IMMUNE RESPONSE IN MOSQUITOES

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ABSTRACT

The primary purpose of this project was to investigate the immune response of the mosquito Aedes aegypti to the filarial worm Brugia pahangi. For comparative purposes responses to the bacterium Escherichia coli and to bacterial lipopolysaccharide (LPS), the ß-1,3-glucan laminarin, and the α-1,6-glucan dextran were also investigated. By transferring haemolymph from donor to recipient mosquitoes, it has been demonstrated that the haemolymph of Ae. aegypti infected with microfilariae of B. pahangi or with E. coli is partially protective against subsequent infection with filarial nematode. When the mosquitoes are infected with B. pahangi or E. coli, or immunized with laminarin, or LPS, followed by a challenge infection with B. pahangi, development of the challenge infection is suppressed. Using an in vitro motility assay, the haemolymph from infected mosquitoes and those treated with laminarin or LPS is found to have a direct inhibitory effect on microfilariae. Infection with B. pahangi produces a substantial increase in the numbers of haemocytes, as does infection with E. coli, while laminarin and LPS initially suppress haemocyte counts, possibly by stimulating release of adhesion factor. The protein content of the cell-free haemolymph was found to rise following injection of microfilariae, E. coli, laminarin and LPS.

The haemolymph proteins were examined following inoculation with microfilariae, bacteria and the cell wall components laminarin, LPS and dextran, using SDS-PAGE followed by silver staining. Twenty-five protein bands were

detected in inoculated mosquitoes; these ranged from 2.5 to 66kDa. Five protein bands of 66, 24, 12, 8 and 4kDa were induced by all treatments. A protein of 42kDa was induced only by microfilariae and bacteria. Another 14 protein bands were sometimes present in the haemolymph of treated mosquitoes. A protein band of 45kDa was specifically inducible by inoculation of living and heat-killed microfilariae but not by microfilariae ingested by membrane feeding. By virtue of their molecular weights and the nature of the stimuli inducing them, several of the proteins have been tentatively identified as homologous to previously described antimicrobial proteins.

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INTRODUCTION

Filariasis results from infection with eight distinct species: Wuchereria bancrofti, Brugia malayi, B. timori, Onchocerca volvulus, Loa loa, Dipetalonema streptocerca, Mansonella ozzardi and M. perstans. Human lymphatic filariasis is caused mainly by W. bancrofti, B. malayi, and B. timori. Of the estimated 90.2 million people infected, more than 90% have bancroftian and less than 10% brugian filariasis (WHO, 1987). The control of filariasis has relied on chemotherapy, vector control and reduction of human-vector contact.

The vectors of filariasis include a wide variety of mosquitoes within the genera Aedes, Anopheles, Culex, and Mansonia. Since the various mosquito species involved in the transmission of different forms of filariasis have different breeding and feeding habits, different approaches have to be made for their control. One of the possible approaches to controlling the mosquito vectors is the introduction of refractory genes into the natural population (Macdonald, 1976). Theoretically, the more that the refractory mechanisms to filarial infections in mosquitoes are understood, the better are the chances of vector manipulation for control.

The mosquito vectors are more than simply passive vectors transporting filarial parasites from infected to non-infected animals and man; they are also an integral part of the parasite life cycle. The filarial parasites must,therefore, be able to survive and develop in two different hosts and consequently deal with two different defence systems. Interactions occur between the vector and the parasite, the defence reaction of the vector attacking the parasite, and the parasite causing pathogenic effects in the vector (Ratcliffe, 1982; Kaaya and

Ratcliffe, 1982). This relatively new area of comparative immunology is providing information that demonstrates a complex, effective, and immune response against foreign invaders.

The recent increase in the number of significant articles and symposia reviewing the knowledge of immune capabilities of arthropods against foreign insults attests to the growing interest in this research area (Lackie, 1980; 1986a; 1986b; Rowley and Ratcliffe, 1981; Ratcliffe, 1982; Söderhall, 1982; Hall, 1983; Götz and Boman, 1985; Brehelin and Zachary, 1986; Dunn, 1986; Gupta, 1986; Boman and Hultmark, 1987; Nappi and Christensen, 1986; Christensen and Tracy, 1989). The data presently available suggest that significant differences exist, not only between arthropods from different classes, orders, genera and developmental life stages, but also between different species within the same genus and different strains within the same species.

The immune response of arthropods against the parasites they transmit to vertebrate hosts has been termed encapsulation (Götz, 1969), melanotic encapsulation (Poinar and Leutenegger, 1971), or melanization response (Christensen et al., 1984). It is likely that an effective immune response in arthropods is dependent on the ability of the host to recognize a parasite as a foreign invader (Gupta, 1986; Lackie, 1980; 1986a, 1986b; Ratcliffe, 1982; Ratcliffe and Rowley, 1983; Götz and Boman, 1985). These recognition signals must then undergo transformation in order to produce the appropriate effector causing parasite destruction.

This study investigates the immune mechanisms of susceptible strains of Aedes aegypti mosquitoes to Brugia pahangi microfilariae, the bacteria Escherichia coli and the microbial components laminarin, LPS and dextran.

LITERATURE REVIEW

1.1 FILARIAE AND FILARIASIS CONTROL

Filarial worms are arthropod-borne parasites of the lymphatic system and body cavity of vertebrates. These nematode parasites can sometimes cause serious diseases of man, such as elephantiasis and hydrocoele. The most important human lymphatic filariae are W. bancrofti, B. malayi and B. timori. The juvenile and adult worms normally live in the lymphatic vessels and lymph nodes, and microfilariae are found in the blood. The adult parasites can live for many years. The life span of microfilariae is about a year at the most. The latter stages are transmitted by mosquitoes which serve as an intermediate host in which microfilariae develop to the infective stage.

Urban W. bancrofti is transmitted mainly by Culex quinquefasciatus in tropical regions and by Cu. pipiens pallens and Cu. p. molestus in subtropical regions. The periodicity of the microfilariae is nocturnal.

Rural W. bancrofti is normally periodic over most of its range. It is mainly transmitted by several species of Anopheles, occasionally by Aedes spp, and rarely by Mansonia uniformis. The diurnally subperiodic type is transmitted predominantly by several species of Aedes, and the nocturnally subperiodic type by mosquitoes of the Ae. niveus group.

The nocturnally periodic type of B. malayi is transmitted in some regions by Mansonia spp, and in others by An. barbirostris or An. campestris. Ae. togoi is a vector in coastal parts of the Republic of Korea and parts of southern China. The nocturnally subperiodic type is transmitted by Mansonia spp.

B. timori is transmitted by An. barbirostris.

The control of vector-borne diseases is complicated and difficult. Filariasis control may have one or other of the following four objectives: reduction of morbidity, reduction of transmission, reduction of morbidity and transmission, and interruption of transmission (Mak, 1987).

While mosquitoes serve as the vector and the intermediate host of filarial nematodes, the mosquitoes are capable of protecting themselves in many ways from the invading parasites. Thus the vector often elicits a successful defensive encapsulation and/or melanization reaction. Laboratory selection experiments clearly demonstrated that a single sex-linked recessive gene, designated f^m, controls the development of Brugia spp. and W. bancrofti in the thoracic muscle of Ae. aegypti (Macdonald, 1962b; Macdonald and Ramachandran, 1965). The resistance to infection is a consequence of a host defence mechanism, but the mode of action of the f^m gene and other possible refractory genes is unclear. Nevertheless, genetic factors may control a variety of processes relevant to acquisition and survival of parasitic infections. A particular interest for the understanding of insect immunity is the genetic basis of the host's ability to respond to and recover from infection.

1.2 INSECT RESPONSE TO NEMATODE

The arthropods, like most invertebrates, display a variety of defence strategies to protect themselves against the harmful effects of parasites and various invading microorganisms. These host defences include the processes of inflammation, clotting, wound repair, phagocytosis, nodule formation and encapsulation (Rowley and Ratcliffe, 1981) and the production of agglutinins,

lysins and other antibacterial factors in the haemolymph (Söderhall and Smith, 1986b).

The insect host also responds to damage of the cuticle and/or midgut, the major sites of entry for nematode parasites. The penetration of the cuticle and epidermal cells may elicit a tissue response by the host but a haemocoele derived cellular or humoral response is usually not involved at that stage. Generally, the initial recognition of a nematode begins when the parasite breaks through the basement membrane and enters the haemocoele. At this time the host recognition of non-self can take one of two pathways, leading to a cellular or humoral response or both, depending on the host.

In nematocerous Diptera which have few haemocytes, a humoral response is usually produced (Lingg, 1976). This response typically results in melanization and death of the parasite. However, nematode parasites have evolved various mechanisms for dealing with such host responses, such as counter mechanisms that involve three possible strategies, entering host tissue or cells, molecular mimicry, and antigen sharing.

Nematode parasites induce responses in insects which can be categorized into three types: Humoral, cellular and tissue responses. The following section discusses areas relating to the humoral response to the nematode parasites which are in need of further research.

1.2.1 Humoral encapsulation

Humoral encapsulation has been found in some species of the following families of Diptera: Chironomidae (non-biting midges), Culicidae (mosquitoes), Psychodidae (moth-flies), Syrphidae (hover-flies) and Stratiomyidae (soldier-

flies) (Lingg, 1976). Encapsulation of nematodes without the participation of haemocytes has been previously reported by Götz (1969), Poinar (1974), Lingg (1976), Götz et al. (1977).

Humoral encapsulation in Culicidae against parasitic nematodes was mentioned by Bronskill (1962) and Esslinger (1962). Esslinger (1962) observed that the encapsulation of microfilariae of B. pahangi in the haemocoele of An. quadrimaculatus began with the deposition of a cell free layer of brown pigmented material or melanic and fibrous material on the surface of the nematodes. This material then increased in size and coalesced to form a melanotic capsule. The host reactions were not directly associated with specific types of cells.

Electron microscopic investigation of humoral encapsulation in Chironomus larvae confirmed the non-cellular origin of the capsule material (Götz, 1969). Electron dense material was found deposited directly from haemolymph on the surface of the mermithid nematode Hydromermis contorta. Debris of disintegrating haemocytes occurred only outside the capsule.

That encapsulation of developing filarial larvae in mosquitoes is a humoral rather than cellular reaction was supported by observations on Di. immitis in Ae. sollicitans and Ae. taeniorhynchus (Nayar and Sauerman, 1975) and B. patei in An. labranchiae atroparvus (Oothuman et al., 1974). However, Burton (1963) observed that the pigmented capsules which enclosed the microfilariae of W. bancrofti in seven species of mosquitoes were associated with varying amounts of tiny granules, globules, plaques and flake like debris. Poinar and Leutenegger (1971) described a biphasic formation of the melanized sheath around the nematode Neoaplectana carpocapsae in larvae of Cu.

pipiens. At the beginning of encapsulation, 25 minutes after invasion of the parasite, a homogeneous deposit with low electron-density was found surrounding the parasites. After one hour, electron-dense granules had appeared within this homogeneous layer. The definitive capsule was found 5-10 hrs after invasion and was composed of an inner region with electron-dense material. Chen and Laurence (1985) studied ultrastructural encapsulation of microfilariae of B. pahangi in An. quadrimaculatus and reported that the microfilariae were first seen enclosed in an acellular electron-dense capsule as early as 10 min after the mosquitoes commenced feeding on a parasitized cat. Two hours later the mosquito plasmatocytes spread around the humoral capsule. The complete capsule, which was seen at 24-48 hrs was composed of an inner humoral layer and an outer cellular layer. These authors suggested that the encapsulation of microfilariae in the haemocoele of mosquitoes combines both a humoral and a cellular reaction, the humoral capsule occurring first and the cellular reaction taking place later.

Humoral encapsulation is usually associated with insects having low total haemocyte counts, i.e. less than 6,000 cells/mm^3. Many Diptera have been found to have blood haemocyte counts of 500-5,100 cells/mm^3 (Lingg, 1976; Götz et al., 1977). The haemocyte count of mosquitoes ranges from 3,700-5,900 cells/mm^3 (Lingg, 1976). In dipteran species which cellular encapsulation occur however, the haemocyte count ranges from 2,300-4,900 cells/mm^3 (Götz and Boman, 1985).

1.2.2 Melanization response in mosquitoes

The melanization response has been noted in mosquito species against filarial parasites, even where those species are highly susceptible to filarial worm development (Intermill, 1973; Christensen, 1981). Such responses have generally been associated with mosquitoes that do not support the development of a particular species of filarioid nematode, e.g. B. patei in An. labranchiae atroparvus (Oothuman et al., 1974). Christensen et al. (1984) also reported that both susceptible and refractory mosquitoes possess the ability to destroy microfilariae by melanization. All microfilariae of Di. immitis and B. pahangi were melanized in Ae. trivittatus following day 2 PI and the response of this species was significantly more rapid and effective than the LVP and RKF strains of Ae. aegypti. The refractory RKF strain showed a significantly greater response against both Di. immitis and B. pahangi than the highly susceptible LVP strain, the authors suggested that the increased responsiveness was due to a physiological incompatibility in the RKF strain of Ae. aegypti.

Bartlett (1984) suggested that the response to Aedes species and the Ma. perturbans response to Di. scapiceps represented a humoral response, since haemocytes were never associated with larvae within the fat body, but in some cases melanization outside the fat body represented haemocyte melanization. Ogura (1987) demonstrated melanin deposition in vitro on microfilariae of B. pahangi and B. malayi exposed to the haemolymph of the mosquito Arm. subalbatus. The live microfilariae of both B. pahangi and B. malayi were melanized in haemolymph samples taken from one day old female adult Arm. subalbatus which had been injected with Hayes saline supplemented with sucrose. In the cell free haemolymph prepared by centrifugation live

microfilariae were only slightly melanized while heat-killed microfilariae were strongly melanized.

1.2.3 Composition of humoral capsule material

The capsule material which formed around fungi in Chironomus larvae was demonstrated by histochemical techniques to consist of a protein-polyquinone complex (Götz and Vey, 1974; Vey and Götz, 1975). Humoral encapsulation is a biphasic process that starts with the deposition of a soft, translucent layer during the first 10-15 minutes and later leads to the formation of solid dark brown pigment material (Chen and Laurence, 1985). Melanin is a copolymer comprising five or more aromatic constituents joined by carbon-carbon bonds of great stability (Lipke, 1975). Biochemically, melanization is not a clearly defined mechanism, and the formation of brown to black pigments generally occurs during quinone sclerotization, which is responsible for the hardening and tanning of the insect cuticle. The enzymes controlling quinone sclerotization, phenoloxidases, are present in cuticle, in the haemolymph and in the haemocytes of insects. Phenoloxidases are able to oxidise phenol derivatives to quinones. Autopolymerization of quinone produces dark pigments or pure melanin. The end-products of phenoloxidase activity, the protein-polyquinone complex, are mechanically stable (Lipke, 1975; Turnbull et al., 1980).

1.2.4 Antibacterial immune protein in insects

Humoral immunity can be induced in insects by an injection of either live, non-pathogenic bacteria or heat-killed pathogens (Götz and Boman, 1985). Mohrig and Messner (1968) reported that lysozyme, a universal substance in

the haemolymph of most invertebrates, was likely to be responsible for all immunity in insects although this view is now known to be incorrect (Boman, 1982; Gupta,1986).

1.2.4.1 Lysozyme

Stephen (1962) demonstrated bactericidal activity in the serum of the wax moth larva Galleria mellonella which had been subjected to injection of Pseudomonas aeruginosa and he showed that the bactericidal activity was correlated with the protective immunity of larvae.

The first antibacterial factor to be identified in insect haemolymph was lysozyme and it has been claimed that this enzyme is the main antibacterial factor (Mohrig and Messner, 1968). Kinoshita and Inove (1977) showed that significant bactericidal activity against E. coli B/SM existed in pooled, cell-free haemolymph from larvae of the silkworm Bombyx mori. They found that two factors were required to kill the organism ; one was a lysozyme-like enzyme and the other an anionic factor of low molecular weight, referred to as cofactor. They believed that the lysozyme-like enzyme was similar to that described by Powning and Davidson (1973), but they did not discuss further the nature of the second factor. Boman (1982) demonstrated that the insect can eliminate many lysozyme-resistant bacteria. Hultmark et al. (1980) isolated the cecropia lysozyme from Hyalophora cecropia during purification of cecropin A and B. Cecropia lysozyme is composed of 120 amino acids, has a molecular weight of 13.8 kDa and shows great similarity with vertebrate lysozyme of the chicken type. Jolles et al. (1979) demonstrated that although the lysozymes from B. mori and G. mellonella and Spodoptera littoralis (Lepidoptera) have a different

molecular weight (23,000) they are structurally related to the chicken type lysozyme. Engström et al. (1985) also reported the amino acid sequence of Cecropia lysozyme and the isolation and structure of cDNA clone containing a complete lysozyme sequence which was described as being of chicken type.

