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Immunoprophylaxis against filarial parasite, *Brugia malayi*: potential of excretory-secretory antigens in inducing immunity

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Abstract. The role of excretory-secretory antigens in inducing immunity in the host against *Brugia malayi* microfilariae and infective larvae was studied by *in vitro* antibody dependent cell-mediated reaction as well as *in vivo* inoculation of filarial parasites within a microchamber in the host. The immune sera of jirds raised against *Brugia malayi* microfilarial and infective larval excretory-secretory antigens (*Bm* Mf ESA and *Bm* L_3 ESA) promoted the adherence of peritoneal exudate cells to *Brugia malayi* microfilariae and infective larvae *in vitro* and induced cytotoxicity to the parasites within 48 h. The anti *Bm* Mf ESA serum was more effective than anti *Bm* L_3 ESA serum in inducing cytotoxicity to microfilariae and both antisera had a similar cytotoxic effect on infective larvae. In the microchambers implanted in the immune jirds, host cells could migrate and adhere to the microfilariae and infective larvae and kill them within 48–72 h. Further, *Mastomys natalensis* immunized against *Bm* Mf ESA and L_3 ESA generated a high degree of protective response against circulating microfilariae. These results suggest that excretory-secretory antigens are effective in inducing resistance against filarial parasites and thus have potential in immunoprophylaxis.

Keywords. *Brugia malayi*; microfilariae; infective larvae; immunoprophylaxis; excretory-secretory antigens.

1. Introduction

Lymphatic filariasis continues to pose a major public health problem in India. The general approach to control filariasis in most endemic areas are reduction of morbidity and interruption of transmission. Despite recent advances both in chemotherapy and in vector control, the filarial disease remains a major cause of morbidity in the developing world. Immunoprophylaxis may serve as an effective adjunct to the existing control measures.

Studies on immunoprophylaxis against filarial infection have been made in several experimental models employing different stages of *Brugia malayi* parasites. The jirds (*Meriones unguiculatus*) and *Mastomys natalensis* are the fully permissive rodent hosts for sub-periodic *B. malayi* and have been found to be very useful in immunological and other studies related to brugian filariasis. Protective immunity against *B. malayi* infection has been demonstrated in jirds using irradiated third stage larvae (Yates and Higashi 1985) or microfilarial antigens (Kazura *et al* 1986). Successful vaccination with attenuated larvae suggested that it would be the excretory-secretory (ES) products or moulting fluid that contains the effective

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Abbreviations used: *Bm* Mf ESA, *Brugia malayi* microfilarial excretory-secretory antigen; *Bm* L_3 ESA, *Brugia malayi* infective larval excretory-secretory antigen; ADCC, antibody-dependent cell mediated cytotoxicity; HBBS, Hank's balanced salt solution; PEC, peritoneal exudate cells; FCS, fetal calf serum.

immunogen. Attempts were therefore made to find host protective antigens amongst the substances released by worms (Clegg and Smith 1978; Rajasekariah *et al* 1988). Vaccination with ES products of third stage larvae of *B. pahangi* showed partial immunity (determined by decreased worm numbers and/or absence of circulating microfilariae) in jirds (WHO 1987). Antibody dependent cell-mediated cytotoxicity (ADCC) is believed to be one of the principal immunological mechanisms functional in man and animals and the disappearance of circulating parasites is mainly attributed to this phenomenon (Mehta *et al* 1981b; Chandrashekar *et al* 1985a,b). This communication reports the potential of ES products of *B. malayi* microfilariae and infective larvae in inducing ADCC reaction both *in vitro* and *in vivo* and killing the microfilariae and infective larvae of *B. malayi.*

2. Materials and methods

2.1 *Filarial infection*

Multimammate rats, *Mastomys natalensis* or *M. coucha* (Kruppa *et al* 1990) (GRA Giessen strain) were infected with *B. malayi* (sub-periodic) by sub-cutaneous (sc) inoculation of 100 third stage infective larvae and the infection was monitored as described by Sanger *et al* (1981).

Jirds (*M. unguiculatus*) were infected with 100 *B. malayi* infective larvae by the intraperitoneal (ip) route (McCall *et al* 1973).

2.2 *Filarial parasites*

Infective larvae (L_3) of *B. malayi* were collected following the Baermann's technique (Suzuki and Seregeg 1979). *Aedes aegypti* mosquitoes were fed on the blood from *B. malayi* infected *Mastomys* and two weeks later, they were dissected to recover infective larvae.

Microfilariae (Mf) were obtained by lavage of the peritoneal cavities of jirds which had been infected with *B. malayi* L_3 larvae, 15–20 weeks earlier.

