EFFECT OF NUTRITIONAL SUPPLEMENTS AND PROTEASE INHIBITOR ON THE YIELD OF ES ANTIGENS OF *BRUGIA MALAYI* MICROFILARIAE *IN VITRO*

Bonny Bhunia, R. S. Alli, M.V.R. Reddy* & B.C.Harinath

Jamnalal Bajaj Tropical Disease Research Centre and Department of Biochemistry, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha 442 102, India

ABSTRACT

Brugia malayi microfilarial excretory-secretory (mf ES) antigens obtained by *in vitro* maintenance of mf are important tools in the immunodiagnosis of bancroftian filariasis. To increase the yield of mf ES products, the effect of nutritional supplements on the culture medium (RPMI 1640) and the maintenance temperature were studied. Supplementation of RPMI 1640 medium with organic acids and sugars of Grace's insect culture medium for *in vitro* maintenance of 10 lakhs of mf in 40 ml medium increased the yield of mf ES antigen from 152 ug to 364 ug of mean protein content and the mean antigen titre from 200 to 400. Supplementation with phenyl methyl sulphonyl fluoride (PMSF) and shift in the culture temperature resulted in a further increase in the yield of mf ES antigen to 502 ug of mean total protein with an antigen titre of 800. The modifications resulted in a net increase of 3 fold in the protein content and 4 fold in the antigen titre of ES products. The above modifications in the *in vitro* maintenance of mf did not affect the diagnostic quality of mf ES antigen, which gave a sensitivity and specificity of 80% and 90% respectively in detection of filarial IgG antibodies in *Wuchereria bancrofti* infected cases.

KEY WORDS : *Brugia malayi*; *Wuchereria bancrofti*; excretory secretory antigens; ELISA.

INTRODUCTION

The tissue dwelling nematodes *Wuchereria bancrofti* and *Brugia malayi* are the causative agents of lymphatic filariasis, which is still a major public health problem in India and many other tropical and subtropical countries in the world. *In vitro* maintenance and cultivation of filarial parasites would facilitate in isolation of their excretory-secretory products as valuable

tools for the immunodiagnosis and immunoprophylaxis of filariasis. Identification of nutritional requirements of the parasites and evaluation of the efficacy of new antifilarial drugs are the other areas dependent on the availability of suitable *in vitro* maintenance system. Earlier we reported about the *in vitro* maintenance of *Wuchereria bancrofti* microfilariae (mf) (1) and infective (L₃) larvae (2) and *Brugia malayi* microfilariae (mf) (3) and infective (L₃) larvae (4,5)

Author for correspondence :

Dr. M.V.R. Reddy
Additional Professor, at above address.
Tel : 84341 Extn.: 262
FAX, 91-7152-44179

to collect ES antigens of immunodiagnostic and immunoprophylactic importance. The poor yield of ES antigen from *in vitro* cultures has been the main limitation in getting sufficient antigen material for their routine use in immunodiagnostic tests or in their further purification or in the generation of specific antibodies. In the present study we have evaluated the effect of nutritional supplements, temperature and enzyme protease inhibitor on the *in vitro* maintenance of *B. malayi* mf in terms of yield of ES antigen.

MATERIALS AND METHODS

Sera Samples

Human sera samples belonging to different groups viz., normal (endemic) and filarial (microfilaraemic and clinical filariasis) were collected from Wardha and the surrounding villages, known to be endemic for *W. bancrofti* infection. The presence of microfilariae was confirmed by the conventional night blood (wet smear) examination. Clinical filarial samples were collected from those suffering from acute and chronic disease manifestations like lymphoedema, funiculitis, epididymo-orchitis, hydrocele, elephantiasis etc. Endemic normal samples were collected from healthy individuals who had lived in a filarial endemic region over 5 years and had no history of filariasis. Sera were separated and stored at -20°C with sodium azide as preservative.