Hughes et al. (1983) have described the synthesis of several haemolymph proteins in larvae of the tobacco hornworm Manduca sexta injected with viable Enterobacter cloacae. One of these proteins has been identified as lysozyme (Hughes et al., 1983; Spies et al., 1986b). Normally, following injection of bacteria, induced lysozyme increased as a proportion of the total haemolymph proteins and there is also a sharp increase in the total haemolymph protein concentration (Dahlman, 1969). Dahlman also reported significantly higher protein concentrations in the haemolymph of fifth instar larvae of M. sexta parasitized by the braconid wasp Apanteles congregatus. In contrast, however, a study by Dunn et al. (1987) demonstrated increased serum lysozyme following injection of either viable or killed cells of Ps. aeruginosa into larvae of M. sexta. Lysozyme increase was not accompanied by a change in total serum protein concentration. They suggested that synthesis of antibacterial protein neither contributes a significant new mass of protein to haemolymph nor requires a compensatory decrease in the synthesis of normal haemolymph proteins.

Zachary and Hoffmann (1984) demonstrated the isolation of lysozyme from the serum of normal adult Locusta migratoria. Immunocytological techniques indicated that lysozyme is synthesized and stored in two granular haemocyte types of this insect species, the typical granulocyte and coagulocyte.

1.2.4.2 Agglutinins

Agglutinins (lectins) are widely distributed in the body fluids of insects and other invertebrates (Lis and Sharon, 1986). The ability of agglutinins to bind to carbohydrates had led to the hypothesis that those found in the haemolymph are involved in the recognition of foreign materials or degraded host tissue (Rowley et al., 1986). The apparent induction of haemolymph agglutinins by wounding has been reported in the flesh fly, Sarcophaga peregrina (Komano et al., 1980, 1981, 1983; Takahashi et al., 1986), in the velvetbean caterpillar Anticarsia gemmatilis infected with the fungus Normuraea rileyi (Pendland and Boucias, 1985), in the tobacco hornworm M. sexta induced by the bacteria En. cloacae (Minnick et al., 1986), and in the blackfly Simulium ornatum induced by the parasite O. lienalis (Ham et al., 1988). On the other hand Castro et al. (1987) specifically stated that the agglutinins of H. cecropia are not inducible.

Agglutinins have been reported to be synthesized in haemocytes of the cockroach Leucophaea maderae (Amirante and Mazzalai, 1978) and in H. cecropia (Castro et al., 1987) and in both the haemocytes and the fat body cells of only S. peregrina (Komano et al., 1983). Agglutinins have been detected on the surfaces of plasmatocytes and granular cells (Komano et al., 1983) but not on phagocytic cells (Ratcliffe, 1986). The active agglutinins of S. peregrina were found to have a molecular weight of 190 kDa and after treatment with mercaptoethanol shown by SDS electrophoresis to consist of four alpha subunits and two beta subunits, with molecular weight of 32 and 30 kDa (Komano et al., 1980). H. cecropia had a molecular weight about 160 kDa and

after treatment with mercaptoethanol two subunits A and B, with weights of 41 and 38 kDa respectively, can be identified (Castro et al., 1987).

Gilliam and Jeter (1970) demonstrated in the honeybee Apis mellifera, following immunization with the bacterium Bacillus larvae, that the agglutinins formed were active in agglutination assays against the microorganism and the closely related B. subtilis, while no reactivity was recorded against Salmonella thompson. Such results suggest a high level of specificity. In contrast, however, in Sc. gregaria, following immunization with the protozoan Leishmania hertigi, the agglutinins formed were active against the unrelated protozoan parasite Trypanosoma cruzi (Ingram et al., 1984). Lackie (1981) reported range specific agglutinins present in different insect species. The agglutinins induction or activation and the specificity of the reaction are unclear. The agglutinins may bind microbial and other non-self material to the coagglutination cells and trigger the prophenoloxidase cascade. This binding may represent the recognition phase in the insect immune defence system.

1.2.4.3 Cecropins

The cecropins form a class of antibacterial peptides produced by the humoral immune response of certain insects (Hultmark et al., 1980). Cecropins and about 10 other immune proteins are induced in the haemolymph of the pupae of the giant silk moth, H. cecropia, following injection of live bacteria. The first cecropins to be purified and identified and for which the tentative primary structures are available, are cecropin A and B forms (Steiner et al., 1981). They each contain 37 amino acid residues, with a basic N-terminal region and hydrophobic C-terminal region ending with a blocked carboxyl

group. Cecropins A and B are strongly homologous but differ significantly in structure and function from other known basic peptides (Merrifield et al., 1982).

The antibacterial activity of cecropin A and B was tested against bacteria; cecropin A did not appear to play a significant role against E. coli but was clearly involved against Ps. aeruginosa, B. megatorium and Mi. luteus (Andreu et al., 1985). Both cecropin A and B were active against Serratia marcescens, Ps. aeruginosa, Xenorhabdus nematophlus and B. megatorium (Steiner et al., 1981). Two years later, cecropin C and D were found as well as three minor forms, believed to be precursors (Hultmark et al., 1982). Cecropin C has an amino acid sequence of cecropin A and it may be a precursor or degradation product of cecropin A. The three principal cecropins A, B and D are small basic proteins with molecular weights around 4000 daltons, and a comparatively ionic hydrophobic region (Boman and Hultmark, 1981). The antibacterial activity of these three main cecropins was assayed against nine different bacteria species, showing that cecropins A and B are highly active against several Gram-positive and Gram-negative bacteria, while cecropin D only showed a high activity against E. coli (Hultmark et al., 1982). Qu et al. (1982) isolated cecropin B and D from pupae of the Chinese oak silkmoth Antheraea pernyi. The antibacterial activity was tested against nine different bacteria species, and it was shown that cecropin B was more highly active than cecropin D. Boman et al. (1985) reported that cecropin B was slightly more potent than cecropin A while cecropin D had the most narrow antibacterial spectrum.

1.2.4.4 Attacins

Attacins have been described as six closely related antibacterial proteins, attacins A-F, in the haemolymph of immunized pupae of the cecropia moth H. cecropia (Hultmark et al., 1983). They were considered to be immunologically identical to the P5 protein previously described by Pye and Boman (1971). Attacins have molecular weights of 20-23 kDa and while similar to that of P5, their molecular weights are significantly lower than 28 kDa found for pre-P5 protein synthesized in vitro (Engström et al., 1984a). Attacin A and B have a molecular weight of 22 kDa, attacin C and D 20 kDa, and attacin E and F 23 kDa (Engström et al., 1984b). The six attacins can be divided into two groups according to basic amino acid composition and amino-terminal sequences. Attacins A-D constitute a basic group and attacins E and F an acidic group. Within each group the forms are similar. The attacins effectively killed E. coli and two other Gram-negative bacteria, Acinetobacter calcoacetcus and Ps. maltophilia, both bacteria isolated from the gut of the Chinese silkworm (Hultmark et al., 1983). Engström et al. (1984a) also demonstrated that the attacins affected the growth of E. coli.

Kaaya et al. (1987) reported attacin-like antibacterial factors induced by E. coli in the haemolymph of Glossina morsitans, and when tested against ten different bacterial species, the spectrum of activity was the same as the antibacterial activity in the immune haemolymph from cecropia.

1.2.4.5 Sarcotoxins

Okada and Natori (1983) described an antibacterial protein, induced by injury and referred to as sarcotoxin I, from the haemolymph of S. peregrina larvae. The molecular weight of the purified protein was 5000 daltons and its amino acid composition was similar to that of the cecropins which are major antibacterial proteins in pupae of H. cecropia. Sarcotoxin I was a mixture of three proteins with almost identical primary structures. The proteins consist of 39 amino acid residues and differ in only two to three residues (Okada and Natori, 1985). The three structurally related proteins are designated sarcotoxin IA, IB and IC.

Ando et al. (1987) purified and identified three bactericidal proteins with almost identical primary structure from the haemolymph of third stage larvae of S. peregrina. These were designated sarcotoxin IIA, IIb and IIC, and their molecular weights are about 24 kDa. These proteins were found to have common antigenicity. Radioimmunoassay techniques using these antibacterial proteins showed that they are induced in the haemolymph in response to injury of the larval body integument. Sarcotoxin IIA consists of 270 amino acids, which have little homology with attacins. However, significant homology was found between these proteins in a region near the carboxyl terminal, suggesting that the prototype gene of these antibacterial proteins existed before the evolutionary branching of Diptera and Lepidoptera (Ando and Natori, 1988).

1.2.4.6 Diptericins

Keppi et al. (1986) reported an antibacterial substance which was induced by injection of live bacteria into third instar larvae of the dipteran Phormia terranovae. There were at least five heat stable, more or less basic proteins showing antibacterial activity against E. coli. One of these proteins has been partially purified and characterized as a non-lysozymic peptide of 9 kDa with an isoelectric point of 7.8. Fat body tissue has been shown to be responsible for the synthesis of the antibacterial protein in P. terranovae.

Dimarcq et al. (1988) demonstrated the amino acid sequence of this antibacterial protein from P. terranovae, and three of the peptides have been purified. The amino acid sequence has been completely established for one of these and partially for the two others. The sequences showed marked homologies indicating that the peptides belong to a common family. They are not related to the other known antibacterial peptides from insects, namely lysozyme, cecropins, sarcotoxin I and II, and attacins. They proposed the name of Diptericin for this new family of antibiotic molecules. Diptericin showed the highest antibacterial activity against E. coli, and it has a basic molecule containing 82 amino acid residues with a relative molecular weight of 8610 daltons.

1.2.4.7 Apidaecins

Apidaecins form a new family of bactericidal proteins isolated from both larval and adult honeybees, A. mellifera, induced by bacteria E. coli. These heat stable antibacterial proteins are detectable in haemolymph of adult honeybees while bee larvae contain considerable amounts of inactive precursor

molecules. Apidaecins have molecular weights of 2100 daltons, making them the smallest proteins purified from the insects (Casteels et al., 1989).

1.2.4.8 Prophenoloxidase activation system

Phenoloxidase, another naturally occurring substance in invertebrates, has also been considered an efficient defence material. Since melanin deposition in invertebrates is associated with wound repair, nodule formation, and melanization, then the prophenoloxidase system which generates the pigment by conversion of aromatic quinones (and its products) may be involved.

Melanin formation around parasites is commonly associated with the cellular and humoral defences of arthropods (Nappi, 1973). Generally melanization has been considered to represent, at least in part, a defence reaction of insects. The reaction of melanin with proteins surrounding foreign materials might serve to physically isolate them. In addition, substances such as quinone, which are intermediate in melanin synthesis, are phenolic substances which are cytotoxic and hence could act as bactericidal or fungistatic agents. Both the inactive precursor of phenoloxidase and the substrate of enzyme, tyrosine, have been shown to be present in the haemolymph of many insect species including the oak silkmoth, A. pernyi, and the silkworm B. mori (Wyatt and Pan, 1978).

Phenoloxidase activity has been identified in the haemolymph of many insects, including G. mellonella (Pye, 1974), P. americana (Fisher and Brady, 1983), and L. migratoria (Hoffmann et al., 1970). The phenoloxidase system of Diptera appears to more complex than that in Lepidoptera, Dictyoptera and

Orthoptera. Ashida et al. (1983) reported that components of the phenoloxidase system are present in the plasma of the silkworm B. mori and that activation of this enzyme is triggered by Gram-negative and Gram-positive bacteria cell walls, and by glucans with ß-1,3-glycosidic linkages such as laminarin and denatured lipophorin. Ashida and Dohke (1980) have purified and identified a serine protease enzyme that stimulates the prophenoloxidase activating system of B. mori. Söderhall (1983) also reported that a serine protease was present in haemolymph lysate of the crayfish Actacus and that it was involved in activation of prophenoloxidase and the coagulation process. He also showed that the serine protease can be specifically activated by ß-1,3-glucan.

More direct evidence for the role of the prophenoloxidase system has been reported. The addition of laminarin or endotoxin E. coli (055;B5) to G. mellonella cell monolayers significantly enhances haemocyte phagocytic capacity (Ratcliffe et al., 1984). Furthermore, the spectrophotometric assay also demonstrated that laminarin is an effective activator of prophenoloxidase (Ratcliffe et al., 1984). Such stimulation of the prophenoloxidase system by certain microbial products may also play an important role in the non-self recognition process in insects. Leonard et al.(1985a) suggested that prophenoloxidase activation by ß-1,3-glucan is dependent upon Ca^{+2} ions, and prophenoloxidase seems to be transformed to phenoloxidase by the proteolytic attack. Blaberus craniifer haemocyte lysate contains several peptidases, one or more of which may be involved in prophenoloxidase activation. This involvement in the prophenoloxidase mechanism is indicated by the enhancement of the activity of several peptidases by laminarin. Protease

inhibitors, such as soybean trypsin inhibitor, benzamidin and p-nitrophenyl-p-guanidobenzoate, blocked laminarin activation of prophenoloxidase, which indicated that prophenoloxidase is converted to phenoloxidase by a limited proteolysis. The biochemical studies on the G. mellonella prophenoloxidase system demonstrated that it was activated by trypsin, laminarin and laminarin G, a highly purified ß-1,3-glucan, but not by dextran. Serine protease activities were also enhanced by adding laminarin to a haemocyte lysate supernatant (Leonard et al., 1985b). Dularay and Lackie (1985) also reported that the prophenoloxidase pathway in the haemocyte lysate supernatant of the locust Sc. gregaria can be partially activated in the presence of Ca^{+2} and strongly activated by ß-1,3-glucan, but the production of phenoloxidase was not enhanced by the presence of bacteria LPS and was inhibited by a serine protease inhibitor. Yoshida and Ashida (1986) also demonstrated that lipopolysaccharide cannot be activated by the serine enzyme in the silkworm B. mori.

Zymosan, ß-1,6-glucan, which was purified from cell walls of the yeast Saccharomyces cervisae, can activate the prophenoloxidase system in Sc. gregaria, the desert locust (Gunnarsson, 1988). Histochemical staining demonstrated that the prophenoloxidase enzyme was located in haemocytes of L. migratoria and G. mellonella (Hoffmann et al., 1970; Schmit et al., 1977). Iwama and Ashida 1986) suggested that prophenoloxidase was synthesized by the haemocytes in the larval haemolymph of the silkworm B. mori. Huxham et al. (1989) reported that the destruxins, which contain 5 amino acid residues, extracted from insect fungus pathogen Metarhizium anisopliae, inhibited or reduced the prophenoloxidase activating system induced by laminarin or

zymosan activation in the cockroach P. americana and locust Sc. gregaria. The mechanism of the interaction between laminarin, destruxin and phenoloxidase production is still unclear, but they suggested that destruxin acted at the level of the intact haemocyte plasma membrane.

Söderhall et al. (1988) activated with laminarin the peptidase and prophenoloxidase in haemocyte lysates in the cockroach B. craniifer. The enzymes were isolated and purified by affinity chromatography on laminarin-Sepharose and found to have a molecular weight of 90 kDa, whereas the reduced forms (by mercaptoethanol) had weights of 63 kDa and 52 kDa. Tsukamoto et al. (1986) purified the latent phenoloxidase from the prepupae of the housefly M. domestica ; the molecular weight of the latent phenoloxidase was estimated to be 178 kDa, while the phenoloxidase formed by the activation of latent phenoloxidase had a higher molecular weight of 340 kDa.

The molecular weight of prophenoloxidase of B. mori was 80 kDa and its subunit or reduced form was 40 kDa. In Calliphora erythocephala the active form has a molecular weight of 115 kDa and its subunits or reduced forms, after treatment with mercaptoethanol, were 77, 70 and 49 kDa (Munn and Bufton, 1975; Wyatt and Pan, 1978).

J.L. Huang and H.Townson (unpublished) demonstrated that strains of mosquitoes with genetically defined differences in filarial susceptibility show slightly but significantly differences in phenoloxidase activity following both injury and infection, and their capacity to melanize filarial parasites.

1.2.4.9 The synthesis of immune haemolymph proteins in insects

Although the insect immune system lacks such important features of vertebrate immunity as lymphocytes and immunoglobulin (Boman and Hultmark, 1981; Götz and Boman, 1985; Dunn, 1986), both cellular and humoral factors play a role in the insect defence system.

Three types of circulating antibacterial proteins, lysozyme, cecropins and attacins, have been described in moths (Boman, 1986) and sarcotoxins and diptericin have been identified in flies (Okada and Natori, 1983; Keppi et al., 1986). However, the site of the haemolymph immune protein synthesis is unclear. Faye and Wyatt (1980) reported fat body of diapausing pupae of the silkmoth H. cecropia immunized with E. cloacae released at least nine haemolymph immune proteins (P1-P9). Postlethwait et al. (1988) demonstrated that the fat body of larvae and adult males of the fruit fly Ceratitis caspitata, inoculated with E. cloacae, released antibacterial immune proteins. Palli and Locke (1987) also reported that the fat body was responsible for synthesis of 50% of different haemolymph polypeptides and about 90% of the total haemolymph protein.

