Excretory-Secretory antigens of rodent filariid, *Acanthocheilonema viteae* and their cross-reaction with human filarial sera

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Abstract: *In vitro* released products of adult filariid *Acanthocheilonema viteae* were characterized according to their SDS-PAGE profiles and immune recognition patterns by infected mastomys and by human sera. Adult worms survived well in the serum free media (HBSS; Sigma) more than ten days. SDS-PAGE analysis of E/S antigens revealed the molecules between 70 and 54.5 kDa highly dominant whereas immune recognition with infected mastomys sera demonstrated the immunoreactivity in molecules present between 48.5 and 58 kDa. Many of these cross-reacted with *W. bancrofti* infected human sera showing the sharing of antigens by the two filariid species. 143, 96, 74, 62, 44 and two below 26.6 kDa of adult *A. viteae* E/S cross-reacted with human filarial sera. A number of molecules between 60 and 44 kDa were found reactive with both mastomys and human sera demonstrating their possible use in diagnosis. Presence of common antigens between the rodent and human filariid and their shared immunoreactivity demonstrate the usefulness of *A. viteae* antigens in diagnosis/prophylaxis of human filariasis.

Key words: *Acanthocheilonema viteae*, *Wuchereria bancrofti*, *Mastomys coucha*, Excretory/Secretory antigen, filaria, characterization.

INTRODUCTION

Filariasis is a chronic disease where parasites live in the host for prolonged period extending into several years even. The long association of host with parasite is expected to induce immune reactions in the former. It is now generally conceived that many of the pathological alterations evoked by this nematode infection are induced by and associated with the nature of host immune response to parasite materials.

The most convenient molecular interactions between host and viable parasite occur via surface and excretory-secretory (E/S) components. The latter plays a major role in host-parasite interaction and may be exploited as tools for diagnosis or foci for protection.

Identification and characterization of these filarial products have attracted world wide attention but lesser attempts have been made to exploit excretory-secretory products which appear to be more responsible for development of immune response of host (Kaushal *et al.*,

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1982; Maizels *et al.*, 1985; Kwan-Lim *et al.*, 1989; Harnett *et al.*, 1989). The present study deals with characterization of E/S products of a subcutaneous dwelling rodent filariid *A. viteae* and identification of the target molecules of functional immunity in order to evolve better immunoprophylactic agents.

Materials and Methods

Host parasite model: The life cycle of *A. viteae* was maintained in *Mastomys coucha,* the rodent host, through tick vector *Ornithodoros moubata* as described earlier (Singh *et al.*, 1985). Six-week old male mastomys were infected subcutaneously by inoculating 50 L_3 of *A. viteae* for isolation of adult parasites and collection of sera samples. Microfilaraemic animals were autopsied and the parasites were recovered from the subcutaneous tissues.

Sera: Pooled sera were prepared from the groups of infected mastomys on day 15 and thereafter on day 30, 60, 90, 120, 150, 180 covering prepatent, patent and latent stages of infection. Sera of uninfected animals served as controls. Each pool was obtained from five identical individual animals.

Sara were also collected from well defined human filarial subjects (two microfilaraemic and two symptomatic) and pooled together.

***In vitro* culture:** Fifty male and fifty female parasites were asceptically culturd at 37℃ in 5% CO_2/air in a protein free defind medium. The worms were washed three times with culture medium (Hank's balanced salt solution (HBSS) 'sigma' pH 7.4, supplemented with $NaHCO_3$, gentamycin 40μg/ml and 1% glucose but no serum) before they were transferred to the flat bottom screw capped vials. The spent media of the cultures were collected at 24 hrs. Interval and replaced with fresh medium till day 7. All worms were alive at the end of incubation. The collected medium was centrifuged to remove microfilariae released during *in vitro* incubation of worms and concentrated 40-fold by Sartorius ultrafilters (retaining proteins above 10,000 mol. wt.). This was stored at −20℃ until used. The total quantity of protein in E/S products was determined by Bradford's method (1976).

Preparation of somatic antigen: Adult parasites of *A. viteae* (both male and female) recovered from the subcutaneous tissues of the host *Mastomys coucha* were washed and homogenized in tris buffered saline (pH 7.2) under cold conditions. Homogenate was then sonicated at 10 kcs for 5 minutes, and protein was estimated by Bradford's method (1976).

