/T were added to the wells and incubated at 37°C for 3 h or at 4°C for overnight. After washing, 200 μl of 3 % bovine serum albumin in PBS/T was added to each well and incubated at 37°C for 2 h. After washing the plate again, 100 μl of optimally diluted conjugate in PBS/T (1:1600 for anti IgG+M+A penicillinase, 1:1600 for anti IgG penicillinase and 1:1000 for anti IgM penicillinase) was added and incubated at 37°C for 3 h. Following the final washing of the plate the immune reaction was revealed by adding 100 μl of the substrate solution to each well and incubating at 37°C for 30 min. The enzyme reaction was then terminated by adding 50 μl of 5 N HCl and the results were evaluated visually. The complete decolorization or decolorization with slight tinge of blue colour denoted the positive reaction while the negative reaction was confirmed by the persistance of blue colour. The antibody titres in the serum was the highest serum dilution showing decolorization with a slight tinge of residual blue colour.

Results

W. bancrofti mf ES antigen from culture fluid was fractionated by membrane filtration into four fractions of different molecular weights namely ES1 ($\leq$ 10,000), ES2 (10,000–30,000), ES3 (30,000–100,000) and ES4 ($\geq$ 100,000).The antigen fractions ES2, ES3 and ES4 showed the presence of antibody in all of the 8 microfilaraemia, 8 clinical filarial and none of the 2 non-endemic and 6 endemic normal sera, when tested in indirect ELISA, using anti-IgG + M + A conjugate (table 1). Two sera from each group of microfilaraemia and clinical filariasis were screened for filarial IgG and IgM antibody titres using the three antigen fractions (ES2, ES3 and ES4). The results (table 2) revealed that the two antigen fractions ES2 and ES4 showed different sensitivities in determining the filarial IgM antibody in microfilaraemia and clinical filariasis. The mean values of reciprocal of filarial IgM antibody titres in microfilaraemia sera were 750 and 12000 with ES2 and ES4 antigens respectively, whereas clinical filarial sera showed mean values of reciprocal of filarial IgM antibody titres of 12000 and 300. No significant difference was observed between the two groups of sera using anti IgG conjugate.

Table 1. Detection of antibody in human filarial sera by indirect ELISA using mf ES antigen fractions and anti IgG + M + A-penicillinase conjugate.

Sera	No Exam.	Number showing positive reaction* employing			
		ES1	ES2	ES3	ES4
Nonendemic normal (mf −ve)	2	0	0	0	0
Endemic normal (mf −ve)	6	0	0	0	0
Microfilaraemia (mf +ve)	8	0	8	8	8
Clinical filariasis (mf −ve)	8	0	8	8	8

* Sera showing positive reaction for filarial antibody at the dilution of 1:300.2

Table 2. Reciprocal of IgM and IgG antibody titres in filarial sera using ES2, ES3 and ES4 antigen fractions.

	Reciprocal of antibody titre (mean of 2 sera) employing					
	ES2		ES3		ES4	
Sera	IgM	IgG	IgM	IgG	IgM	IgG
Microfilaraemia (mf +ve)	750	19200	4800	19200	12000	19200
Clinical filariasis (mf −ve)	12000	19200	1200	12000	300	12000

Table 3 shows the analysis of 12 microfilaraemia and 12 clinical filarial sera for filarial IgM antibody titres using ES2 and ES4 antigen fractions. Statistical analysis of the reciprocal of antibody titres was carried out using Mann-Whitney U test. ES2 antigen fraction showed higher geometric mean of reciprocal of antibody titre (GMRT) in clinical filariasis (7620) than in microfilaraemia (2138) and the difference was significant ($P < 0{\cdot}05$). ES4 antigen fraction showed higher GMRT in microfilaraemia (9600) than in clinical filariasis (1347) and the difference was found to be significant ($P < 0{\cdot}01$). The differences in the antibody titres with ES2 and ES4 antigens in microfilaraemia ($P < 0{\cdot}05$) as well as in clinical filariasis ($P < 0{\cdot}025$) were also found to be significant.

The ES2 and ES4 antigen fractions were found to be highly reactive in detecting IgM antibody from clinical filarial and microfilaraemia sera respectively and hence these sera were used to assess the reactivity of concerned antigen fraction after treatment with different agents. The ES2 and ES4 antigen fractions when treated with buffer alone showed filarial IgM antibody titres of 9600 and 18200 respectively (table 4). After chymotrypsin treatment or heating at 100°C the ES2 antigen showed an antibody titre

Table 3. Distribution of filarial IgM antibody titre in microfilaraemia and clinical filariasis using ES2 and ES4 antigen fractions.

Antigen fraction	Sera	No Exam.	Reciprocal of filarial IgM antibody				GMRT*
			300	1200	4800	19200	
ES2†	Microfilaraemia (mf +ve)	12	3	4	2	3	2138[a]
	Clinical filariasis (mf −ve)	12	1	—	5	6	7620[b]
ES4†	Microfilaraemia (mf +ve)	12	—	1	4	7	9600[c]
	Clinical filariasis (mf −ve)	12	6	2	1	3	1347[d]

† Sera used were same for both the antigens.

* Geometric mean of reciprocal of antibody titres.

a vs. b ($P < 0{\cdot}05$); *c vs. d* ($P < 0{\cdot}01$); *a vs. c* ($P < 0{\cdot}05$) and *b vs. d* ($P < 0{\cdot}025$)– statistically significant.

of 300 with pooled clinical filarial serum and ES4 antigen showed an antibody titre of 1200 with pooled microfilaraemia serum. When treated with sodium periodate or α-amylase ES2 antigen showed antibody titre of 600 and ES4 antigen showed antibody titre of 300 with pooled clinical filarial and microfilaraemia sera respectively. However, there was no change in antibody titres in the control experiments where the ES2 and ES4 antigens were treated with heat inactivated enzymes. None of these treated and untreated antigen fractions showed reaction with non-endemic normal sera.