Lysozyme has been isolated from and appears to be synthesized by the granulocytes and coagulocytes of normal and immunized adults of L. migratoria (Zachary and Hoffmann, 1984). Trenczek and Faye (1988) demonstrated that fat body from injured or immunized pupae and from untreated pupae of H. cecropia synthesized and released in vitro immune proteins, namely protein P4 (48 kDa), attacins, cecropins and lysozyme, which previously had been reported as proteins P1-P9. When these haemolymph proteins were purified, P5 was

identified as attacins (Hultmark et al., 1983), P7 as lysozyme (Hultmark et al., 1980); Engström et al., 1985), and P9 as cecropins (Hultmark et al., 1980; Hultmark et al., 1982).

Agglutinins have been reported to be synthesized in haemocytes of the cockroach L. maderae (Amirante and Mazzalai, 1978), H. cecropia (Castro et al., 1987) and in both haemocytes and fat body cell in S. peregrina (Komano et al.,1983).

Thus the synthesis of immune haemolymph proteins occurs in the fat body and the haemocytes of normal and bacteria-infected insects, but the primary inducing agent(s) remains unknown.

1.2.5 Cellular Immunity

There have been a number of reviews published recently on invertebrate cellular immunity (Gupta, 1986; Lackie, 1980, 1981, 1986a; Götz and Boman, 1985). Insects and other invertebrates lack the immunoglobulin cell surface receptors that characterize the immune competent cells of vertebrates, yet they manifest an efficient cell-mediated response against a diversity of nonself components. Unfortunately, little is presently understood of the mechanisms of cellular immunity and nonself recognition by insect haemocytes.

The cellular defences of insects include wound healing (Yeaton, 1983; Ratcliffe et al., 1985), haemolymph coagulation, phagocytosis and an encapsulation-type response (Ratcliffe and Rowley, 1983, 1984). The circulating haemocytes immobilize invasive parasites and microorganisms, and injected foreign particles (Götz, 1986a, 1986b; Ratcliffe et al., 1985). The smaller entities are phagocytosed (Brehelin and Hoffmann, 1980), or at higher concentrations

entrapped in haemocyte aggregates (nodule formation)(Ratcliffe and Gagen, 1977; Brookman et al., 1989), whereas larger particles and nematode parasites are humorally encapsulated, followed by several layers of flattening haemocytes (Chen and Laurence, 1985; Götz, 1986a, 1986b; Ratcliffe, 1986; Stoffolano, 1986).

The ultrastructural studies have clearly demonstrated the active participation of haemocytes in the encapsulation reaction of Ae. trivittatus against Di. immitis microfilariae (Forton et al., 1985) and the lysis of Ae. aegypti haemocytes at the surface of inoculated microfilariae prior to the deposition of melanotic material (Christensen and Forton, 1986). Chen and Laurence (1985) also used transmission electron microscopy to illustrate the active involvement of haemocytes in adult An. quadrimaculatus during the response to B. pahangi. Nappi and Christensen (1986) reported that some of the circulating haemocytes in immune reactive Ae. aegypti acquire surface modification that can be identified by enhanced binding of wheat germ agglutinin. These activated haemocytes were the only cells found adhering to inoculated Di. immitis microfilariae. Subsequently, Chen and Laurence (1987) determined that an in vitro system required the presence of intact haemocytes from An. quadrimaculatus for the successful melanization of B. pahangi microfilariae. In adult Ae. aegypti inoculated with Di. immitis microfilariae, total haemocyte populations were found to be 2-3 fold greater than populations from saline inoculated controls during the early stages of melanotic encapsulation (Christensen et al., 1989).

The plasmatocyte is the main circulating phagocytic cell in many insects and is the main structural component of the capsule formed around invading

parasites or other nonself material (Salt, 1970; Ratcliffe and Rowley,1979). Chain and Anderson (1981,1982) demonstrated that subsequent to injection of different species of bacteria into the larvae of the wax moth G. mellonella, plasmatocytes, but not other haemocytic cell types, disappeared very rapidly from the circulation, probably by increased adherence to the haemocoele lining. Sharma et al. (1986) also demonstrated that the predominant cell involved in phagocytosis, in the response to injection of yeast cells into the haemocoele of Poecilocerus pictus, was the plasmatocyte with granular haemocytes playing a lesser role.

Gunnarsson and Lackie (1985) compared the cellular response of the locust Sc. gregaria with that of the cockroach P. americana. The effect of injection of solutions of LPS, zymosan (ß-1,6-glucan, from yeast cell walls) solution and a ß-1,3-glucan laminarin into cockroaches was investigated and found to induce the same order of response as in the locust. Nodule formation was not induced in insects injected with saline or a solution of dextran (an α-1,6 glucan).

Söderhall and Smith (1986a, 1986b) demonstrated that the cell adhesion activity in crustaceans was only present in haemocyte lysates in which the prophenoloxidase activating system was activated. Johanson and Söderhall (1988) showed that the prophenoloxidase activating system of the crayfish could be activated, either by LPS, the ß-1,3-glucan laminarin, or by preparing the lysate in Ca^{+2}. Both lysates of the granular or semigranular haemocytes could mediate adhesion. Nevertheless, the connection between the prophenoloxidase system and the activities of the various haemocyte type have

so far been mostly studied in crustaceans, whereas in the insects, especially Diptera, the functional retationships are unclear.

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CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

2.1.1 Mosquitoes

The Aedes aegypti (Linnaeus) Liverpool strain was originally selected for filarial susceptibility in the Department of Medical Entomology of L.S.T.M. (Macdonald, 1962a) and it has been maintained continuously since then. The stage of mosquitoes used in this study were 5-7 day-old virgin females. Mosquitoes in all experiments were maintained in an environment of 25 ± 1°C and 75 ± 10% R.H.

2.1.2 Filariae

The filarial parasite Brugia pahangi was maintained in jirds, Meriones unguiculatus, in the animal unit and it has been kept in continuous laboratory culture in the Ae. aegypti susceptible strain designated ref^{m}.

2.1.3 Bacteria

The bacteria Escherichia coli H.B.101 a streptomycin-resistant mutant was obtained from the Molecular Genetics laboratory. The bacteria was grown in Difco nutrient broth.

2.1.4 Mosquito injection techniques

2.1.4.1 Inoculation with microfilariae

Adult virgin female mosquitoes were inoculated intrathoracically with B. pahangi microfilariae. The microfilariae were isolated and concentrated from jird

washings by diluting with chilled distilled water and centrifuging at 1500 rpm for 5 min. The supernatant was discarded, and washing and centrifuging were repeated at least three times with TC199 mixture with antibiotic streptomycin sulphate 100 μg/ml (Townson, 1974; Sucharit and Chuchote, 1982). The final sediment was resuspended in 0.5 ml of TC199 and streptomycin sulphate mixture. The heat-killed microfilariae were killed by boiling the microfilariae suspension in a hot water bath at 100°C for 5 min.

Mosquitoes were inoculated using a capillary pipette drawn out to a tip diameter of 60-80 μm (Nelson, 1962). Movement of the pipette was controlled by means of a manipulated handle. The microfilariae were drawn into the pipette in a small volume of medium of approximately 1 μl under a binocular dissecting microscope. The glass needle was then inserted laterally into the thorax via a small plate at the base of the wing axis and the microfilariae expressed into the carbon dioxide anaesthetized mosquito. At the completion of the experimental procedures, these mosquitoes were dissected in Hayes saline and 1% methylene blue mixture to assess how many microfilariae had developed and to record the stage of development.

2.1.4.2 Bacteria injection

The virgin female mosquitoes were injected with E. coli H.B.101, a streptomycin resistant mutant. The bacteria was grown in Difco nutrient broth to the early logarithmic stage of growth. Then the bacteria cells were centrifuged and suspended in ice cold 0.1M phosphate buffer pH 6.4 to give a density on the colorimeter of 0.3 A650, representing $1x10^8$cells/ml. The suspension was diluted with TC199 to give $1x10^4$cells/μl and 100 μg/ml

streptomycin sulphate was added (Boman et al., 1974; Natori, 1977). The heat-killed bacteria were killed by boiling the suspension in a hot water bath at 100°C for 5 min.

The carbon dioxide anaesthetized mosquitoes were injected with 1 μl of E. coli suspension containing $1x10^4$cells/μl into the thorax via a small plate at the base of the wing axis, using a finely extruded microcapillary glass tube, under a binocular microscope.

2.1.4.3 TC199 control injection

For a control 1 μl of TC199 and 100 μg/ml streptomycin sulphate was injected into the thorax of anaesthetized mosquitoes.

2.1.4.4 Collection of mosquitoes haemolymph

At defined intervals following initial inoculation, haemolymph was withdrawn from the mosquitoes using the technique described by Ham (1986). The mosquitoes were first injected with a further 1 μl of Hayes saline and streptomycin sulphate mixture. Then, after 30 seconds using the same needle without having withdrawn it, approximately the
same volume of fluid was removed from the thorax by suction under a binocular microscope. The haemolymph and Hayes saline mixture was collected in a clean plastic vial and stored at -70°C.

2.2 CHEMICALS

2.2.1 Laminarin

Laminarin, a ß-1,3-glucan from Laminaria digitata (Sigma Chemical Company): laminarin 1 mg/ml solution, was dissolved in double distilled water, heated to aid dissolution, and then diluted with o.15M NaCl solution to the final concentration (Ratcliffe et al., 1984; Leonard et al., 1985a).*

* ß-1,3-glucans are common components of microbial cell walls. Laminarin is the participal commercially available ß-1,3-glucan.

2.2.2 Lipopolysaccharide (LPS)

Lipopolysaccharide (lyophilized powder) from E. coli serotype 026:B6, from Sigma Chemical Company, in a solution of 100 μg/ml was added to double distilled water and diluted with 0.15M NaCl solution to the final concentration required (Ratcliffe et al., 1984; Leonard et al., 1985a).

2.2.3 Dextran

Dextran, an α-1,6-glucan (Clinical grade Av.mol.wt.60-90 kDa) from the Leuconostoc mesenteroides strain of Sigma Chemical Company, was obtained in a 100 μg/ml solution. This was diluted in double distilled water to the required concentration (Ratcliffe et al., 1984; Leonard et al., 1985a).

2.3 ANALYSIS OF PROTEIN BY SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis is a powerful tool for protein separation and characterization. Polyacrylamide has

several advantages over other materials, mainly because of its superiority as a molecular sieve. Pore size can be varied over a wide range by varying the acrylamide concentration and extent of cross-linking.

Polyacrylamide gels are generated by the free radical polymerization products of the acrylamide monomer and the cross linking CO-monomer. The polymerization reaction is initiated by a catalyst redox system which furnishes free radicals (Maurer, 1971; Gordon, 1975). The most commonly used system utilized the tertiary amine TEMED(N,N,N'-N'-tetramethylethylenediamine) as the catalyst and ammoniumpersulphate as initiator.

The treatment of proteins with SDS and a reducing agent beta-mercaptoethanol or ditiothreitol changes their three dimensional shape into rod-like structures (Reynolds and Tanford, 1970a). Since SDS binds to polypeptides, as a constant weight ratio, the charge per unit weight is constant and electrophoretic mobility becomes a function of molecular weight (Reynold and Tanford, 1970b). In addition to protein separation, the SDS-polyacrylamide gel electrophoresis technique has been widely used to determine the molecular weight of unknown proteins (Weber and Osborn 1969,1975; Gordon, 1975). A straight line is obtained in a plot of log molecular weight as a function of relative

electrophoretic mobility.

Several systems for SDS-polyacrylamide gel electrophoresis have been described. This present study describes the discontinuous system introduced by Laemmli (1970) for disc gel electrophoresis, which was later adapted to slab gels by Studier (1973). The system is characterized by a discontinuity in the buffer pH and in the polyacrylamide pore size. Two kinds of gel are prepared,

"a stacking gel" with large pores, at pH 6.8, where the sample is concentrated, and "a running gel" with small pores, at a more basic pH 8.8, where the sample is separated into its components.

The concentration gradient of acrylamide and the cross-link agent is polymerized to produce a gel with gradually decreasing pore size, the high concentration of acrylamide, 10-15%, giving better separation of low molecular weight proteins. The proteins are electrophoresed into the gel and eventually reach the part of the matrix where the pores are small enough to reduce their mobility to zero. Therefore, proteins with different sizes stop at different regions of the gradient gel giving sharp and separated bands.

A cooled vertical slab gel apparatus from LKB Instruments Ltd, manufactured by Hoefer Scientific Instruments, was used. Each slab gel was formed between glass-plates 16.0x8.0x0.3 cm, neither of which was notched, and which were held apart by PVC spacers 0.15 cm thick, placed down each of two vertical sides of the sandwich.

The spacers were held in position by one piece plastic clamps. The bottom of the sandwich was sealed using polycarbonate cams which pressed the base against a silicone rubber gasket in a casting stand. After gel pouring, polyarylamization, sample well formation and sample loading, the slab gel sandwich was released from the casting stand and locked in position against another silicone rubber gasket in the upper buffer reservoir using the same cams system. The upper and lower reservoir were filled with reservoir buffer and that in the lower reservoir was cooled by coolant passing through a glass tube heat exchanger, placed between the glass slab gels, and stirred by a magnetic stir bar. The water cooling system had a flow of 2 litres/min and

the slab gel electrophoresis apparatus was cooled to 4°-8°C (Hames and Drickurd, 1988).

A power pack LKB 2197 electrofocusing constant power supply model was used.

2.3.1 Reagents and solution

2.3.1.1 Polyacrylamide slab gel electrophoresis Stock solution

2.3.1.1.1 Acrylamide-bisacrylamide

Acrylamide-bisacrylamide (29.2:0.8) was prepared by dissolving 29.2 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml double distilled water. The solution was filtered through Whatman No.1 filter paper, and stored at 4°C in a dark bottle.

2.3.1.1.2 TEMED

TEMED (N,N,N'-N'-tetramethylethylenediamine) was used as supplied from BDH.

2.3.1.1.3 Ammonium persulphate (Kodak)

10% ammonium persulphate (w/v) and 0.1 gm of ammonium persulphate were dissolved in 1 ml double distilled water. The solution was made fresh just before use.

2.3.1.1.4 Sodium dodecyl sulphate (SDS)

10% SDS (w/v) was prepared by dissolving 10 gm SDS in double distilled water to 100 ml it was stored at room temperature for several weeks.

2.3.1.1.5 Electrophoresis buffer

2.3.1.1.5.1 Separating gel buffer

1.5 M Tris pH 8.8 was prepared by dissolving 18.5 gm Tris base in 50 ml double distilled water, adjusting to pH 8.8 with 1N HCl, then adding double distilled water to a total volume of 100 ml.

2.3.1.1.5.2 Stacking gel and samples buffer

0.05 M Tris pH 6.8 was prepared by dissolving 3.0 gm Tris base in 20 ml double distilled water, adjusting to pH 6.8 with 1N HCl, then adding double distilled water to a total volume of 50 ml.

2.3.1.1.5.3 Running buffer

Running buffer pH 8.3 was prepared by dissolving 12.12 gm Tris base, 57.68 gm of glycine, and 4.0 gm SDS in 4 litres of double distilled water and adjusting to pH 8.3.

2.3.1.2 Sample preparation

2.3.1.2.1 Sample cocktail solution

The mixture of 1.88 ml Tris-HCl pH 6.8, 6.0 ml 10% SDS, 3.0 ml glycerol and 2.12 ml double distilled water was prepared before use. To it was added 0.1 ml 1.5 M ditiothreitol and 0.1%(w/v) bromophenol blue (BDH) which was stored in aliquots at -20°C or 1.39 gm DTT in 6 ml 0.1% bromophenol blue. The cocktail sample solution and unknown protein solution were used in a ratio of 1:4 (V:V).

2.3.1.2.2 Molecular weight marker

Molecular weight marker is prepared by adding 2 ml of the sample preparation buffer to the contents of the vial. The total proteins (1 mg/ml) are completely dissociated by immersing the vial in boiling water for 5 min. The 2 µl of treated marker solution provided a suitable gel loading.

2.3.1.3 Staining

2.3.1.3.1 Silver stain

The staining was performed using the procedure for Bio-Rad silver stain and modified by the staining procedure outline in Table 2.

2.3.1.3.2 Silver stain reagent preparation

2.3.1.3.2.1 Oxidizer

The 20 ml oxidizer concentrate was mixed with 180 ml of double distilled water. This solution must be prepared on the same day that staining is to be performed.

2.3.1.3.2.2 Silver reagent

The 20 ml silver reagent concentrate was mixed with 180 ml of double distilled water. The solution must be prepared on the same day that staining is to be performed.

2.3.1.3.2.3 Developer

The 32 gm developer was dissolved in 1000 ml of double distilled water. The contents will dissolve in 15 min at room temperature with constant stirring. The solution can be stored at 4°C for 1 month.