Sample preparation: Somatic and E/S antigens were solubilised in sample buffer (63 mM Tris-HCl, pH 6.8; 10% (V/V) glycerol; 3% (W/V) SDS; 5% (V/V) B-ME; 0.1% (W/V) bromophenol blue) containing protease inhibitors (benzamidine E-aminocaproic acid and PMSF, 100 mM each). Somatic and E/S antigens in sample buffer were heated at 95℃ in a water bath for 5 minutes and centrifuged at 2000 rpm for 7 minutes. The supernates were isolated and stored at −20℃ until use.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE): Antigens in sample buffer subjected to one-dimensional polyacrylamide gel electrophoresis (PAGE) using 1 mm thick linear gradient (7−15%) slab gel and the discontinuous buffer system of Laemmli (1970) with the

modifications of Ruppel and Cioli (1977) in the presence of sodium dodecyl sulfate (SDS).

Samples were run at 25 mA for 4−6 hrs. Proteins of known molecular weights (prestained molecular weight marker kit; Sigma) were used for molecular weight determination. Separated proteins were stained with 0.1% (W/V) Coomassie brilliant Blue-R-250 (Sigma) followed by background destaining. Silver staining (Silver stain Kit; Sigma) of the gel was also done whenever required.

Immunoreactivity of separated proteins: The electrophoretic transfer of proteins onto nitrocellulose filter sheets was performed as described by Towbin *et al* (1979) at 4℃ overnight in Tris-glycine buffer (25 mM Tris; 186 mM glycine, 20% (V/V methanol). The immunorecognition was performed according to Burnette (1981).

In brief, to saturate the unspecific protein binding on the nitrocellulose, the filter sheets with adherent proteins were blocked in a solution of 3% (W/V) Bovine serum albumin (BSA) in Tris buffer saline (TBS; pH 7.4) for 90 min at room temperature. After a short rinsing in TBS nitrocellulose strips were incubated for 90 min with test sera prepared in BSA-TBS. The strips were washed thoroughly 3× with TBS, 2× with TBS containing 0.05% (V/V) Triton X-100 and then again 3× with TBS. Each step required 5 minutes and was performed under constant shaking. Subsequently the strips were incubated for 90 min with 1 : 600 dilution of second antibody (goat-anti-mouse polyvalent; Sigma) conjugated with enzyme (HRP) in BSA-TBS. For observing the cross-reaction with human sera, goat antihuman IgG + IgM (Sigma) was used. To remove unbound conjugate the strips were washed as above. Substrate solution consisting of 10 mM H_2O_2 and diaminobenzidine (DAB) in TBS was added lastly to the strips and antigen-antibody reaction was visualized as brown bands. Strips containing individual antigens were also incubated with nonendemic human sera from Kashmir to serve as controls. Strips were air dried, preserved and photographed. Relative molecular mass (Mr) of the separated protein molecules were determined on the basis of standard protein markers.

RESULTS

Maintenance of parasites in vitro: Both sexes of adult parasites servived well in the serum free medium and E/S products were collected till 120 hrs of incubation. Nevertheless parasites remained viable and actively motile even beyond 15 days. The maximum amount of protein released by a single male worm was 1.020 μg at 96 hrs. Whereas a female released 1.573μg at 72 hrs of incubation (Fig. 1).

SDS-PAGE analysis: In SDS-PAGE E/S products of male and female parasites (pooled separately) were resolved into lesser number of components whereas their somatic counterparts revealed comparatively much higher number of bands (Fig. 2a). Silver staining of gel proved to be superior to Coomasie blue and a few more bands could be visualized with the former staining method (Fig. 2b). Majority of the dominant bands in E/S antigens were confined between 84 and 40 kDa region, but a few were also distributed in the lower and higher Mr regions as well (Fig. 2b). Amongst all the molecules released by live females throughout