2.3 *Antigen preparation*

2.3a *B. malayi Mf ESA*: Microfilariae were washed with normal saline and RPMI 1640 medium and then plated on a sterile disposable petriplate. The plate was incubated at 37°C for 1 h to remove, the hosts' peritoneal cells. An hour later, the microfilariae were recovered, washed and maintained in the same medium at 28°C. After 48 h, culture fluid was centrifuged and the supernatant was dialyzed, freeze-dried and stored at –20°C until use. The protein content was estimated by the method of Lowry *et al* (1951).

2.4 *L_3 ESA*

The infective larvae of *B. malayi* were maintained in Hank's balanced salt solution (HBSS) at 28°C for 48 h as described by Kharat *et al* (1989). The *Bm* L_3 ESA was obtained by processing the culture fluid as described above.

2.5 *Immunization of jirds with filarial antigens*

Two groups of jirds were immunized ip, each group with *Bm* Mf ESA or L_3 ESA. The first dose consisted of 25 μg of antigenic protein emulsified in Freund's complete adjuvant. The second and third doses of similar amount, emulsified in Freund's incomplete adjuvant were given at intervals of 10 days. Control animals received only saline emulsified in Freund's adjuvant as above. A week after the last dose of antigen, the animals were bled from retro-orbital plexus and immune sera were isolated.

2.6 *Peritoneal exudate cells*

Peritoneal exudate cells (PEC) from normal jirds were collected by peritoneal cavity washings with sterile medium RPMI 1640. The cells were washed with the same medium supplemented with 10% fetal calf serum (FCS) and the viability of the cells was assessed by trypan blue dye exclusion.

2.7 *In vitro cytotoxicity assay*

The cytotoxicity assay was carried out as described by Chandrashekar *et al* (1985a, 1990). Briefly, 100 mf or 6 L_3 in 50 μl of RPMI 1640 were incubated with 50 μl of PEC (2×10^3 cells/mf (or) 5×10^4 cells/L_3 larva) and 50 μl of normal or immune jird serum in the 96 well culture plate (Costar Inc, MA, USA). The plate was incubated in an atmosphere of 5% CO_2 at 37°C. At different periods of incubation (24 and 48 h) the samples were examined microscopically for cellular adherence and cytotoxicity to microfilariae and infective larvae. Percentage of cytotoxicity was expressed by considering the number of immobile or dead parasites within the experimental period.

2.8 *Micropore chambers technique*

Micropore chambers were assembled using 14 × 2 mm plexi glass rings (Millipore Filter Corp., Bedford, MA, USA) and 3 μm nucleopore polycarbonate membrane (Thomas Scientific, USA) as described by Weiss and Tanner (1979). The micropore chambers were loaded with 100 mf or 10 L_3 larvae in RPMI 1640 medium via an aperture (diameter -1 mm) at the side of the plexi glass ring and then sealed with paraffin wax and subsequently with MF cement (Millipore Filter Corp., USA). The chambers were implanted intraperitoneally into the immunized and control jirds through an incision of 2–3 cm and the skin was sutured. After 24, 48 and 72 h, the chambers were taken out and the contents were removed with pasteur pipette and examined microscopically.

2.9 *In vivo cytotoxicity assay*

Two different groups of *Mastomys,* with five in each group were immunized ip, with *Bm* Mf ESA or L_3 ESA as described above. A week after giving the last dose of immunization, the two groups of *Mastomys* along with the control group were inoculated (ip) with 1×10^5 *B. malayi* microfilariae in RPMI 1640 medium. The

Mastomys were monitored for microfilaraemia for the next 10 days at intervals of 2 days.

3. Results

In the *in vitro* cytotoxic assays, the immune sera of jirds raised against *Bm* Mf ESA and L_3 ESA promoted the adherence of PEC to *B. malayi* microfilariae and infective larvae and induced cytotoxicity to the parasites within 24–48 h (table 1). The anti *Bm* Mf ESA serum was more effective than anti *Bm* L_3 ESA serum in killing microfilariae and the difference was statistically significant ($P < 0{\cdot}05$) by Student's *t*-test. However, against infective larvae, both the antisera had a similar cytotoxic effect and there was no (statistically) significant difference.

The microscopic observation of the nucleopore chambers implanted in jirds immunized with *Bm* Mf ESA or L_3 ESA showed the migration of host lymphocytes, polymorphonuclear cells and few macrophages into the chambers leading to their adherence and killing of microfilariae or infective larvae within 48–72 h (figures 1 and 2 and table 2). The cytotoxicity in the immunized jirds varied between 38 to 75% in 48 h and 95–100% in 72 h. On the other hand, in the chambers implanted in control jirds, the cytotoxicity to the parasites was only 8–15% in 48 h and 20–35 % in 72 h.