Table 4. Reciprocal of lgM antibody titre of pooled filarial sera with ES2 and ES4 antigens before and after treatment with different agents.

Treatment of antigen fraction with	Reciprocal of ELISA IgM antibody titre employing	
	ES2*	ES4*
Tris-HCl buffer	9600	18200
Sodium periodate	600	300
α-Amylase	600	300
Chymotrypsin	300	1200
Heat inactivation	300	1200
Lipase	9600	18200

* Pooled clinical filarial serum and pooled micro filaraemia serum were used for ES2 and ES4 antigen fractions respectively.

Discussion

W.bancrofti mf ES antigen from culture fluid was found to be highly sensitive when used in ELISA for the diagnosis of filariasis and only as little as 0·35 ng antigen per well was found to be sufficient (Kharat *et al.*, 1982). But no correlation was observed between antibody titres and disease status of the infection and mf ES antigen could not distinguish between microfilaraemia and clinical filariasis. This has necessitated the fractionation of mf ES antigen by membrane filtration, the method better suited for separation of minute amounts of ES antigens. Out of the four fractions obtained, three showed the antigenic activity. Nash *et al.* (1974) have sequentially passed the *S. mansoni* homogenate through Amicon Diaflo ultra filter membranes and found the antigen activity in only one fraction, having molecular weight of above 100,000. Mok *et al.* (1977) fractionated *H. capsulatum* yeast cell antigens by membrane filtration and observed the polydispersed nature of the antigens in several fractions.

Two of the three antigen fractions namely ES2 and ES4 showed different sensitivities while determining the filarial antibody titres in clinical filariasis and microfilaraemia (table 3). ES2 antigen detected elevated filarial IgM antibody (GMRT = 7620) in clinical filariasis than in microfilaraemia (GMRT = 2138) and ES4 in microfilaraemia (GMRT = 9600) than in clinical filariasis (GMRT = 1347). The differences were statistically significant. Similar observations were made with the two antigen fractions

mfS1b and mfS3e isolated from soluble antigens (mfS) of *W. bancrofti* microfilariae (Kaliraj *et al*., 1982). It would be of interest to identify and characterize these antigens.

Studies have been carried out in many parasite infections to identify the chemical nature of the antigens (Nash *et al*., 1974; Carlier *et al*., 1978; Tanner, 1963). Treatment of mf ES antigen fractions ES2 and ES4 with lipase did not reduce their reactivity. The reactivity of both the antigen fractions was reduced as observed from reduction in ELISA antibody titres, after treating with chymotrypsin or heating at 100°C, due to the degradation or inactivation of the protein part of the antigens. The loss in reactivity of both the antigen fractions was also observed after treatment with sodium periodate or α-amylase. This might be due to the destruction of the carbohydrate part of the antigens. These observations suggest that both the mf ES antigen fractions ES2 and ES4 might be glycoproteins.

Acknowledgement

This work was supported in part by Indian Council of Medical Research, New Delhi and by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. The authors are grateful to Dr. Sushila Nayar, Director and Dr. K. S. Sachdeva, Dean of this Institute for their keen interest and encouragement. Thanks are also due to Mr. Tekade and Miss Ranade for technical assistance.

References

Avrameas, S. (1969) *Immunochemistry*, **6,** 43.
Carlier, Y., Bout, D. and Capron, A. (1978) *Ann Immunol*. (*Inst. Pasteur*), **129C,** 811,
de Savigny, D. H. and Tizard, I. R. (1977) *Trans. Roy. Soc. Trop. Med. Hyg*., **71,** 501.
Kaliraj, P., Ghirnikar, S. N. and Harinath, B. C. (1982) *Indian J. Exp. Biol.*, **20,**440·
Kharat, I., Ghirnikar, S. N. and Harinath, B. C. (1982) *Indian J. Exp. Biol*., **20,** 378.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randal, R. J. (1951) *J. Biol. Chem*., **193,** 265.
Malhotra, A., Reddy, M. V. R., Naidu, J. N., Ghirnikar, S. N. and Harinath, B. C. (1982) *J. Biosci*., **4,** 507.
Marcoullis, G., Salonen, E. M. and Grasbeck, R. (1978) *Tropenmed. Parasit*., **29,** 39.
Mok, W. Y., Buckley, H. R. and Campbell, C. C. (1977) *Infect. Immun*., **16,** 461.
Nash, T, E., Prescott, B. and Neva, F. A. (1974) *J. Immunol.* **112,** 1500.
Paul, J. (1975) in *Cell and tissue culture*, (Edinburg, London, New York: Churchill Livingstone), p. 439.
Rolfe, R. D. and Fingold, S. M. (1979) *Infect. Immun.* **25,** 191.
Sawada, T. and Sato, K. (1969) *J pn. J. Exp. Med*., **39,** 427.
Schiller, E. C, D'Antonio, R. and Marroquin, H. F. (1980) *Am. J. Trop. Med. Hyg.,* **29,** 1215.
Tanner, C. E. (1963) *Exp. Parasitol*., **14,** 337.
Tarrant, C. J., Fife, E. H. and Anderson, R. L (1965) *J. Parasitol*., **51,** 277.