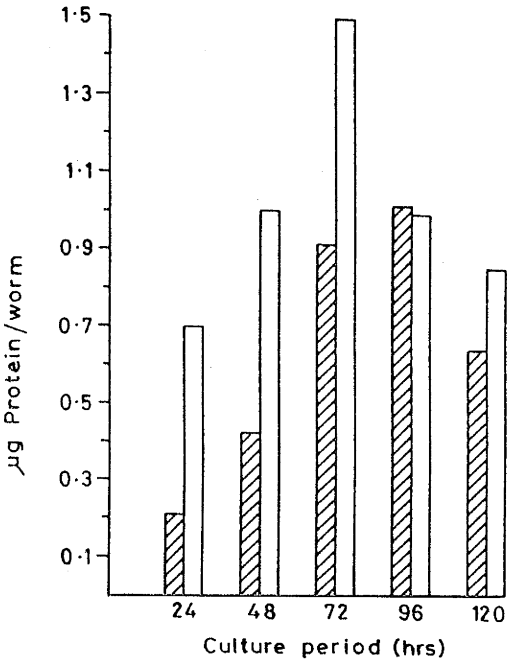

Fig. 1. Release of E/S products by adult *A. viteae in vitro.*

the culture period, molecules between 70 and 54.5 kDa were the most prominent ones (Fig. $3b_1$). However a few other molecules were also released along with, having the molecular mass (Mr) of >180, 132, 54.5 to 43, 17.5 to 14.4 kDa (Fig. 2a), most of which were also released by male parasites though in lesser quantity. The dominant molecule released by the male parasites was Mr 67 kDa which was also present in their somatic extracts (Fig. 2a). Comparative study indicated that there were clear cut difference in the electrophoretic pattern of E/S and somatic products of worms of both sexes. A molecule of 43 kDa which was most prominent in somatic products was not exported out by the live parasites *in vitro.*

Molecules released at different hrs of culture: Majority of the molecules were released throughout the period of culture and a few could be detected only at definite time periods. Fig. $3a_1$, a_2, b_1, b_2 show the release of different molecules in the E/S products of male and female parasites at different hrs of culture on SDS-PAGE. In female E/S products a cluster of molecules could be well detected between 54.5 and 70 kDa throughout the period of culture with maximum release from 72 hrs onwards when gel was stained with Coomassie blue (Fig. $3b_1$). However, silver staining revealed much higher number of bands extending up to 108 kDa region with two above 180 kDa (Fig. $3b_2$). In case of male worms very faint bands were observed on Coomassie blue staining (Fig. $3a_1$); however, the same gel on silver staining showed a whole range of bands between 180 and 36.6 kDa (Fig. $3a_2$).

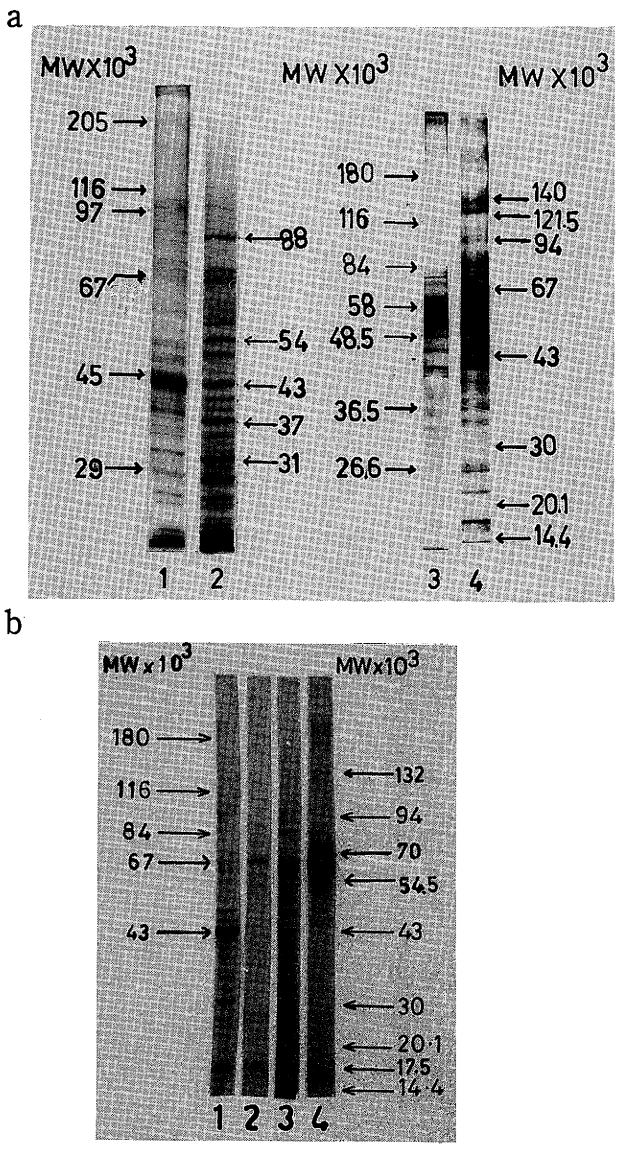

Fig. 2. SDS-PAGE profile of *A. viteae* adult E/S and somatic proteins (a) coomassie-blue stained gels. lane 1, male somatic; lane 2, male E/S; lane 3, female somatic; lane 4, female E/S. (b) silver stained gel, lane 1, male somatic; lane 2, female somatic, lane 3, male E/S, lane 4, female E/S.