B. malayi microfilariae

Patent *B. malayi* infection was maintained in jirds, *Meriones unguiculatus* as described by Mc. Call, et.al. (6). Mf were obtained by lavage of the peritoneal cavities of jirds with

intraperitoneal filarial infection of 3 months or more duration. Mf were washed with normal saline and RPMI 1640 medium, plated on sterile petridishes and incubated at 37°C for 1 hr. to remove peritoneal cells. The mf were collected from petridishes and used for *in vitro* maintenance.

In vitro maintenance of *B. malayi* microfilariae

B. malayi mf were maintained *in vitro* in RPMI 1640 medium (10 lakhs mf in 40 ml medium) at 5% CO₂ for 48 hrs. The culture fluid was centrifuged at 14000 g, the supernatant was concentrated 40 fold by ultramembrane filtration, dialysed at 4°C against 0.01M sodium phosphate buffer, pH 7.2 and labelled as mf excretory-secretory (ES) antigen. The protein was estimated by Lowry's method (7) and stored at -20°C with protease inhibitors 0.2 mM N- α -p-tosyl-L-lysine chloromethyl ketone HCL (TLCK), 0.1 mM N-tosylamide-L-phenylalanine chloro-methyl ketone (TPCK), 0.1 mM phenyl methyl sulphonyl fluoride (PMSF), 1 mM ethylene diamine tetracetic acid, disodium salt (EDTA) and 0.1% sodium azide.

In separate experiments mf were maintained with and without supplementation of RPMI 1640 medium with organic acids (malic acid, α -ketoglutaric acid, D-succinic acid and fumaric acid at concentrations of 670, 370, 60 and 55 mg/liter respectively) and sugars (sucrose and fructose at concentrations of 26,680 and 400 mg/litre respectively) of Grace's insect culture medium (1). The mf were also maintained separately at 28°C and 37°C and with and without enzyme protease inhibitor, PMSF (0.1mM). The protein content and antigenic activity of ES antigen obtained from different experiments were determined.

IgG fraction of filarial serum immunoglobulins (FSIgG)

Filarial serum immunoglobulins (FSIgG) were isolated from pooled clinical filarial sera by 33% saturation with ammonium sulphate followed by DEAE cellulose ion exchange (Whatman DE 52) chromatography as described by Reddy, et al. (8).

Microtitre plate Inhibition ELISA

Bm mf ES antigen was conjugated with enzyme penicillinase (Hindustan Antibiotics Ltd., Pune) following the single step method of Avrameas (9). The inhibition ELISA was carried out as previously described (10) with few modifications. FSIgG sensitized wells (1.5 ug/50 ul/well) in polyvinylchloride plates (Dynatech, USA), serially diluted Bm mf ES antigen (starting with 25 ng/50ul/well), optimally diluted Bm mf ES antigen-penicillinase conjugate (1:1000), and starch-iodine-penicillin 'V' substrate were used in the assay. The final dilution of Bm mf ES antigen which delayed the decolorization of the blue substrate by at least 5 min. compared to decolorization in the wells coated with FSIgG and incubated directly with penicillinase conjugate was considered as inhibitory titre.

Stick Indirect ELISA

Stick ELISA for detection of filarial IgG antibodies in different groups of human sera was carried out as described by Reddy et. al. (11).

Statistical analysis

Student's un-paired 't' test was used to compare the yield of mf ES antigen from

different experiments.

RESULTS AND DISCUSSION

Excretory-secretory (ES) products of different stages of filarial parasites maintained *in vitro* have diagnostic and prophylactic use in filariasis. *B. malayi* mf ES antigens obtained by maintaining the parasite in RPMI 1640 medium at 37°C were shown to be useful for detection of filarial IgG antibodies (3) and circulating filarial antigen (3) in *W. bancrofti* infected cases. In the present study the effects of nutritional supplements and addition of protease inhibitor in the medium and alteration in temperature were evaluated in terms of increasing the yield of *in vitro* released ES antigens.