2.3.1.3.3 Coomasie Brilliant Blue G-250

Coomassie Brilliant Blue G-250 was obtained from Sigma Chemical Comp., and 1.25 gm Coomassie Brilliant Blue G-250 was dissolved in 454 ml 50 methanol and 46 ml glacial acetic acid prefiltered through Whatman No.1 paper. Molecular weight standard marker were obtained from BDH, and are detailed below ;

Component	Molecular weight
Marker I	
1. Ovotransferin (Hen eggs)	76-78,000
2. Albumin (Bovine Serum)	66,000
3. Ovoalbumin (Hen eggs)	45,000
4. Carbonic anhydrase (Bovine erythrocyte)	30,000
5. Myoglobulin (Equine)	17,200
6. Cytochrome (Equine)	12,300
Marker II	
1. Myoglobulin	16,900
2. Myoglobulin I+II	14,404
3. Myoglobulin I	8,159
4. Myoglobulin II	6,214
5. Myoglobulin III	2,512

TABLE 1. RECIPE FOR GEL PREPARATION USING THE SDS DISCONTINUOUS BUFFER SYSTEM.

STOCK SOLUTION	STACKING GEL		SEPARATING GEL			UNITS
	3%	5%	10%	12.5%	15%	
Acrylamide-bisacrylamide	1.0	1.67	10	12.5	15.0	ml
0.125M Tris-HCl pH 6.8	1.25	1.25	-	-	-	ml
0.37M Tris-HCl pH 8.8	-	-	11.2	11.2	11.2	ml
Double distilled water	7.7	7.03	8.7	6.2	0.7	ml
Glycerol	-	-	-	-	3.0	ml
10% SDS	100	100	300	300	300	µl
10% Ammonium persulphate	50	50	100	100	100	µl
TEMED	10	10	20	20	20	µl

TABLE 2. SILVER STAIN PROTOCOL.

REAGENTS	VOLUME(ml)	DURATION
1. Fixative 40% methanol 12% acetic acid(v/v)	400	Overnight
2. Fixative 10% ethanol 5% acetic acid (v/v)	400	60 min
3. Fixative 10% ethanol 5% acetic acid (v/v)	400	60 min
4. Oxidizer	200	10 min
5. Double distilled water	400	60 min
6. Double distilled water	400	60 min
7. Double distilled water	400	60 min
8. Double distilled water	400	60 min
9. Double distilled water	400	60 min
10. Silver reagent	200	30 min
11. Double distilled water	200	2 min
12. Developer	200	2 min
13. Developer	200	8 min
14. Developer	200	8 min
15. Stop 5% acetic acid (v/v)	400	15 min
stored at 4°C. Double distilled water.		

CHAPTER 3

IN VIVO STUDIES OF THE ACQUIRED IMMUNE RESPONSE TO BRUGIA PAHANGI IN AEDES AEGYPTI

3.1 ACQUIRED IMMUNE RESPONSE TO BRUGIA PAHANGI IN AEDES AEGYPTI BY IMMUNIZED WITH MICROFILARIAE, BACTERIA AND CELL WALLS COMPONENTS

3.1 Introduction

The mechanisms by which mosquitoes protect themselves against invading parasites have been of interest since the reports of melanotic encapsulation of filarial nematodes by mosquitoes (Esslinger, 1962; Intermill, 1973; Oothuman et al., 1974; Nayar and Sauerman, 1975; Christensen, 1981; Christensen et al., 1984; Sutherland et al., 1984; LaFond et al., 1985; Christensen and LaFond, 1986; Christensen et al., 1986). Most of the literature dealing with mosquito defence mechanisms is primarily descriptive and deals almost exclusively with observations on the encapsulation and/or melanization of filarial worms. Little effort has been made to determine what effect, if any, encapsulation and/or melanization reaction might have on reducing the parasite load in the vector, thereby increasing the vector's chances of survival.

Clearly, both humoral and cellular components are features of insect immunity, but mosquitoes have a somewhat limited number of haemocytes in comparison to other insects (Lingg, 1976). It is therefore possible that humoral immunity mechanisms may play a large part in the variation in susceptibility to filarial worms in mosquitoes. Ham (1986) reported the acquired resistance to O. lienalis infection in S. ornatum and S. lineatum following passive transfer of haemolymph from previously infected simuliids. The data showed that some factor in the haemolymph of O. lienalis infected flies, when injected into the

thorax of previously untreated recipient flies, was capable of preventing development of O. lienalis larvae.

The role and specificity of humoral immunity to intrathoracically inoculated B. pahangi microfilariae was investigated by means of a series of experiments involving passive transfer of haemolymph between treated donor and microfilariae inoculated recipient mosquitoes, and immune suppression in the mosquitoes injected with microfilariae, bacteria and cell walls components followed by challenge infection with B. pahangi.

3.1.2 Materials and Methods

3.1.2.1 Haemolymph extraction

The mosquitoes were injected with 1 μl of TC199 in the membranous area below the paratergite of the wing axis. Then, after 30 sec, using the same needle without having withdrawn it, approximately the same volume of fluid was removed from the thorax by suction. On microscopic examination, the fluid was observed to contain haemocytes. Their presence demonstrated that haemolymph could be successfully withdrawn from the injected mosquitoes using this technique.

In order to estimate the proportion of haemolymph in the withdrawn fluid, the mosquitoes were injected with 1 μl of 0.5% methylene blue, and after 30 sec the same volume of fluid removed by suction and diluted in 1 ml Hayes saline solution. The methylene blue concentration was then estimated by measuring absorbance at 290 nm in a spectrophotometer. The haemolymph dilution factor was calculated by reference to standard curves of the absorbance of 0.5%, 0.25%, 0.125% and 0.10% methylene blue in Hayes saline solution. By this process it was estimated that approximately 75% of the withdrawn fluid comprised haemolymph.

3.1.2.2 Acquired immune resistance to B. pahangi infection in mosquitoes following passive transfer of immunized haemolymph.

3.1.2.2.1 Experiment I

Experiment I. was performed to investigate the acquired resistance of Ae. aegypti to B. pahangi infection, following passive transfer of haemolymph from previously infected mosquitoes.

Four days after infection with either living microfilariae or heat-killed microfilariae or injection of TC199, the mosquitoes were injected with 1 μl of TC199, then after 30 seconds, a 1 μl sample of haemolymph withdrawn.

The haemolymph sample was then injected into the thorax of the recipient mosquitoes. Recipient mosquitoes had previously been injected with B. pahangi microfilariae approximately 2 hours earlier. The procedure was repeated with successive pairs of mosquitoes, 1 donor to 1 recipient. The recipients were placed individually in 75x25 mm labelled glass tubes covered with cotton mesh. They were fed on 5%(w/v) sugar solution soaked in small cotton wool pads placed on the mesh.

The donor mosquitoes were dissected immediately in drops of Hayes saline with 1% methylene blue solution and the numbers and stages of the larvae recovered from thorax and abdomen were recorded.

Four days after haemolymph transfer (8 days after initial inoculation of donor mosquitoes), the recipients were all dissected and the number and stages of larvae per mosquito were recorded.

Five trials (A-E) were conducted with donors receiving 5 heat-killed and 5, 10, 20, 20 and 60 living microfilariae respectively.

3.1.2.2.2 Experiment II

Experiment II involved the transfer of haemolymph from mosquitoes infected with B. pahangi microfilariae, with living and heat-killed bacteria E. coli and from TC199 inoculated controls.

This experiment was performed to investigate the induced specificity of the induced protective response observed in experiment A.

Four groups of mosquitoes were used.

i. Donor injected with TC199-sham treated control (T1);

ii. Untreated donor control (T2);

iii. Inoculated recipient control (no prior treatment)(T3);

iv. Donor inoculated with B. pahangi microfilariae (T4);

v. Donor injected with living (T5.L) or heat-killed (T5.HK) E. coli $1x10^4$cells/μl;

Four days after inoculation of donors, the haemolymph was transferred from the donor mosquitoes to 4 groups of previously infected recipients as described earlier. The subsequent dissection of donors and recipients followed the procedures described in 3.1.2.2.1 .

3.1.2.3 Acquired immune suppression to B. pahangi infection in Ae. aegypti mosquitoes previously immunized with microfilariae, bacteria, laminarin, LPS and dextran.

3.1.2.3.1 Experiment III

Experiment III involved the injection of Ae. aegypti with B. pahangi microfilariae, or E. coli or immunization with laminarin and LPS followed by challenge injection with B. pahangi.

Six groups of mosquitoes were used. On day 0 treatment 4 mosquitoes were injected with B. pahangi microfilariae and treatment 3 served as control. On day 3 treatment 1 sham control was injected with TC199 and antibiotic, and treatment 5 received 1 μl of E. coli HB 101 $1x10^4$cells/μl , treatment 8 received 1 μl of 1mg/ml laminarin, and treatment 9 1 μl of 100 μg/ml of LPS

(E. coli). On day 4 all of the treatments were inoculated with B. pahangi microfilariae; another group of untreated mosquitoes was inoculated with microfilariae for the challenge infection. On day 4 after the second injection the mosquitoes were dissected and the number of larvae recorded.

3.1.2.3.2 Experiment IV

Experiment IV involved immunization of Ae. aegypti with laminarin (β-,1-3,glucan) or dextran (α-1,6-glucan) followed by challenge infected with B. pahangi.

Experiment IV was performed to determine whether the acquired immunity derived from injection of laminarin extended to the α-glucan dextran.

Four treatments of mosquitoes were used. On day 0 treatment 1 mosquitoes were injected with 1 μl of TC199, treatment 8 with 1 μl of 1mg/ml laminarin, and treatment 10 with 1 μl of 100 μg/ml of dextran.

On day 4 treatments 1, 8 and 10 were further inoculated with B. pahangi microfilariae and untreated mosquitoes, treatment 2 , were inoculated with microfilariae as a control group.

On day 4 after the second injection the mosquitoes were dissected and the number of larvae recorded.

3.1.3 Results

Experiment I

The results of five trials (A, B, C, D and E) within Experiment I are presented in Table 3. They show the mean numbers of developing B. pahangi larvae, and all B. pahangi parasites recovered from both the donor and recipient groups of Ae. aegypti, together with the respective survival and infection rates. The results of statistical tests comparing the numbers of total parasites recovered and numbers of developing larvae for the different donor treatment groups are given in Tables 4 and 5 respectively. The mean percentage recoveries are shown in Fig.1-5.

In all five trials, there were highly significant reductions in the number of both the numbers of total parasites recovered and larvae developing in the recipient mosquitoes given haemolymph from the infected donor mosquitoes as compared to the recipients given haemolymph from the TC199 'sham control' and compared with control infection in untreated mosquitoes. The results show that haemolymph from the TC199 treated donors significantly reduces both the total number of parasites and the number of developing larvae recovered from recipients. However, the reductions in the numbers of larvae developing and the parasites recovered from the recipients given haemolymph from infected donor mosquitoes were significantly greater than for recipients given haemolymph from the TC199 (sham) control donors.

Furthermore, the survival rates of the mosquitoes receiving haemolymph from the infected donor mosquitoes were similar to those receiving haemolymph from sham control donors and the infected recipient controls. Therefore, there was no mortality-related bias associated with treatment groups.

The infection rates of the mosquitoes receiving haemolymph from the immunized donors were less than those receiving haemolymph from sham controls and infected recipient controls. The infection rates of mosquitoes receiving haemolymph from living microfilariae infected donors (93%) were less than the recipients receiving haemolymph from heat-killed microfilariae infected donors (100%) (Table 3). However, the reductions in the number of parasites recovered from, and in the larvae developing in, the recipients receiving haemolymph from donor mosquitoes inoculated with heat-killed microfilaria were similar to those in recipients receiving haemolymph from donors inoculated with live microfilariae in Trial A (Tables 4 and 5).

There was no correlation between dosages of microfilariae injected into the donors and the percentage reduction in the number of larvae developing ($P>0.05$) nor with parasites recovered from recipients ($P>0.05$). Furthermore, there was no correlation between the dosages of microfilariae injected into the donor and the percentage infection rates ($P>0.05$).

There appears to be an inherent capacity for the immune haemolymph to adversely effect large numbers of microfilariae as shown when 20 microfilariae were inoculated into the recipient in trial D, but when compared with the recipients in Trial C which received 10 microfilariae, the proportional reductions in the numbers of parasites recovered and of developing larvae was less in recipients receiving 20 microfilariae (48.6% and 37.6% in Trial C, 26.6% and 15.9% in Trial D) (see Tables 4 and 5).

TABLE 3. EXPERIMENT I: THE DEVELOPMENT OF BRUGIA PAHANGI IN DONOR AEDES AEGYPTI AND IN THE RECIPIENT MOSQUITOES FOLLOWING TRANSFER OF HAEMOLYMPH FROM INFECTED DONORS.

Treatment	No. inf^n	%surv. No.diss	$\%inf^n$ rate	Larvae developing M±SE	Parasites recovered M±SE	Range
Trial A						
Donor						
I.5 L.mff/m.	50	94.0(40)	100	2.92±0.16	3.02±0.15	1-5
II.5 HKmff/m.	50	92.0(40)	-	-	-	-
Recipient						
TC199	40	72.5(29)	100	4.31±0.19	4.51±0.20	3-7
Recp.Cont.	30	97.0(29)	100	6.31±0.18	6.51±0.20	5-8
I.10 mff/m.	40	75.0(30)	93	2.76±0.25	3.06±0.20	0-5
II.10 mff/m.	40	72.0(29)	100	3.13±0.25	3.27±0.24	1-7
Trial B						
Donor						
10 mff/m.	160	77.6(90)	100	3.96±0.18	4.10±0.18	1-7
Recipient						
TC199	95	70.5(67)	100	3.13±0.16	3.79±0.18	0-7
Recp.Cont.	85	71.7(61)	100	4.60±0.14	4.75±0.16	2-7
10 mff/m.	90	78.8(70)	87.1	1.93±0.17	2.50±0.22	0-6
Trial C						
Donor						
20 mff/m.	150	80.0(80)	100	11.33±0.25	11.57±0.27	7-18
Recp.Cont.						
TC199	80	50.0(40)	100	3.25±0.32	4.02±0.40	0-7
Recp.Cont.	55	69.0(30)	100	5.02±0.37	5.36±0.40	2-8
10 mff/m.	80	48.7(36)	86.1	1.88±0.25	2.61±0.32	0-5
Trial D						
Donor						
20 mff/m.	75	53.3(40)	100	12.70±0.31	13.20±0.34	9-20
Recipient						
TC199	40	47.5(19)	100	5.84±0.31	7.00±0.44	4-8
Recp.Cont.	30	60.0(18)	100	11.90±0.46	12.39±0.49	9-16
20 mff/m.	40	50.0(20)	95	1.90±0.37	3.30±0.35	0-5
Trial E						
Donor						
60 mff/m.	200	69.0(105)	100	28.30±0.64	50.20±0.44	36-60
Recipient.						
TC199	100	74.0(74)	100	3.89±0.12	4.48±0.11	2-6
Recp.Cont.	95	83.2(78)	100	5.82±0.15	6.03±0.14	3-9
10 mff/m.	105	82.8(87)	94.2	2.15±0.15	3.04±0.15	0-4

L.mff/m. = Living microfilariae/mosquito
HKmff/m. = Heat-killed microfilariae/mosquito
Recp.Cont. = Recipient control

TABLE 4. EXPERIMENT I: STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY TEST OF THE TOTAL NUMBERS OF B. PAHANGI RECOVERED FROM RECIPIENT AE. AEGYPTI MOSQUITOES FOLLOWING TRANSFER HAEMOLYMPH FROM DONORS PREVIOUSLY INFECTED WITH MICROFILARIAE OR INOCULATED WITH TC199 MEDIUM (SHAM CONTROLS).

Treatments Compared with	Donor Treatment Group Sham TC199 (T1)	Living mff (T4L)	Heat-killed mff (T4HK)
Trial A			
Recp.Cont.	P<0.00003 (30.7)	P<0.00003 (52.9)	P<0.00003 (49.7)
TC199	- -	P<0.00020 (32.1)	P<0.00040 (27.5)
Living mff.	- -	- -	P<0.77910 (6.4)
Trial B			
Recp.Cont.	P<0.00020 (20.3)	P<0.00003 (47.4)	- -
TC199	- -	P<0.00003 (34.1)	- -
Trial C			
Recp.Cont.	P<0.01190 (25.1)	P<0.00003 (51.4)	- -
TC199	- -	P<0.00530 (35.2)	- -
Trial D			
Recp.Cont.	P<0.00003 (43.5)	P<0.00003 (73.4)	- -
TC199	- -	P<0.00003 (52.9)	- -
Trial E			
Recp.Cont.	P<0.00003 (25.7)	P<0.00003 (49.5)	- -
TC199	- -	P<0.00003 (32.1)	- -

Footnote : In the body of the table, P values are given for the different group comparisons together with the % reduction compared the treatments given in column 1.