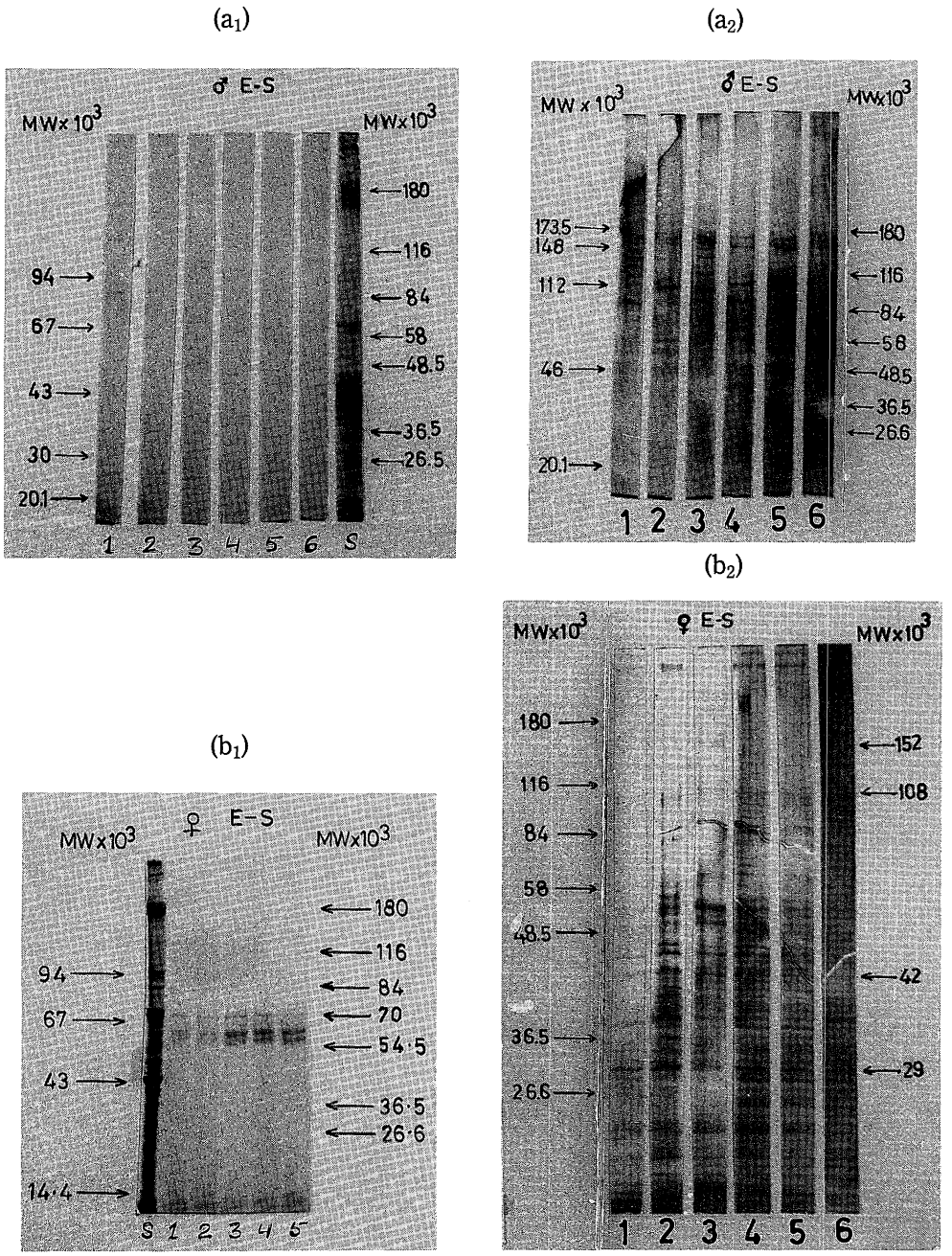

Fig. 3. SDS-PAGE profile of E/S products released at different hrs of culture: a_1, b_1 Coomassie blue stained gels of male and female E/S respectively;* a_2, b_2 silver stained gels of male and female E/S respectively. Lanes 1 to 6 contain E/S products collected at 24, 48, 72, 96, 120 and 144 hrs respectively. * Lane S, somatic proteins.