The supplementation of RPMI 1640 medium with organic acids (malic acid, α -keto glutaric acid, succinic acid and fumaric acid) and sugars (sucrose and fructose) of Grace's Insect culture medium increased the ES antigen mean protein content from 152 ug to 364 ug ($P < 0.001$) from the *in vitro* maintenance of 10 lakhs mf in 40 ml medium (Fig. 1.). The mean antigenic titre against FSIgG in inhibition ELISA also increased by 2 fold (from 200 to 400). Kharat, et. al.(1) used medium 199 supplemented with organic acids and sugars of Grace's insect culture medium to maintain *W. bancrofti* mf *in vitro*.

When the temperature of *in vitro* maintenance was altered from 37°C (human body temperature) to 28°C (mosquito body temperature) the ES antigen mean protein content increased from 364 ug to 435 ug ($P < 0.001$) corresponding with increase in the ES antigen mean titre from 400 to 666 (Fig. 1.).

Fig 1. The yield of ES antigen in terms of total protein and antigen titre when mf were maintained (A) in RPMI 1640 medium at 37°C (B) in medium supplemented with organic acids and sugars of Grace's insect culture medium at 37°C (C) in organic acids and sugars supplemented medium at 28°C and (D) in medium further supplemented with protease inhibitor PMSF (0.1 mM conc.) at 28°C. The results shown are mean $\pm$ S.D. of three individual experiments.

While Chenthamarakshan, et. al. (3) maintained *B. malayi* mf at 37°C, Kharat et. al. (1) kept the *W. bancrofti* mf culture at 28°C to prepare ES products. The significant increase in the yield of ES antigen at lower temperature (28°C) compared to at 37°C may possibly be due to decreased activity of enzymes proteases in mf ES products at lower temperature during the 48 hrs. maintenance period. The presence of proteases has been demonstrated in *B. malayi* mf extracts (12) and ES products of L_3 larvae and adult worms (13). Protease activity was demonstrated in *B. malayi* mf ES products using azocoll as substrate (Chenthamarakshan, V., Reddy, M.V.R. and Harinath, B.C. unpublished observations).

The addition of enzyme protease inhibitor PMSF to the culture medium at a concentration of 0.1mM did help in arresting the protease degradation of ES antigen. In the presence of PMSF, the ES antigen mean protein content from 10 lakhs mf increased to 502 ug ($P < 0.001$) and the mean antigen titre increased to 800 (Fig. 1.). Supplementation of medium with still higher conc. of PMSF or other inhibitors (TPCK, EDTA) did have toxic effect on mf (data not shown). The supplementation of RPMI 1640 medium with organic acids and sugars and PMSF and shift in the culture temperature to 28°C resulted in a net 3 fold increase in the mean protein content and 4 fold increase in the mean antigen titre of ES products.

The mf ES antigens prepared in RPMI 1640 medium without any supplements at 37°C and with supplements at 28°C were compared for their diagnostic use for detection of filarial IgG antibodies by stick-indirect ELISA. Both the ES antigen preparations were required to be used at an optimal concentration of 100 pg/5 ul/stick and the results of immunoscreening of different groups of sera were almost similar (Table 1.). Both the ES antigens detected filarial IgG antibodies in 2 of 20 (10%) endemic normal sera. While the mf ES antigen as such showed 75% of microfilaraemic and 83% of clinical filarial sera as positive for filarial antibodies, the high yield preparation detected filarial antibodies in 80% of these sera. The filarial antibody assay using the high yield ES antigen showed a sensitivity of 80% and specificity of 90%. These results are comparable with the data shown earlier from our laboratory (11) on the diagnostic use of Bm mf ES antigens for detection of filarial IgG antibodies by stick-indirect ELISA.

Table 1. Detection of filarial IgG antibodies in human sera by stick indirect ELISA using Bm mf ES antigen.

Sera	No. examined	No. showing positivity* for filarial IgG antibodies (% positivity) using	
		Bm mf ES Ag.	Bm mf ES Ag. (High yield preparation)
Microfilaraemia (mf +ve)	20	15 (75%)	16 (80%)
Clinical filariasis (mf -ve)	30	25 (83%)	24 (80%)
Endemic normal (mf -ve)	20	2 (10%)	2 (10%)

* Sera showing filarial IgG antibody titre of > 1:300

Thus in the present study the supplementation of RPMI 1640 medium (with organic acids and sugars and PMSF and maintenance of mf at 28°C (instead of at 37°C) was shown to result in an increased yield of mf ES products without affecting their diagnostic utility.