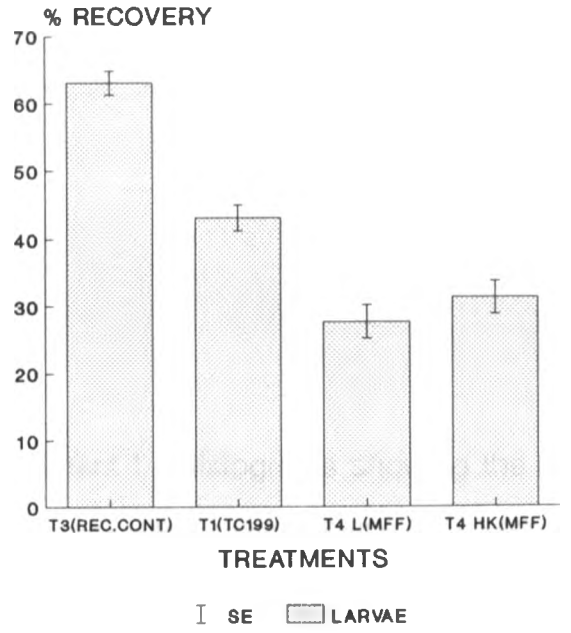
TABLE 5. EXPERIMENT I: STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY TEST, OF THE NUMBER OF DEVELOPING LARVAE OF B. PAHANGI IN RECIPIENT AE. AEGYPTI MOSQUITOES FOLLOWING TRANSFER HAEMOLYMPH FROM DONORS PREVIOUSLY INFECTED WITH MICROFILARIAE OR INOCULATED WITH TC199 (SHAM CONTROL).

Treatments Compared with	Donor Treatment Group Sham TC199 (T1)	Living mff. (T4L)	Heat-killed mff. (T4HK)
Trial A			
Recp.Cont.	P<0.00003	P<0.00003	P<0.00003
	(31.7)	(56.2)	(50.3)
TC199	-	P<0.00010	P<0.00080
	-	(35.8)	(27.2)
Living mff.	-	-	P<0.43940
	-	-	(12.8)
Trial B			
Recp.Cont.	P<0.00003	P<0.00003	-
	(32.0)	(58.2)	-
TC199	-	P<0.00003	-
	-	(38.5)	-
Trial C			
Recp.Cont.	P<0.00120	P<0.00003	-
	(35.3)	(62.4)	-
TC199	-	P<0.00003	-
	-	(41.9)	-
Trial D			
Recp.Cont.	P<0.00003	P<0.00003	-
	(51.1)	(84.1)	-
TC199	-	P<0.00003	-
	-	(67.5)	-
Trial E			
Recp.Cont.	P<0.00003	P<0.00003	-
	(33.2)	(60.1)	-
TC199	-	P<0.00003	-
	-	(44.8)	-

Footnote : In the body of the table, P values are given for the different group comparisons together with the % reduction compared with the treatments given in column 1.

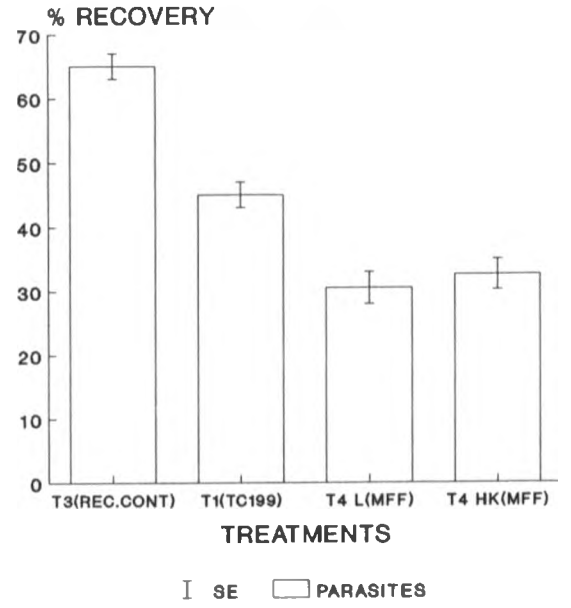
FIG.1. Experiment I : Histograms showing the mean percent recovery of developing larvae and of all stages of Brugia pahangi from adult virgin female Aedes aegypti mosquitoes 4 days after treatment with haemolymph from donors infected with 5 living microfilariae, 5 heat-killed microfilariae or form donor given TC199 compared with a control (T3 Recp.cont). Recipient mosquitoes received 1 μl haemolymph approximately 2 hrs after inoculation with 10 microfilariae.

HAEMOLYMPH TRANSFER TRIAL A
DEVELOPING LARVAE



5 MFF/DONOR:10 MFF/RECIPIENT

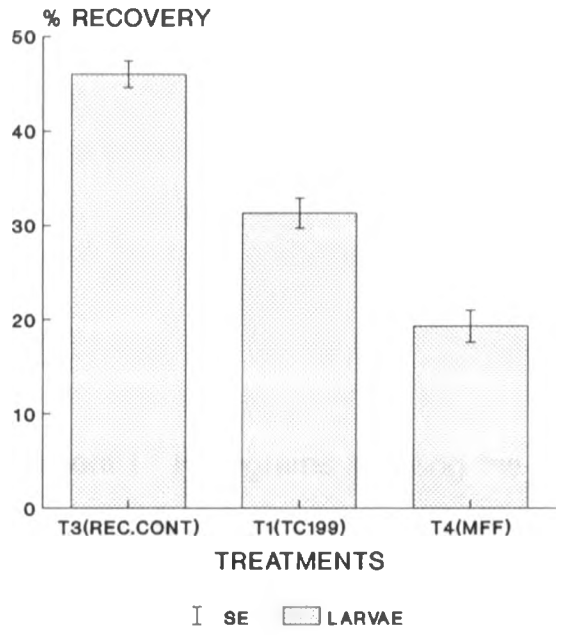
TOTAL PARASITE RECOVERY



5 MFF/DONOR:10 MFF/RECIPIENT Fig.1

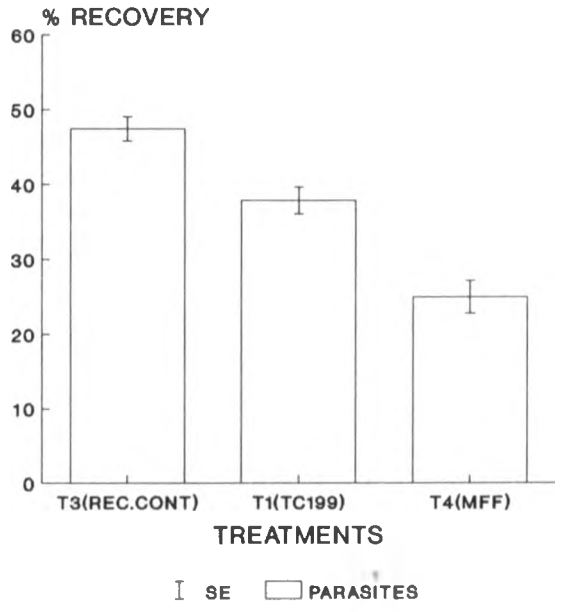
FIG.2. Experiment I : Histograms showing the mean percent recovery of developing larvae and of all stages of Brugia pahangi from adult virgin female Aedes aegypti mosquitoes 4 days after treatment with haemolymph from donors infected with 10 living microfilariae (T4) or from donors given TC199 compared with a control (T3 Rec.Cont). Recipient mosquitoes received 1 μl haemolymph approximately 2 hrs after inoculation with 10 microfilariae.

HAEMOLYMPH TRANSFER TRIAL B
DEVELOPING LARVAE



10 MFF/DONOR:10 MFF/RECIPIENT

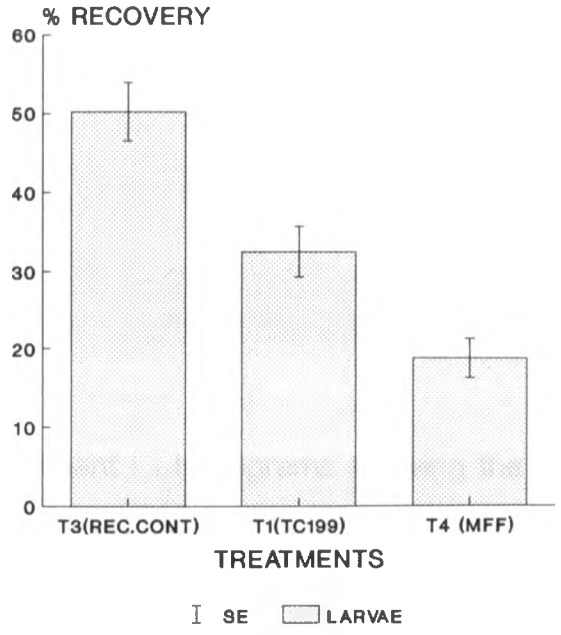
TOTAL PARASITE RECOVERY



10 MFF/DONOR:10 MFF/RECIPIENT Fig.2

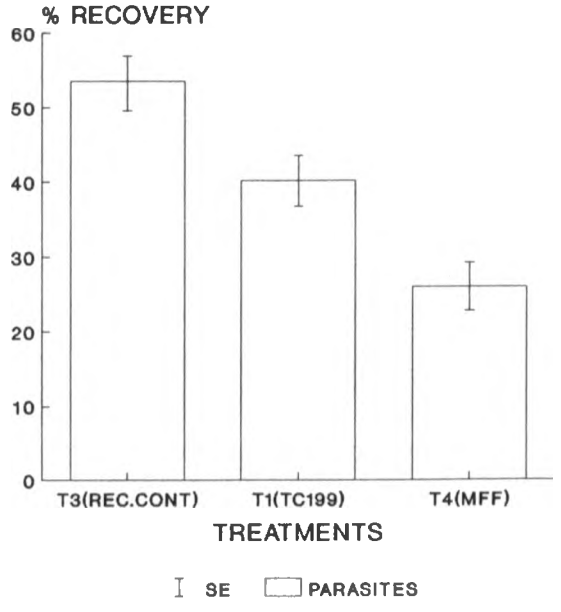
FIG.3. Experiment I : Histograms showing the mean percent recovery of developing larvae and of all stage of Brugia pahangi from adult virgin female Aedes aegypti mosquitoes 4 days after treatment with haemolymph from donors infected with 20 microfilariae or from donors given TC199 compared with a control (T3 Rec.Cont). Recipient mosquitoes received 1 μl haemolymph approximately 2 hrs after inoculation with 10 microfilariae.

HAEMOLYMPH TRANSFER TRIAL C
DEVELOPING LARVAE



20 MFF/DONOR:10 RECIPIENT

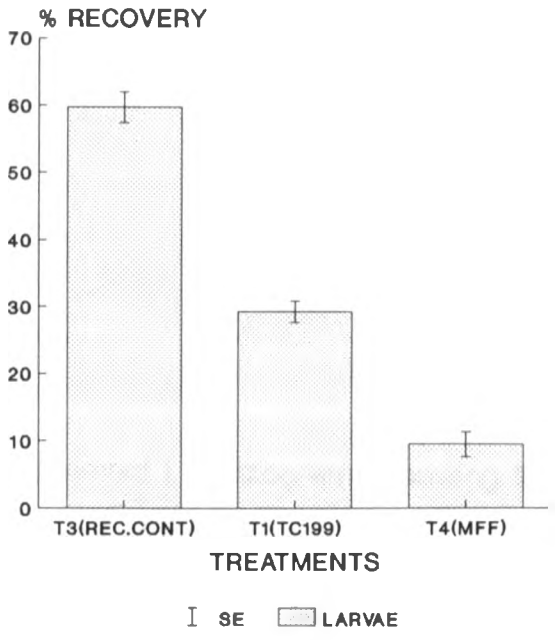
TOTAL PARASITE RECOVERY



20 MFF/DONOR:10 MFF/RECIPIENT Fig.3

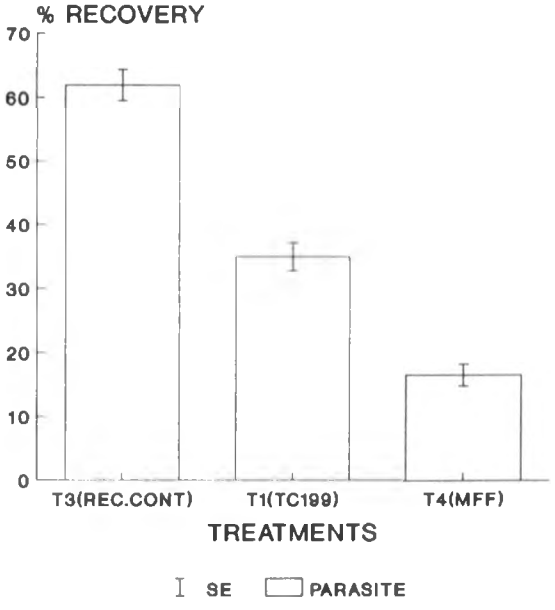
FIG.4. Experiment I : Histograms showing the mean percent recovery of developing larvae and of all stages of Brugia pahangi from adult virgin female Aedes aegypti mosquitoes, 4 days after treatment with haemolymph from donors infected with 20 microfilariae (T4) or from donors given TC199 compared with a control (T3 Rec.Cont). Recipient mosquitoes received 1 µl haemolymph approximately 2 hrs after inoculation with 20 microfilariae.

HAEMOLYMPH TRANSFER TRIAL D
DEVELOPING LARVAE



20 MFF/DONOR:20 MFF/RECIPIENT

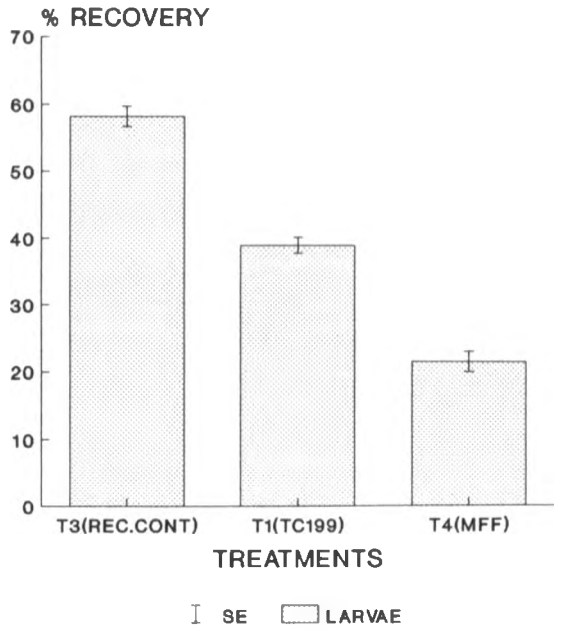
TOTAL PARASITE RECOCERY



20 MFF/DONOR:20 MFF/RECIPIENT Fig.4

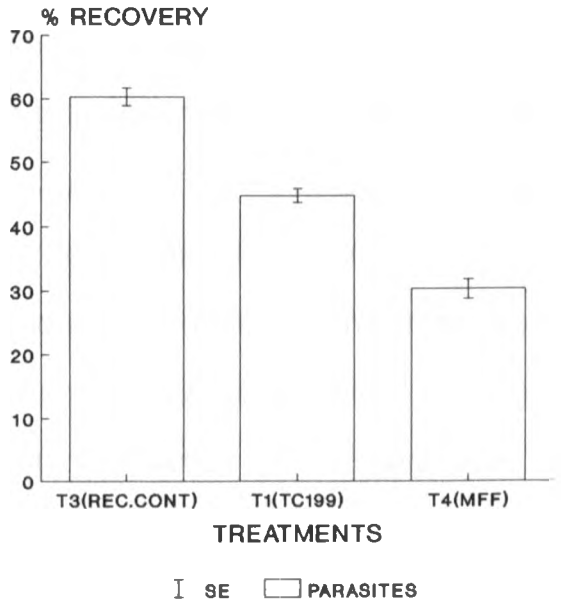
FIG.5. Experiment I : Histograms showing the mean percent recovery of developing larvae and of all stages of Brugia pahangi from adult virgin female Aedes aegypti mosquitoes 4 days after treatment with haemolymph from donors infected with 60 microfilariae (T4) or from donors given TC199 compared with a control (T3 Rec.Cont). Recipient mosquitoes received 1 μl haemolymph approximately 2 hrs after inoculation with 10 microfilariae.

HAEMOLYMPH TRANSFER TRIAL E
DEVELOPING LARVAE



60 MFF/DONOR:10 MFF/RECIPIENT

TOTAL PARASITE RECOVERY



60 MFF/DONOR:10 MFF/RECIPIENT Fig.6

Experiment II

The results of Experiment II, Trials A and B, are presented in Table 6. The table shows the mean numbers of developing larvae and of total parasites recovered from both donor and recipient groups, with the survival and infection rates of the mosquitoes. The results of statistical tests comparing the numbers of total parasites recovered and the numbers of developing larvae for the different donor treatment groups are shown in Tables 7 and 8. Mean percentage recoveries are shown in Fig. 6.