Immune recognition pattern: Western blotting of the SDS-PAGE separated antigens and their identification with antibodies in infected sera revealed markedly strong reaction with the antigens present between 48.5 and 58 kDa region in the form of a single thick band which was present in both male and female E/S products whereas the molecules between 58 and 48.5 kDa in the somatic products of female were found reactive to comparatively lesser extent. However in case of male somatic products molecules between 58 and 43.5 kDa were strongly reactive unlike the female somatic. The molecules in this region were recognized by the sera of mastomys whether taken at prepatent, patent or latent stage. Though maximum number of E/S molecules were identified by the patent serum (Fig. 4). Faint reaction was noticed in low Mr regions in case of latent serum.

There were certain differences in the release of immunoreactive molecules by male and female worms at various periods of culture. An immunoreactive molecule of 84 kDa was released exclusively by males only at 48 hrs of culture whereas low Mr components of 36.5 and 29 kDa were released by females between 72 and 120 hrs which reacted strongly with pooled infected sera (Fig. 5). Besides exporting out 44 kDa at 72 hrs females also released high MW functional molecules of >180 and 137 kDa throughout the period of cultures. Most of the immunoreactive molecules released by male worms were below 116 kDa. Besides these immunoreactive molecules which were common with somatic extracts, molecule of 67 kDa in both the somatic and E/S products of male was strongly recognized at patent and latent stages of infection but not in the E/S products of females.

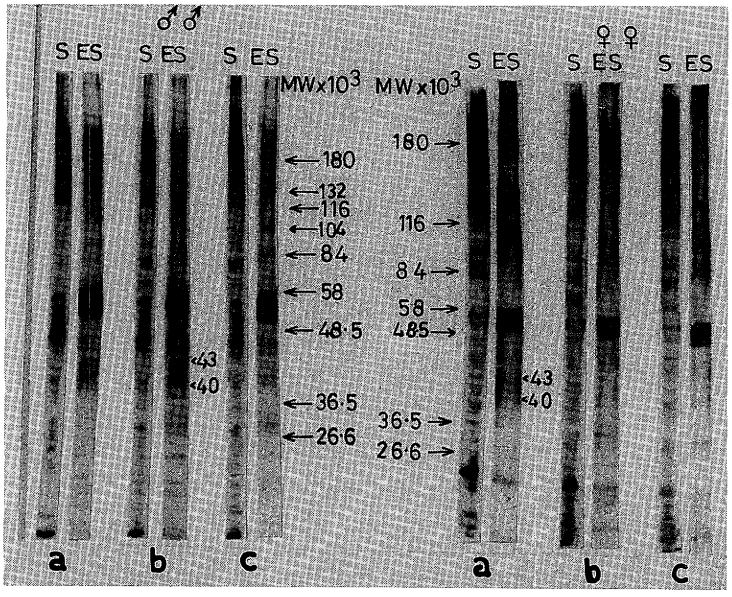

Fig. 4. Immunoreactivity of E/S with infected mastomys serum
(a) prepatent serum, (b) patent serum, (c) latent serum.

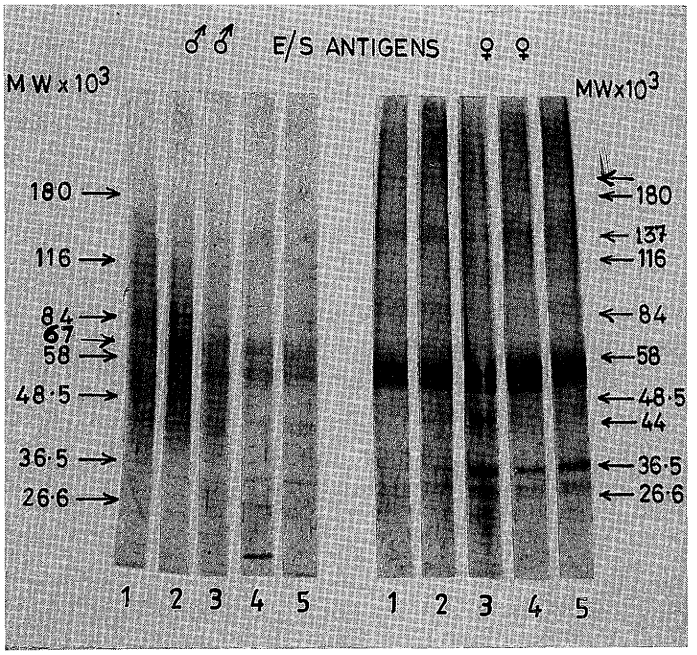

Fig. 5. Immunoreactivity of E/S released at different hrs of culture with infected mastomys serum. Lane 1, 24 hrs; lane 2, 48 hrs; lane 3, 72 hrs; lane 4, 96 hrs; lane 5, 120 hrs.