ACKNOWLEDGEMENTS

This work was supported by the Department of Biotechnology, New Delhi.

The authors are grateful to Dr. Sushila Nayar, Shri Dhirubhai Mehta and Dr. O.P. Gupta, for their keen interest and encouragement and Mr. Naik for technical assistance.

REFERENCES

1. Kharat, I., Ghirnkar, S. N. and Harinath, B.C. (1982) Antibody analysis in human filarial sera by ELISA using *W. bancrofti* mf culture antigen. Ind. J. Exptl. Biol. 20, 378-380.
2. Malhotra, A. and Harinath, B. C. (1984) Comparative efficiency of *Wuchereria bancrofti* microfilarial and larval excretory- secretory antigens in ELISA for the diagnosis of tropical eosinophilia and bancroftian filariasis. Ind. J. Exptl. Biol. 22, 520-522.
3. Chenthamarakshan, V., Reddy, M. V. R. and Harinath, B. C. (1996b) Diagnostic potential of fractionated *Brugia malayi* microfilarial excretory-secretory antigen for bancroftian filariasis. Trans. Roy. Soc. Trop. Med. Hyg. 90, 252-254.

4. Kharat, I., Cheirmaraj, K., Prasad, G.B.K.S. and Harinath, B.C. (1989) Antigenic analysis of excretory-secretory products of *Wuchereria bancrofti* and *Brugia malayi* infective larval forms by SDS-PAGE. Ind. J. Exptl. Biol. 27, 681-684.
5. Cheirmaraj, K., Chenthamarakshan, V., Reddy, M.V.R. and Harinath, B. C. (1991) Immunoprophylaxis against filarial parasite, *Brugia malayi*: Potential of excretory-secretory antigens in inducing immunity. J. Biosci. 16, 209-216.
6. Mc. Call, J.W., Malone, J.B., Ah, H.S. and Thompson, P.E. (1973) Mangolian jirds (*Meriones unguiculatus*) infected with *Brugia malayi* by intraperitoneal route : a rich source of developing larvae, adult filariae and microfilariae. J. Parasitol. 59, 436-439.
7. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Rundall, R. J. (1951) Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193, 265-275.
8. Reddy, M.V.R., Malhotra, A. and Harinath, B. C. (1984) Detection of circulating antigen in bancroftian filariasis by Sandwich ELISA using filarial serum IgG. J. Helminthol. 58, 259- 262.
9. Avrameas, S. (1969) Coupling of enzymes to proteins with glutaraldehyde - use of conjugates for the detection of antigen and antibody. Immunochemistry. 6, 43-52.
10. Chenthamarakshan, V., Reddy, M.V.R. and Harinath, B. C. (1996a) Detection of filarial antigen by Inhibition Enzyme Linked Immunosorbant Assay using fractionated *B. malayi* microfilarial excretory-secretory antigen. J. Biosci. 21, 27-34.
11. Reddy, M.V. R., Alli. R., Devi, K. K., Narayan, R., Harikrishnan, R., Cheirmaraj, K. and Harinath, B. C. (1996) Comparative evaluation of microtitre plate peroxidase and stick penicillinase enzyme immuno assays for detection of filarial antibodies using *Brugia malayi* microfilarial excretory-secretory antigen. J. Parasitic. Dis. 20, 173-176.
12. Bianco, A.E., Mustafa, M. B. and Ham, P. J. (1989) Fate of developing larvae of *Onchocerca lienalis* and *O. volvulus* in micropore chambers implanted into laboratory hosts. J. Helminthol. 63, 218-226.
13. Petralanda, I., Yarzabai, L. and Piessens, W. F. (1986) Studies on a filarial antigen with collagenase activity. Mol. Biochem. Parasitol. 19, 51-59.