In both Trials A and B, the numbers of larvae developing and parasites recovered from the recipients given haemolymph from bacteria injected donors were lower than the numbers from recipients given haemolymph from TC199 control donors or from the infected recipient control group.

Although the reduction in the number of larvae developing in the recipients given haemolymph from the live bacteria immunized donors was greater than that in recipients given haemolymph from heat-killed bacteria immunized donors, the differences were not significant.

However, although haemolymph from bacteria infected donors was effective in reducing parasite survival and development in recipient mosquitoes, the reductions in the number of larvae developing and parasites recovered from recipients given haemolymph from microfilariae infected donors was significantly greater than that in recipients given haemolymph from bacteria infected donors.

The results demonstrated that the haemolymph of mosquito donors which were immunized with bacteria can reduce both survival and development of Brugia parasites in the recipient mosquitoes.

TABLE 6. EXPERIMENT II: DEVELOPMENT OF B. PAHANGI MICROFILARIAE IN DONOR AE. AEGYPTI AND IN THE RECIPIENT MOSQUITOES FOLLOWING TRANSFER OF HAEMOLYMPH FROM DONORS PREVIOUSLY INJECTED WITH MICROFILARIAE, E. COLI OR TC199.

Treatments	No. inf^n	%surv. rate	$\%inf^n$ rate (No.diss)	Larvae developing M±SE	Parasites recovered M±SE	Range
Trial A						
Donor						
10 mff/m.	260	82.3(180)	100	6.69±0.06	6.78±0.07	5-9
Recipient						
TC199	180	81.6(147)	100	5.05±0.10	5.15±0.10	0-8
Recp.Cont.	180	89.4(161)	100	6.73±0.08	6.83±0.09	5-10
10 mff/m.	180	88.9(161)	93.7	1.76±0.09	1.92±0.09	0-6
L.bacteria	180	86.7(156)	100	3.27±0.09	4.43±0.09	2-7
Trial B						
Donor						
10 mff/m.	60	86.7(30)	100	6.87±0.19	7.03±0.09	5-9
Recipient						
TC199	30	90.0(27)	100	4.18±0.18	4.52±0.18	3-6
Recp.Cont.	30	93.3(28)	100	6.87±0.22	6.46±0.23	4-9
10 mff/m.	30	86.7(26)	93.3	2.04±0.24	2.31±0.23	0-5
L.bacteria	30	86.7(26)	100	2.88±0.22	3.69±0.17	2-5
HK.bacteria	30	90.0(27)	100	2.78±0.20	3.07±0.20	2-5

L.bacteria = Living bacteria E. coli $1x10^4$cells/µl.
HK.bacteria = Heat-killed bacteria E.coli $1x10^4$cells/µl.

TABLE 7. EXPERIMENT II: STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY TEST OF THE TOTAL NUMBERS OF B. PAHANGI PARASITES RECOVERED FROM RECIPIENT AE. AEGYPTI MOSQUITOES FOLLOWING TRANSFER OF HAEMOLYMPH FROM DONORS PREVIOUSLY INJECTED WITH MICROFILARIAE, E. COLI OR TC199 (SHAM CONTROL).

Treatments Compared with	Donor Treatment Group Recp.Cont. (T3)	10 mff/m. (T4.L)	L.bacteria (T5.L)	HK.bacteria (T5.HK)
Trial A				
TC199	P<0.00003 (24.5)	P<0.00003 (62.7)	P<0.00003 (33.5)	- -
Recp.Cont.	- -	P<0.00003 (71.8)	P<0.00003 (49.8)	- -
10 mff/m.	- -	- -	P<0.00003 (43.8)	- -
Trial B				
TC199	P<0.00003 (30.1)	P<0.00003 (48.3)	P<0.00010 (27.7)	P<0.00003 (31.9)
Rec.Cont.	- -	P<0.00003 (64.3)	P<0.00003 (49.4)	P<0.00003 (52.5)
10 mff/m.	- -	- -	P<0.00283 (29.4)	P<0.03060 (24.9)
L.bacteria	- -	- -	- -	P<0.35030 (5.9)

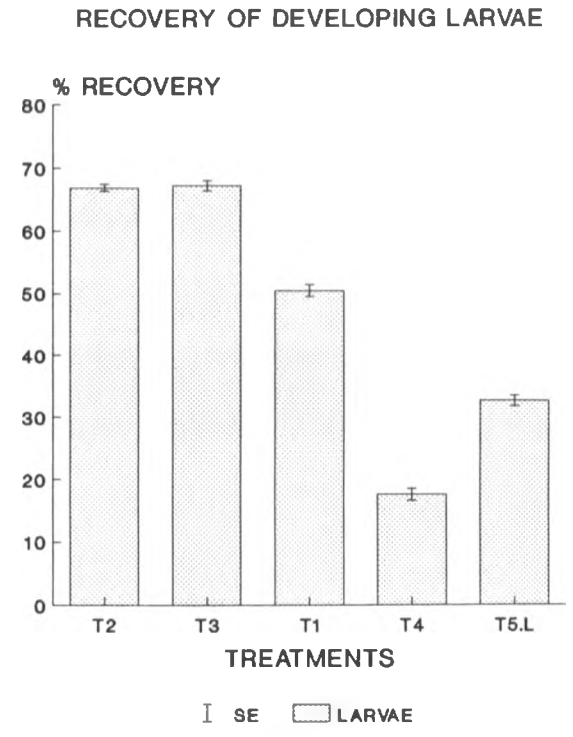
L.bacteria = Living bacteria E. coli $1x10^4$cells/µl.
HK.bacteria = Heat-killed bacteria E. coli $1x10^4$cells/µl.
10 mff/m. = 10 microfilariae inoculated/mosquito
Footnote : In the body of the table, P value are given for the different group comparisons together with the % reduction compared with the treatments given in column 1.

TABLE 8. EXPERIMENT II: STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY TEST, OF THE NUMBERS OF DEVELOPING LARVAE OF B. PAHANGI IN RECIPIENT AE. AEGYPTI MOSQUITOES FOLLOWING TRANSFER OF HAEMOLYMPH FROM DONORS PREVIOUSLY INJECTED WITH MICROFILARIAE, E. COLI OR TC199 (SHAM CONTROL).

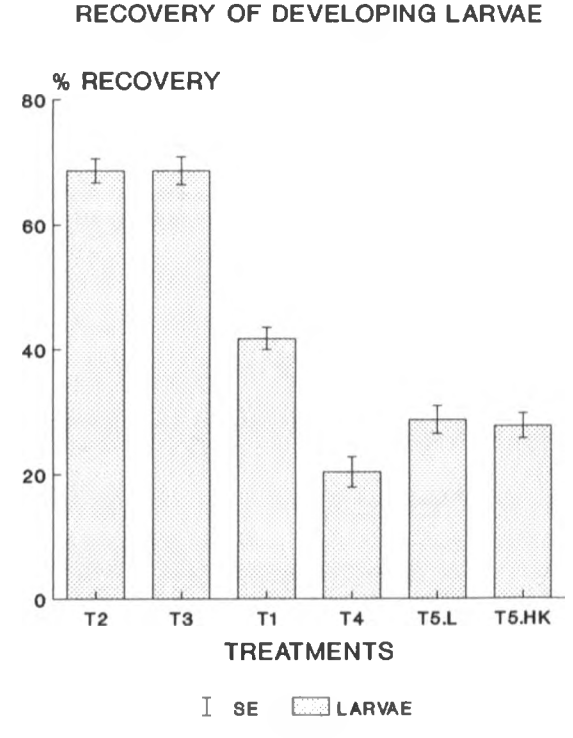
Treatments Compared with	Donor Treatment Group Recp.Cont. (T3)	10 mff/m. (T4.L)	L.bacteria (T5.L)	HK.bacteria (T5.HK)
Trial A				
TC199	P<0.00003 (25.1)	P<0.00003 (65.1)	P<0.00003 (35.1)	- -
Recp.Cont.	- -	P<0.00003 (73.8)	P<0.00003 (51.4)	- -
10 mff/m.	- -	- -	P<0.00003- (46.2)	-
Trial B				
TC199	P<0.00003 (39.1)	P<0.00003 (51.3)	P<0.00020 (31.1)	P<0.00020 (33.6)
Recp.Cont.	- -	P<0.00003 (70.3)	P<0.00003 (56.9)	P<0.00003 (59.5)
10 mff/m.	- -	- -	P<0.01610 (27.3)	P<0.02930 (26.6)
L.bacteria	- -	- -	- -	P<0.65000 (3.7)

L.bacteria = Living bacteria E. coli $1x10^4$cells/µl.
HK.bacteria = Heat-killed bacteria E. coli $1x10^4$cells/µl.
10 mff/m. = 10 microfilariae inoculated/mosquito
Footnote : In the body of the table, P values are given for the different group comparisons together with the % reduction compared with the treatments given in column 1.

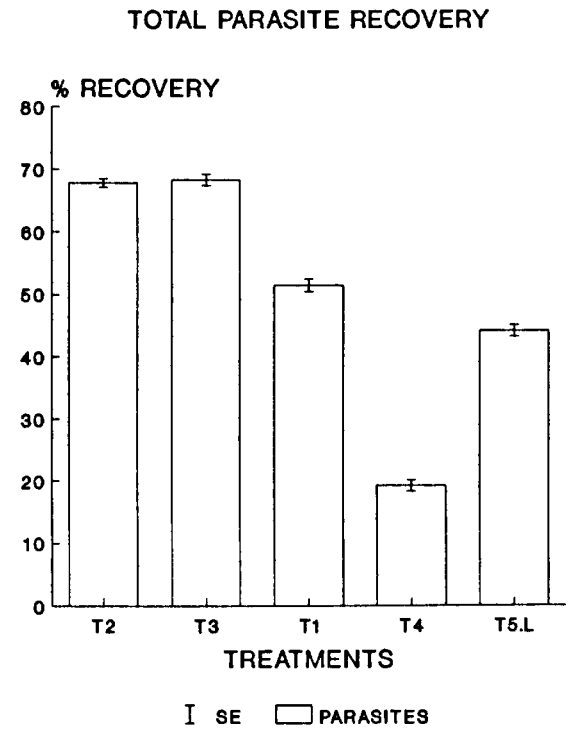
FIG.6. Experiment II ;Trial A and B : Histograms showing the means percent recovery of developing larvae and of all stages of Brugia pahangi from adult virgin female Aedes aegypti mosquitoes 4 days after treatment with haemolymph from donor infected with 10 microfilariae (T4), 10^4cells living Escherichia coli (T5L), 10^4cells heat-killed E. coli (T5HK) or inoculated with TC199 (T1) compared with donor control(T2) and recipient control (T3) groups. Recipient mosquitoes received 1 μl haemolymph approximately 2 hrs after inoculation with 10 microfilariae.



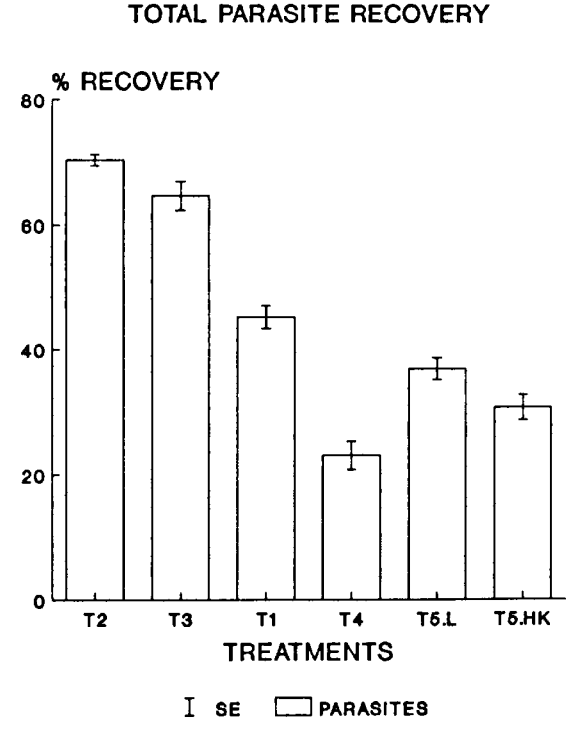
TRIAL A



TRIAL B Fig.6



TRIAL A



TRIAL B Fig.6

Experiment III

The results of Experiment III, Trials A and B, are presented in Table 9. The results of statistical tests comparing the numbers of total parasites and the numbers of developing larvae for the different treatment groups and given in Tables 10 and 11. The mean numbers of larvae developing and parasites recovered from the challenge infection of mosquitoes previously injected with B. pahangi, E. coli, laminarin or LPS were significantly lower than those in both the challenge controls and mosquitoes which were injected first with TC199 and then with microfilariae.

The reduction in the number of larvae developing and total parasites recovered from the mosquitoes previously inoculated with microfilariae was significantly greater than for mosquitoes previously injected with laminarin or LPS (Tables 10 and 11).

The reductions in the number of challenge infection parasites recovered from the mosquitoes previously inoculated with microfilariae was not significantly different from that of the mosquitoes previously injected with bacteria but the reduction in the number of larvae developing was significantly greater.

The reductions in the number of larvae developing and total parasites recovered from the mosquitoes previously injected with laminarin was similar to that of the mosquitoes previously injected with LPS.

The reductions in the number of larvae developing and total parasites recovered from the mosquitoes previously injected with bacteria was significantly greater than that of mosquitoes previously injected with laminarin or LPS.

In Trial B the reductions in the number of larvae developing and total parasites recovered in the mosquitoes previously inoculated with microfilariae were significantly less than those of control.

The reductions in the number of challenge parasites recovered from mosquitoes previously inoculated with microfilariae were similar to those of the mosquitoes previously injected with bacteria (93.3%,$P<0.5931$) and laminarin (86.3%,$P<0.1285$) but significantly greater than in mosquitoes previously injected with LPS.

The reduction in the number of larvae developing in the mosquitoes previously inoculated with microfilariae was significantly greater than that of the mosquitoes previously injected with laminarin or LPS. The reduction in the numbers of larvae developing in the mosquitoes previously injected with laminarin was significantly greater than of the mosquitoes previously injected with LPS.

In these trials melanization of microfilariae was recorded only in the mosquitoes previously injected with laminarin. In Trial A 17.4% (8/46) microfilariae were melanized and 10% (8/80) in Trial B (Table 9).

Fig. 7 shows the mean percent recovery of the larvae developing and the parasites recovered from the challenge infections in Experiment III, Trial A and Trial B. The mean percent recoveries of developing larvae and total parasites recovered from the mosquitoes which had been previously inoculated with microfilariae were lower than the larvae developing and parasites recovered from the mosquitoes previously injected with bacteria,laminarin, LPS, sham TC199 control and the secondary inoculated control.

The results further demonstrate that an acquired immune response in mosquitoes can be induced by bacteria, laminarin and LPS. The inducible immune response from microfilariae is stronger than from bacteria when the mosquitoes were challenged with the lower number of microfilariae (10 mff.)but no different when the mosquitoes were infected with the large number (20 mff.). The inducible immune response from live bacteria is stronger than that from laminarin and LPS at the concentrations used. The inducible immunity response to bacteria, laminarin and LPS partially inhibits both survival and development of microfilariae in the mosquitoes.

TABLE 9. EXPERIMENT III: DEVELOPMENT OF A CHALLENGE INFECTION OF B. PAHANGI IN AE. AEGYPTI GIVEN 4 DAYS AFTER AN INITIAL INOCULATION OF MICROFILARIAE, OR 1 DAY AFTER AN INITIAL INOCULATION OF E. COLI, LAMINARIN, LPS OR TC199. MOSQUITOES WERE DISSECTED 4 DAYS AFTER THE CHALLENGE INFECTION .