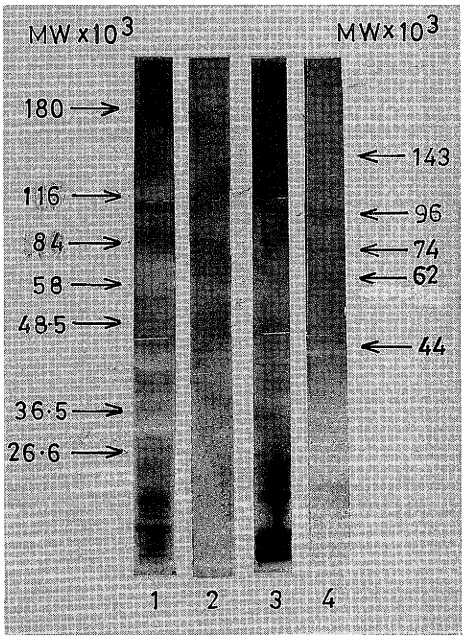

Fig. 6. Immunoreactivity of somatic and E/S proteins with anti *W. bancrofti* human serum pool. Lane 1, male somatic; lane 2, male E/S; lane 3, female somatic; lane 4, female E/S, arrows indicate molecular weights in kilodaltons.

Cross-reaction with human filarial sera: Both somatic as well as E/S products of male and female *A.viteae* cross-reacted with human *W. bancrofti* sera. Approximately fourteen molecules in the E/S products of female and eleven in E/S products of male *A. viteae* were recognized by infected human sera. Still larger number of (24 in male and 28 in female) molecules in somatic products of *A. viteae* were identified by human filarial sera (Fig. 6). Human filarial serum reacted with 143, 74, 62, 44 and two molecules below 26.6 kDa of female E/S (Fig. 6, lane 4). While 96 kDa of both somatic and E/S antigens of both sexes were recognized by the infected patients serum, 62 kDa of male and 44 kDa of female somatic antigen molecules were also reactive though very faintly . A number of molecules between 60 and 44 kDa were also found reactive with both mastomys and human sera (Fig. 4 and 6).

Sera from human subjects from filaria free zome (Jammu and Kashmir) did not reveal any reaction with these antigens in our study (data not shown).

DISCUSSION

Identification and characterization of parasite derived excretory-secretory products are especially important to understand functional components of filariids which might be useful in diagnosis or prophylaxis of the disease. Recently few efforts have been made to isolate and characterize E-S products (Kwan-Lim *et al.*, 1989; Harnett *et al.*, 1989; Kaushal *et al.*, 1984; Collette *et al.*, 1993). but their immunogenic nature and involvement in the development of acquired resistance still remains poorly understood. Parasite products may also play a critical role in parasite survival in host's hostile environment, and may be responsible for development of immunopathological effects.

In earlier studies although there were investigations on different aspects of E/S of *Brugia* and other filarial species (Kaushal *et al.*, 1984; Kwan-Lim *et al.*, 1989) the conditions of culture were not clear and viability of parasites in the medium was poorly defined. The most commonly used media for *in vitro* culture of nematodes have been RPMI-1640 and MEM. Our study reveals HBSS containing L-glutamine and HEPES to be more effective for *in vitro* maintenance of parasites and collection of E/S antigens with daily change of medium and fortification with glucose. Adult *A. viteae* survived well even beyond 10 days without serum supplement thereby allowing collection of E/S products for longer period.