The results of the *in vivo* studies on the effect of immunization on the microfilaraemic state are summarized in table 3. An intraperitoneal injection of 1×10^5 mf in *Mastomys* resulted in the peripheral circulation of microfilariae within 24 h, which was maintained throughout the observation period of 10 days. However in *Mastomys* immunized with *Bm* Mf ESA, very few microfilariae were present in the peripheral circulation, which were also eliminated by day 5 and did not reappear again till day 10. In *Mastomys* immunized with *Bm* L_3 ESA, though the mf count was less in the peripheral circulation compared to control *Mastomys,* they continued to exist till day 10.

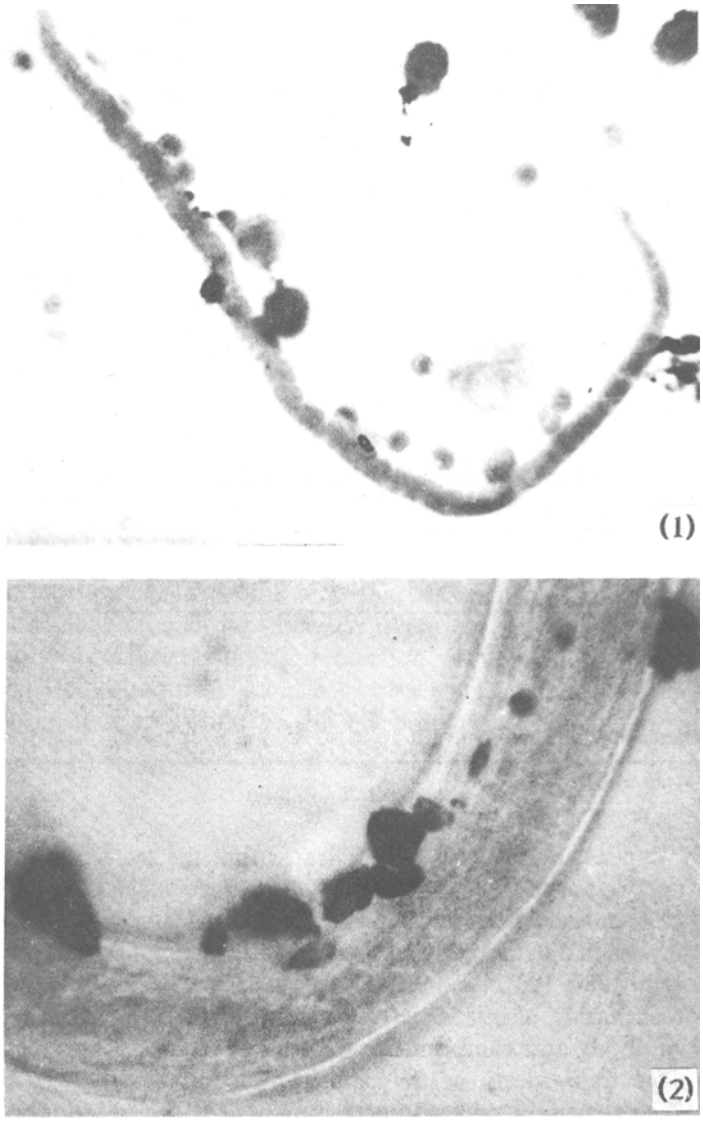
4. Discussion

The present study was undertaken to evaluate the role of ES antigens in producing immunity in the host against filarial infection *in vitro* and *in vivo*. Since, in preliminary experiments, no difference in cytotoxicity was observed with intact or

Table 1. Serum dependent cellular cytotoxicity to *B. malayi* parasites *in vitro.*

	Cytotoxicity (%)*			
	24 h		48 h	
Jird serum†	Mf	L_3	Mf	L_3
Control	3 ± 3	–	6 ± 5	33 ± 14
Anti Mf ESA	63 ± 21	8·3 ± 8	100	62·3 ± 24
Anti L_3 ESA	51 ± 22	21·5 ± 13	78 ± 20	82 ± 17

†Sera were collected from 4 animals and tested separately for cytotoxicity.
*Mean ± SD.



Figures 1 and 2. Adhesion of PEC to *B. malayi* microfilariae (**1**) and infective larvae (one part) (**2**) in the micropore chamber implanted (ip) in the immunized jird.

decomplement serum, the experiment for ADCC reaction was therefore carried out with sera without heat inactivation. The observations in *in vitro* ADCC reaction showed that the antibodies raised against *Bm* Mf ESA and L_3 ESA induced cytotoxicity to the parasites. This observation, which to our knowledge is the first observation, envisages the definite role of *B.* malayi ES products in inducing immunity in the fully permissive host. The non-living parasites or their extracts have generally failed to confer resistance in fully permissive hosts, although they may do so in animals that are semi-permissive for parasite development (Mehta *et al* 1981a; Carlow and Philipp 1987). The observations in prophylactic studies

Table 2. Cytotoxicity to *B. malayi* parasites in micropore chambers implanted into jirds.