Treatments	No. Inf^{n}	%surv. rate (No.diss)	Immunizing infection with Brugia pahangi Larvae developing M±SE	Parasites recovered M±SE	Range	%melanized mff.
Trial A						
20 mff/m.*	80	70.0(56)	14.34±0.23	14.41±0.23	9-19	-
20 mff/m.#	115	73.0(77)	14.32±0.21	14.32±0.21	10-19	-
Trial B						
20 mff/m.*	45	91.1(41)	16.19±0.19	16.41±0.22	13-18	-
20 mff/m.#	90	52.2(47)	7.64±0.24	7.64±0.24	4-12	-
Initial treatment			Challenge infection with Brugia pahangi			
Trial A						
TC199	90	76.7(69)	5.56±0.09	5.98±0.09	4-7	-
2ndinf.Cont.♦	85	82.4(70)	6.88±0.01	7.23±0.10	5-9	-
10 mff/m.	115	73.3(77)	1.76±0.08	3.62±0.13	1-3	-
Bacteria	105	60.9(54)	2.81±0.09	3.41±0.01	1-4	-
Laminarin	90	73.3(66)	3.59±0.01	4.32±0.01	2-5	17.4(8/46)
LPS	90	73.3(66)	3.68±0.01	4.41±0.01	2-7	-
Trial B						
TC199	77	76.6(59)	13.17±0.20	14.31±0.23	9-16	-
2ndinf.Cont.♦	45	71.1(32)	17.22±0.18	17.56±0.23	16-19	-
20 mff/m.	90	52.2(47)	5.49±0.22	7.81±0.26	3-9	-
Bacteria	77	45.5(35)	4.97±0.41	7.29±0.44	1-11	-
Laminarin	88	53.4(47)	7.20±0.43	9.04±0.54	2-14	10.0(8/80)
LPS	87	50.6(47)	8.23±0.43	9.75±0.55	3-14	-

* = First inoculation control mosquitoes dissected on day 8
# = First inoculation in treated mosquitoes following secondary inoculation, dissected on day 8
♦ = No previous treatment

TABLE 10. EXPERIMENT III : STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY TEST OF THE TOTAL NUMBERS OF PARASITES OF B. PAHANGI RECOVERED FROM AE. AEGYPTI GIVEN 4 DAYS AFTER AN INITIAL INOCULATION OF MICROFILARIAE OR 1 DAY AFTER AN INITIAL INOCULATION OF E. COLI , LAMINARIN, LPS OR TC199. MOSQUITOES WERE DISSECTED 4 DAYS AFTER THE CHALLENGE INFECTION.

Initial treatments	Treatment TC199	Mff.	Bacteria	Laminarin	LPS
Trial A					
2nd inf.Cont.♦	P<0.00003 (17.2)	P<0.00003 (49.9)	P<0.00003 (52.9)	P<0.00003 (40.3)	P<0.00003 (39.1)
TC199	- -	P<0.00003 (31.24)	P<0.00003 (42.9)	P<0.00003 (27.9)	P<0.00003 (26.4)
Microfilariae	- -	- -	P<0.28070 (5.8)	P<0.00070 (16.1)	P<0.00001 (17.9)
Bacteria	- -	- -	- -	P<0.00003 (21.2)	P<0.00003 (22.8)
Laminarin	- -	- -	- -	- -	P<0.67010 (2.1)
Trial B					
2nd inf.Cont.♦	P<0.00003 (18.6)	P<0.00003 (55.5)	P<0.00003 (58.5)	P<0.00003 (51.49)	P<0.00003 (44.5)
TC199	- -	P<0.00003 (45.4)	P<0.00003 (49.1)	P<0.00003 (36.8)	P<0.00003 (31.9)
Microfilariae	- -	- -	P<0.59310 (6.7)	P<012850 (14.6)	P<0.01320 (19.1)
Bacteria	- -	- -	- -	P<0.03920 (19.9)	P<0.00310 (25.3)
Laminarin	- -	- -	- -	- -	P<0.3132 (7.3)

♦ = No previous treatment

Footnote : In the body of the table, P values are given for the different group comparisons together with the % reduction compared with treatments given in column 1.

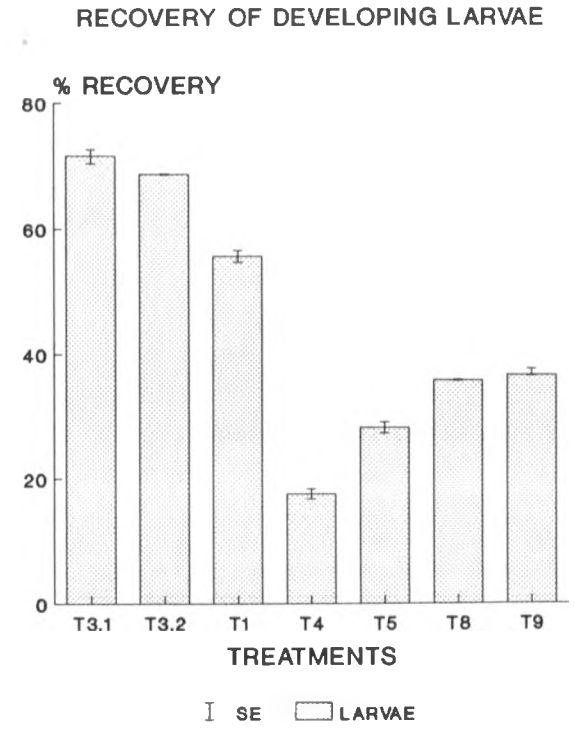
TABLE 11. EXPERIMENT III : STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY TEST OF THE NUMBERS OF DEVELOPING LARVAE FROM A CHALLENGE INFECTION OF B. PAHANGI RECOVERED FROM AE. AEGYPTI GIVEN 4 DAYS AFTER AN INITIAL INOCULATION OF MICROFILARIAE AND 1 DAY AFTER AN INITIAL INOCULATION OF E. COLI, LAMINARIN, LPS OR TC199. MOSQUITOES WERE DISSECTED 4 DAYS AFTER THE CHALLENGE INFECTION.

Initial treatments	Treatment TC199	Mff.	Bacteria	Laminarin	LPS
Trial A					
2nd inf.Cont.♦	P<0.00003 (19.2)	P<0.00003 (74.4)	3P<0.00003 (59.5)	P<0.00003 (47.8)	P<0.00003 (46.5)
TC199	- -	P<0.00003 (68.8)	P<0.00003 (50.2)	P<0.00003 (36.5)	P<0.00003 (34.8)
Microfilariae	- -	- -	P<0.00030 (37.2)	P<0.00003 (50.8)	P<0.00003 (52.1)
Bacteria	- -	- -	- -	P<0.00003 (21.7)	P<0.00003 (23.6)
Laminarin	- -	- -	- -	- -	P<0.89500 (2.5)
Trial B					
2nd inf.Cont.♦	P<0.00003 (23.6)	P<0.00003 (68.2)	P<0.00003 (71.2)	P<0.00003 (58.2)	P<0.00003 (52.3)
TC199	- -	P<0.00003 (58.3)	P<0.00003 (62.3)	P<0.00003 (45.4)	P<0.00003 (37.5)
Microfilariae	- -	- -	P<0.34130 (9.4)	P<0.00290 (23.7)	P<0.00003 (33.3)
Bacteria	- -	- -	- -	P<0.00130 (30.9)	P<0.00003 (39.6)
Laminarin	- -	- -	- -	- -	P<0.00003 (12.6)

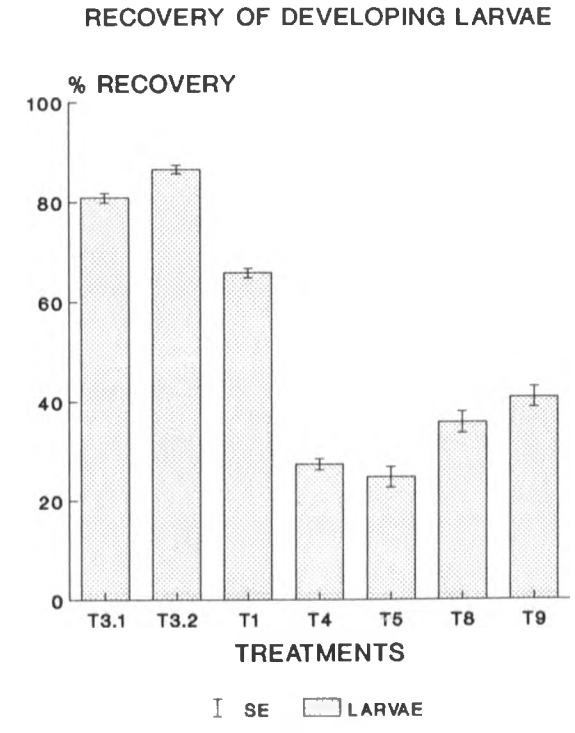
♦ = No previous treatment

Footnote: In the body of table, P values are given for the different group comparisons together with % reduction compared with the treatment given in column 1.

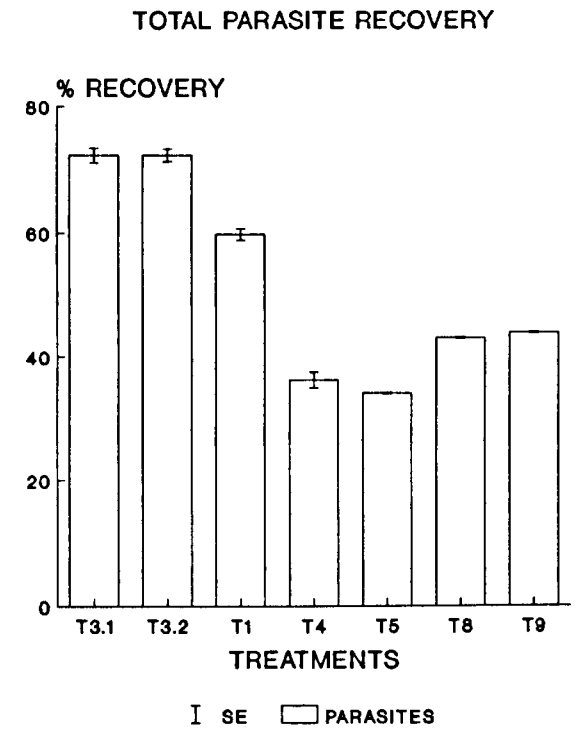
FIG.7. Experiment III : Histograms showing the mean percent recovery both total parasites and developing larvae of Brugia pahangi from challenge infection of adult virgin female Aedes aegypti mosquitoes given 4 days after an initial infection with 10 microfilariae (T4), or 1 day after an initial injected with 10^4cells E. coli (T5), 1 µl of 1 mg/ml laminarin (T8), 1 µl of 100 µg/ml of LPS (T9) or 1 µl TC199 (T1) compared with the immunizing (T3.1) and challenge infection controls (T3.2). Mosquitoes of each treatment group were given a challenge infection of 10 microfilariae by inoculation and dissected 4 days after challenge.



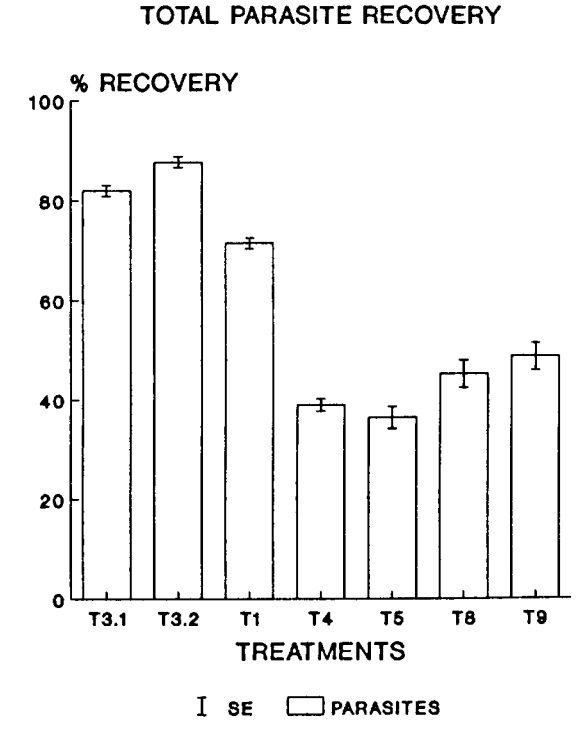
TRIAL A



TRIAL B Fig.7



TRIAL A



TRIAL B Fig.7

Experiment IV

The results from Experiment IV are presented in Table 12, which shows the mean number of larvae developing and the parasites recovered from the challenged infections of mosquitoes previously injected with laminarin, dextran, TC199 control and from an untreated control group.

The reductions in the number of larvae developing and the parasites recovered from the mosquitoes previously injected with laminarin and dextran were significant when compared with the mosquito control group.

The reductions in the number of larvae developing and parasites recovered from the challenge infection mosquitoes previously injected with the dextran were similar to those in the mosquitoes previously injected with TC199, whereas the reductions in mosquitoes previously injected with laminarin were significantly greater than in mosquitoes previously injected with dextran.

Fig. 8 shows the mean percent recovery of the larvae developing and the parasites recovered from the secondary inoculated mosquitoes previously injected with laminarin, dextran, TC199 control and from the control group. The mean percent recovery of developing larvae and total parasites recovered from the mosquitoes previously injected with laminarin were lower than the larvae developing and parasites from the mosquitoes previously injected with dextran, TC199 control and the secondary inoculation mosquitoes control group.

The results demonstrated that the effect of dextran is more or less identical to that of the TC199 control. This suggests that the α-1,6-glucan dextran, unlike the ß-1,3-glucan laminarin, dose not stimulate any significant the immune response within the mosquito.

TABLE 12. EXPERIMENT IV : DEVELOPMENT OF A CHALLENGE INFECTION OF B. PAHANGI IN AE. AEGYPTI MOSQUITOES GIVEN 1 DAY AFTER AN INITIAL INJECTION OF LAMINARIN, DEXTRAN OR TC199. MOSQUITOES WERE DISSECTED 4 DAYS AFTER THE CHALLENGE INFECTION OF 10 MICROFILARIAE.

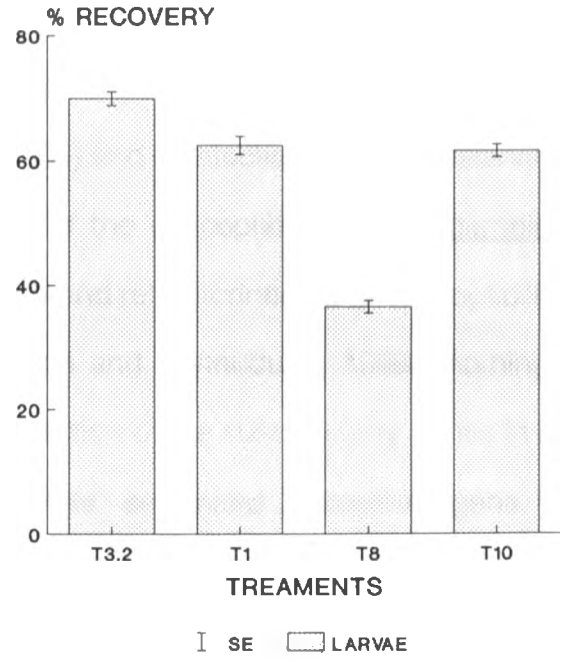
Treatments	No.	%surv. Infn rate (No.diss)	Larvae developing M±SE	Parasites recovered M±SE	Range	%Melanized mff.
Sham TC199	30	93.3(28)	6.25±0.14	6.57±0.13	5-8	-
Laminarin	70	71.4(50)	3.66±0.10	4.30±0.08	2-6	15.6(5/32)
Dextran	70	74.3(52)	6.17±0.10	6.52±0.09	5-8	-
Mff.Inoc.Cont.	70	75.7(53)	7.00±0.11	7.17±0.10	5-9	-

TABLE 13. EXPERIMENT IV : STATISTICAL ANALYSIS BASED ON THE MANN-WHITNEY OF THE NUMBERS OF DEVELOPING LARVAE AND TOTAL NUMBERS OF PARASITES B. PAHANGI RECOVERED IN AE. AEGYPTI MOSQUITOES 1 DAY AFTER AN INITIAL INJECTION OF LAMINARIN, DEXTRAN OR TC199. MOSQUITOES WERE DISSECTED 4 DAYS AFTER THE CHALLENGE INFECTION.

Treatment	Treatment TC199	Laminarin	Dextran
Larval development			
Mff.Inoc.Cont.	P<0.00050 (10.7)	P<0.00003 (47.3)	P<0.00003 (11.8)
TC199	- -	P<0.00003 (41.4)	P<0.73540 (1.3)
Laminarin	- -	- -	P<0.00003 (40.7)
Parasites recovered			
Mff.Inoc.Cont.	P<0.00300 (8.4)	P<0.00003 (40.1)	P<0.00003 (8.1)
TC199	- -	P<0.00003 (34.6)	P<0.76990 (0.8)
Laminarin	- -	- -	P<0.00003 (34.1)

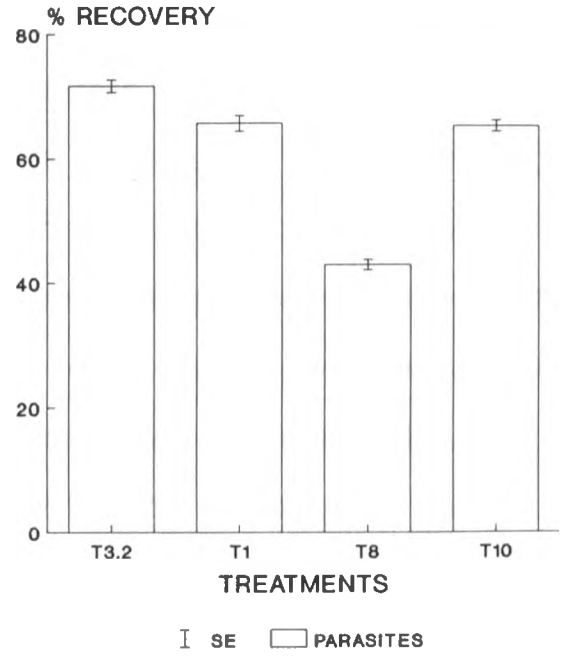
FIG.8. Experiment IV : Histograms showing the mean percent recovery both developing larvae and total parasites of Brugia pahangi from challenge infection of adult virgin female Aedes aegypti mosquitoes given 1 day after an initial injection with laminarin (T8), dextran (T10), TC199 (T1) and challeng infection control(T3.2). Mosquitoes of each treatment groups were given a challenge infection of 10 microfilariae by inoculation and dissected 4 days after challenge.