In the present sutdy E/S was collected under conditions in which parasites remained highly active, as dead or disintegrating parasites, under stress, release products which may not form part of their normal secretion or excretion. Our study also reveals that a number of molecules are exported out continuously by live parasites, and a few but not all are immunogenic and provoke immune response in host at various stages of infection. There are differences in the SDS-PAGE separated components of E/S of adult male and female *A. viteae*. It may be mentioned that no difference in the E/S products of female and male *B. pahangi* was observed (Kaushal *et al.*, 1984) but clear differences were detected between the E/S products of both sexes of *B. malayi* (Kwan-Lim *et al.*, 1989). The major antigen secreted by both

the sexes of adult *A. viteae* is a very thick band extending between 70 and 54.5 kDa. These molecules are predominantly exported out by the females and to a lesser extent by the males. The profile of proteins released is consistent throughout the period of culture but maximum release occurs at 72 and 96 hrs respectively by female and male parasites. Once the parasites are kept *in vitro* they take 2−3 days to get adjusted to their new environment and this may possibly account for maximum release of proteins on or beyond 48 hrs (Kwan-Lim *et al.*, 1989).

A thick band present between 54.5 and 70 kDa was found dominant in SDS-PAGE under reducing conditions, but western blotting and immune recognition pattern with infected mastomys sera reveals only those molecules highly immunoreactive which are present between 48.5 and 54 kDa region. This is true for both male and female parasites. The exact reason is not clear but it may be due to formation of immune complexes and that is why antibodies are not available to react with these molecules.

Harnett *et al.*, (1989) reported the release of 62 kDa molecule by adult *A. viteae in vitro* which was located predominantly in parasite gut and excreted out in host urine in non-antigenic form. This molecule was detected in E/S of L_4 stage of *A. viteae* and cross-reacted with human filarial sera. The molecule was reported to have phosphorylcholine (PC) epitope and appear to be important as circulating filarial antigen.

Possession of PC moiety is a common featur of many parasitic nematodes especially filariids (Dissanayake *et al.*, 1984; Maizels *et al.*, 1987). A number of studies have shown that PC bearing antigens are common metabolites of filariids, and could be readily detected in human and animal filarial sera (Forsyth *et al.*, 1985; Selkirk *et al.*, 1986; Weil *et al.*, 1987; Wegner *et al.*, 1988). The level of these molecules in host may therefore provide an accurate estimation of presence of live parasites in patients (Wegner *et al.*, 1988) and therefore may prove to be the target circulating antigen molecule. In case of human lymphatic filariid (*W. bancrofti*) 17, 21 and 51 kDa molecules have been reported to be dominant molecules released by mf and L_3 of which 51 kDa molecule is also shared by *B.malayi* (Harnette *et al.*, 1989).

The present study carried out with adult *A. viteae* E/S products and homologous mastomys sera revealed the most dominant immunoreactive molecules between Mr 58 and 48.5 kDa. These molecules also reacted with human fialrial serum.

The immunoreactivity of E/S molecules with homologous infected sera observed in the present study implies that E/S molecules released *in vitro* may represent the E/S molecules exported out *in vivo* and are recognised by the respective infected sera. Most of the molecules are possibly released by live parasites throughout their life-span in the host as antibodies from prepatent, patent and latent stages of infection interact with them in blots. Nevertheless maximum molecules are functional during patent stage of infection.

The presence of immunoreactive molecules between 58 and 48.5 kDa derived from both sexes of *A. viteae* adults and their identification by sera of all stages of infection in mastomys may point towards the importance of these molecules for diagnosis. It is worth mentioning that these molecules are also identified by infected human sera and thus deserve

further study for their potential in immunodiagnosis of human filariasis.

Cross-reaction between E/S products of filarial parasites and heterologous sera has been reported earlier (Kaushal *et al.*, 1982; Kwan-lim *et al.*, 1989; Harnett *et al.*, 1989). It is interesting to note that molecules between 58 and 48.5 kDa in E/S products of *A. viteae* react predominantly with homologous sera, cross-reaction of these molecules with human filarial sera was not so intense. It was observed that a large number of molecules reacted with human filarial sera are between 60 and 44 kDa. This similarity in antigen recognition of *A. viteae* homologous mastomys sera as well as *W. boncrofti* human sera demonstrate the practical usefulness of *A. viteae*-mastomys model for immunological investigations of human filariasis.

Thus in conclusion it may be stated that certain components derived from E/S products of adult *A. viteae* might be important in the detection and/or protection against filarial infection. The most important molecules which deserve further investigation for their probable use in human filarial infection are 143, 96, 74, 62 kDa. A few molecules between 60 and 44 kDa which have also been recognized by the filaria -infected human sera also deserve attention for their possible use in diagnosis and/or prophylaxis of filariasis.

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