	Cytotoxicity (%)†					
	24 h		48 h		72 h	
Jirds*	Mf	L_3	Mf	L_3	Mf	L_3
Control	–	–	8 ± 3	15 ± 5	20 ± 8	35 ± 5
Bm Mf ESA immunized	20 ± 5	5 ± 5	48 ± 2	75 ± 5	100	95 ± 5
Bm L_3 ESA immunized	8 ± 2	15 ± 5	38 ± 6	70	100	100

*Experiments were performed in 6 animals of each group.
†Mean ± SD.

Table 3. Effect of immunization on circulating microfilariae in *M. natalensis.*

	Microfilariae* in 10 μl of blood				
Mastomys	Day 1	Day 3	Day 5	Day 7	Day 10
Control (n = 3)	10 ± 2	7 ± 3	8 ± 1	9 ± 3	7 ± 2
Bm Mf ESA immunized (n = 5)	0·2 ± 0·1	1 ± 1	0	0	0
Bm L_3 ESA immunized (n = 5)	3 ± 2	2 ± 2	2 ± 1	1 ± 1	1 ± 1

*Mean ± SD

conducted by Rajasekariah *et al* (1988) indicated that the secretory products are a source of protective antigens.

Both antibody and complement mediated effector mechanisms have been shown to operate on microfilariae and infective larvae *in vitro* (Subrahmanyam *et al* 1976, 1978; Mehta *et al* 1980, 1981b; Tanner and Weiss 1978; Haque *et al* 1981; Sim *et al* 1982; Chandrashekar *et al* 1985a, b, 1990). The nematode epicuticle may act as substrate for antibody and complement mediated adherence of host cells that may result in damage to or death of the parasite (Capron *et al* 1982). Neutrophils, macrophages and eosinophils are all known to possess surface receptors for the Fc part of IgG (Rabellino and Metcalf 1975; Wong and Wilson 1975). The predominant isotope responsible for ADCC reaction was found to be IgG (Chandrashekar *et al* 1985a, b; WHO 1987).

Micropore chamber technique was employed to check whether a similar ADCC reaction can occur *in vivo.* These chambers implanted in animals are advantageous since they provide a closer physiological environment than *in vitro* cultures for larval growth and survival and thus for assessing the host effector mechanisms (Weiss and Tanner 1979; Rajasekariah *et al* 1989). In the present study the microfilariae and infective larvae were attacked by host cells which migrated into the chambers implanted in immunized jirds within 48-72 h. In the earlier studies Chandrashekar *et al* (1990) showed that *Bm* L_3 larvae were attacked by the host macrophages and polymorphs in microchambers, 16–24 h after implantation in the immunized rats. Abraham *et al* (1986) observed that it took 10 days for the host

cells to kill *Dipetalonema viteae* L_3 in diffusion chambers implanted in the infected jirds suggesting a necessary developmental change in the larvae for generating susceptibility to immune attack. These variations in cytotoxicity reactions in *in vivo* conditions suggest that the responsible factors seem to be dependent on parasite species and the host (permissive and non-permissive).

The effect of antibodies raised against *BM* Mf ESA and L_3 ESA on circulating microfilariae was studied *in vivo* in *Mastomys.* The *Mastomys* immunized against *Bm* Mf ESA generated a high degree of protective response against circulating microfilariae compared to control *Mastomys* and those immunized with *Bm* L_3 ESA. In the earlier studies conducted by Rajasekariah *et al* (1987, 1988) immunization of mice with microfilarial antigens induced an absolute degree of protection against mf and comparatively a highly significant level of immunity against L_3. Kazura and Davis (1982) showed that soluble antigenic preparation derived from *B. malayi* microfilariae has the capacity to induce a high degree of resistance in Swiss-Webster mice against the circulating microfilariae. Rajasekariah *et al* (1988) showed that immunization of Balb/c mice with *B. pahangi* Mf ESM antigens produced partial but significant levels of protection against the establishment of Mf in mice. A vaccine against microfilariae will be useful in blocking transmission and thus in the control of filariasis.

These results suggest that functional antigens are contained in the filarial ES product. Failure to achieve complete protection with the ES products may be due to the presence of substance (s)/antigen (s) which presumably mask the activity of protective antigens. Purification of the ES product may be useful in enhancing protection.

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