RECOVERY OF DEVELOPING LARVAE



TRIAL C

TOTAL PARASITE RECOVERY



TRIAL C Fig.8

3.1.4 Discussion

Since the demonstration of the genetic control of susceptibility of Ae. aegypti to filarial worms (Macdonald,1962a,1962b; Macdonald and Ramachandran, 1965) and to infection with the malarial parasite P. gallinaceum (Ward, 1963), and of the susceptibility of An. gambiae to malarial parasites (Collins et al., 1986) and refractoriness and susceptibility of An. stephensi to P. falciparum (Fedmann and Ponnudurai, 1989), nothing has been established about the mode of action of the susceptibility genes in relation to infections. In Ae. aegypti a single sex-linked recessive gene, denoted f^m, controls susceptibility to both B. pahangi and B. malayi (Macdonald, 1962b; Macdonald and Ramachandran, 1965). A refractory strain of Ae. aegypti does not support filarial worms development. Microfilariae are able to penetrate the midgut and migrate to the thoracic muscle, but further development does not occur. However, Am. subalbatus is resistant to infection with B. malayi because of an active immune response (Beerntsen et al., 1989) and acquired immune response resistance to O. lienalis infection in S. ornatum and S. lineatum following passive transfer of haemolymph from previously infected simuliids (Ham, 1986). Possible strategies for immune regulation by parasites in insect hosts have been highlighted in several recent reviews (Lackie, 1986a; Stoffolano, 1986; Christensen and Tracy, 1989).

It is quite clear from the present study that increased resistance to B. pahangi infection occurred in Ae. aegypti given haemolymph from previously infected mosquitoes. The majority of earlier investigations have described the melanization and encapsulation processes which often occur around developing larvae of various species of parasites within the tissues of mosquitoes (Burton,

1963; Poinar and Leutenegger, 1971; Oothuman et al., 1974; Lehane, 1978; Ho et al., 1984; Bartlett, 1984; Sutherland et al., 1984; Christensen et al., 1984; Bradley and Nayar et al., 1985; Forton et al., 1985; Chen and Laurence, 1985; Nayar et al., 1989) and in vitro (Chen and Laurence, 1987; and Ogura, 1987). The present study has demonstrated, on the other hand, that factors can be transferred in the haemolymph of infected mosquitoes that will protect recipient mosquitoes from subsequent infection.

Boman et al. (1981) drew attention to the problem of distinguishing a response to an infection from a response caused by sham injection or injury. They demonstrated the production of 'injury RNA' and 'immune RNA'. In the present study this non-specific 'sham response' has been allowed for by transferring haemolymph from sham injected controls as well as from infected donor mosquitoes, and by injecting the same mosquitoes first with TC199 followed after 24 hrs by challenge microfilariae. Furthermore, this study has shown that there is no reduction in the infection rates in TC199 controls compared with untreated control mosquitoes, and that the reduction in the parasites recovered and larvae developing in the TC199 controls are highly significantly different from the microfilariae, bacteria, laminarin and LPS injected mosquitoes. On the other hand, the dextran injected mosquitoes showed similar results to TC199 controls but they were significantly different from those with laminarin operated mosquitoes. Leonard et al. (1985b) demonstrated that laminarin but not dextran activated phenoloxidase production.

The relationships between the humoral response to injuries and that to infections are not yet understood, but the current state of knowledge can be summarized as follows. At the RNA level there is a qualitative difference

between the injury and immune responses that can be detected with the methods currently available (Boman et al., 1981). At the protein level, there are qualitative similarities but quantitative differences in the pattern of newly-synthesized proteins (Fay et al., 1975; Boman and Steiner, 1981). There is a significant difference at the level of immune response activity. The immune response is a potent antibacterial activity, while the injury response induces either no or only a weak antibacterial activity (Gõtz and Boman, 1985).

Therefore the injury or TC199 control and dextran injection might or might not induce some or all of such enzymes as lysozyme, phenoloxidase and protein lectins, which may play a role in the defence mechanisms by destroying the foreign invaders by melanization, encapsulation, haemocyte-coagulation, agglutination and digestion. But the level of the injury or sham response is lower than that of the immune response.

Nevertheless, it is clear that some factor(s) in the haemolymph of mosquitoes injected with laminarin or LPS have a partial effect on the development and growth of microfilariae in the subsequent infection. This demonstrated that laminarin and LPS can induce an immune response in the mosquitoes and have an effect on microfilariae infection in the mosquitoes.

The reductions in the number of parasites recovered and larvae developing in the recipient mosquitoes subsequently infected with a large number of microfilariae seems to be a result of the immune response. There may be inadequate nutrition coupled with the immune response. Christensen et al. (1984) indicated that the death of B. pahangi microfilariae was caused by physiological incompatibility with the Rockefeller Ae. aegypti mosquitoes, with subsequent melanization, and was not a direct result of the immune response.

It has also been suggested that the immune response of Ae. trivittatus against Di. immitis might function by reducing the parasite load in heavy infected mosquitoes, thereby helping the survival of both parasite and vector. Therefore, the defence mechanisms of immune response and adequate physiological nutrition may be the natural defence mechanisms of the vector to reduce the intensity of infection and to aid the survival of the vector for the extrinsic incubation period of the parasite.

3.2 THE EFFECT OF THE INNATE AND ACQUIRED IMMUNE RESPONSE OF MOSQUITOES ON MICROFILARIA MIGRATION

3.2.1 Introduction

During the early investigations of host-parasite relationships, the development of microfilariae and their migration were studied in various mosquitoes (Esslinger, 1962; Schacher, 1962; Ramachandran, 1966). Esslinger (1962), studying the behaviour of B. pahangi in An. quadrimaculatus, described homogeneous brown plaque intermingled with a cellular fibrous material adhering to the surface of the microfilariae. He suggested that this was a type of humoral melanization which was a defence reaction of the mosquito host. Schacher and Khalil (1968) showed that a cellular reaction was mounted against the lizard filarioid nematode Foleyella philistinae by the fat body cells and fibroblast-like cells of Cu. pipiens molestus. Ho et al. (1982) demonstrated melanization and encapsulation in Ae. aegypti in response to parasitization by a filarioid nematode, Breinlia booliati, the haemocytes of the mosquitoes appearing to be involved in the encapsulation of filariae by adherence of cells to the cuticle of the larvae and the formation of a translucent envelope which contained intact cells. Chen and Laurence (1985) also reported that humoral melanization only occurs in insects such as mosquitoes that have few circulating haemocytes. Forton et al. (1985) provided information concerning the humoral response in an elegant study of the host response of Ae. trivittatus against inoculated Di. immitis microfilariae. The melanization response elicited by the host 2-6 hrs postinjection represented cellular material on the surface of the parasite. By 24 hrs postinjection, the parasite was completely encased in

melanin. Two days postinjection, there was an increase in remnants of haemocytes around the parasite and a membrane-like structure began to form around the parasite. Christensen and Forton (1986) showed that the haemocytes mediated melanization of Di. immitis microfilariae in Ae. aegypti, the membrane-like structure forming around the melanized microfilariae and cellular debris during the later stages of reaction. The present experiments were designed to observe;

i. The time course of microfilarial migration for microfilariae inoculated into mosquitoes compared with microfilariae derived from a membrane blood feed (Experiment I).

ii. The time course of microfilarial migration in the mosquitoes following transfer of haemolymph from microfilaria infected donors (Experiment II).

iii. The effect of the immune response induced by laminarin on microfilarial migration for microfilaria inoculated mosquitoes compared with microfilariae derived from a membrane blood feed (Experiment III).

3.2.2 Materials and Methods

3.2.2.1 Experiment I. The migration of microfilariae in membrane-fed mosquitoes and in inoculated mosquitoes.

Virgin female mosquitoes 5-7 days of age were starved for 24 hrs before feeding. Infected jird washings were diluted with rabbit blood to achieve a microfilaraemia of 100-250 microfilariae/20 μl. The mosquitoes were allowed to feed on the microfilaria-blood mixture with an estimated 206 mff/20 μl through a water jacketed membrane feeder at 37°C fitted with a chicken-skin membrane (Rutledge et al., 1964; Wade, 1976). The mosquitoes that fed fully were separated into small plastic labelled cups at different time intervals and maintained in the insectary until dissected.

The mosquitoes were dissected at different time intervals after feeding. In all dissections, wings and legs of individual mosquitoes were removed and the thorax and abdomen were teased aparted thoroughly in a drop of Hayes saline with 1% methylene blue. The midgut (with foregut, hindgut and Malpighian tubules attached) was removed and placed in a drop of Hayes saline with 1% methylene blue. The numbers of microfilariae in the thorax, midgut and abdomen in each mosquito were recorded.

The migration of microfilariae in inoculated mosquitoes.

Microfilariae of B. pahangi were isolated from jird washings as described previously (Sucharit and Chuchote, 1982) and 1 μl of TC199 containing 10 microfilariae was inoculated intrathoracically into individual 5-7 days old virgin female mosquitoes, using finely drawn glass capillary pipettes (Ham, 1986). The infected mosquitoes were dissected at different time intervals. The numbers of

microfilariae recovered from the thorax and abdomen in individual mosquitoes were noted.

3.2.2.2 Experiment II. The migration of microfilariae in recipient mosquitoes injected with microfilariae following the transfer of haemolymph from an infected donor.

Four days after being inoculated with 10 microfilariae/mosquito, the donor mosquitoes were injected with a further 1 μl of TC199. Then, after 30 sec, using the same needle without having withdrawn it, approximately the same volume of fluid was removed from the thorax by suction. The haemolymph/TC199 mixture was then injected into the thorax of recipient mosquitoes. These recipient mosquitoes had been inoculated with 10 microfilariae/mosquito approximately 2 hrs beforehand. The procedure was repeated with pairs of mosquitoes, 1 donor to 1 recipient. Then the recipient mosquitoes were dissected at different time intervals. The numbers of microfilariae recovered in the thorax and abdomen in individual mosquitoes were recorded.

3.2.2.3 Experiment III. The immune response to B. pahangi microfilariae in Ae. aegypti mosquitoes injected with laminarin or TC199, followed 4 days later by infection with microfilariae by membrane feeding.

The 5-7 days old virgin female mosquitoes were injected with 1 μl of 1 mg/ml laminarin or 1 μl of TC199 on day 0. On day 4, the 22 mosquitoes were membrane-fed as previously described. Infected jird washings were diluted with rabbit blood to achieve a microfilaraemia of 100-250 microfilariae/20 μl. The mosquitoes were exposed to the microfilaria-blood mixture which had an

estimated 142 mff/20 μl. The mosquitoes that fed fully were separated into small plastic labelled cups, and separated into two groups.

The mosquitoes of the first group were dissected 6 hrs after being fed in a drop of Hayes saline with 1% methylene blue. The numbers of microfilariae in the thorax, abdomen and midgut were recorded.

On day 4 after feeding the mosquitoes of the second group in each treatment were dissected and the numbers of larvae and microfilariae were recorded.

3.2.3 Results

Experiment I

The mean numbers and percentage of microfilariae migrating at the various time intervals after inoculation and membrane feeding are presented in Table 14 and Table 15 respectively. At the 10-minute interval, 8% of the microfilariae in inoculated mosquitoes and 0.63% in membrane-fed mosquitoes had reached the thorax. At 2 hrs, the results from the inoculated and membrane-fed mosquitoes were similar, with 20% of the microfilariae having migrated to the thorax. After 4 and 6 hrs there was a slightly higher proportion of microfilariae in the thorax of inoculated mosquitoes (30% and 51%) than in the membrane-fed mosquitoes (23% and 34%). However, the percentage migration to the thorax in the membrane fed group is heavily weighted by the large number of microfilariae in the blood meal. At 6 hrs 84.9% of microfilariae were in the thorax of the membrane blood fed group compared with 51% in the inoculated group (Table 14).

Figs. 9 and 10 show the results graphically.

Experiment II

Table 16 shows the mean number and percentage of microfilariae migrating at the various time intervals in the recipient mosquitoes given haemolymph from the infected donors and the percentage of melanized microfilariae recovered from the recipient mosquitoes.

At the 0-30 min interval, 25% of the microfilariae migrated to the thorax and 1.1% (1/88) were found melanized. after 1 hr 4.9%(2/41) of the microfilariae were melanized in the thorax and 9.6%(5/52) in the abdomen. After 2 and 4 hrs

9.5%(9/95) and 13.9%(13/93) respectively of the microfilariae were found melanized in the thorax and abdomen.

Fig. 11 shows the mean numbers of microfilariae migrating in the recipient mosquitoes. The mean numbers in the thorax increased with the time while the numbers in the abdomen decreased.

The results demonstrate that the acquired immune response in recipients given haemolymph from infected donors has an effect on microfilariae migration and development. Melanized microfilariae were found in the abdomen within 30 min of exposure to the immune haemolymph from the infected donors.

This result contrasts with the experiments I in which no melanized microfilariae were recorded. On dissection, it was noted that the fat body cells and haemocytic cells were aggregated 2 hrs after induced immune haemolymph was transferred to recipient mosquitoes, compared with the normally infected mosquitoes in which fat body cells were observed to float and haemocytic cells to spread.

Experiment III

The migration of membrane fed microfilariae after 6 hrs and 4 days in the mosquitoes given an injection of laminarin or sham TC199 is illustrated in Table 17.

The migration patterns to the thorax of two groups after 6 hrs were significantly different, 14.4% in TC199 group compared with 9.9% in laminarin group ($P<0.014$, AOV test). But there was no different significant recovery of microfilariae in the thorax, abdomen and midgut, and in the total recovered in the laminarin-treated mosquitoes compared with TC199 group.

Furthermore, there was a significant reduction of larvae developing in the laminarin treatment group compared with TC199 treatment group 4 days after the blood meal, ($P<0.00003$ Mann-whitney test) Table 17 and Fig.12.

The number of microfilariae recovered from the thorax after 6 hrs compared with the number of larvae recovered in thorax after 4 days was not significantly different in the TC199 control mosquitoes, but there was significant difference ($P<0.014$) in laminarin treated mosquitoes. Thus the laminarin induced immune response had a marked effect on the microfilariae migrating to the thorax, and on their survival and development.

Fig. 12 compared the numbers of microfilariae in the thorax, abdomen and midgut in the laminarin treated mosquitoes and those with sham TC199. The numbers of microfilariae in the thorax of the laminarin treated group declined, while the numbers of microfilariae in the abdomen and midgut were similar. Fig. 13 shows the frequency distribution of larvae recovered from the thorax of two groups of mosquitoes 4 days after their infecting meal. There was a mark shift towards zero larvae recovery per mosquito in laminarin treatment group, whereas the frequency distribution of larvae recovered from TC199 group is approaching a normal distribution.

TABLE 14. THE MIGRATION OF B. PAHANGI MICROFILARIAE IN AE. AEGYPTI MOSQUITOES AT VARIOUS TIME INTERVALS FOLLOWING INOCULATION OF 10 MFF/MOSQUITO.

Times	No.dissed	Mean ± SE			%Migration
		Thorax	Abdomen	Total	to thorax
0-10 min.	10	0.8 ± 0.36	9.1 ± 0.35	9.9 ± 0.10	8
2 hr.	10	2.0 ± 0.26	7.8 ± 0.13	9.8 ± 0.13	20
4 hr.	10	3.0 ± 0.21	6.8 ± 0.29	9.8 ± 0.13	30
6 hr.	10	4.8 ± 0.25	4.6 ± 0.37	9.4 ± 0.22	51

TABLE 15. THE MIGRATION OF B. PAHANGI MICROFILARIAE IN AE. AEGYPTI MOSQUITOES AT VARIOUS TIME INTERVALS FOLLOWING MEMBRANE FEEDING (206 MFF/20 μl).

Times	No.dissed	Mean ± SE				%Migration
		Thorax	Abdomen	Midgut	Total	to thorax
0-10 min.	10	0.4 ± 0.31	5.2 ± 1.43	57.6 ± 5.10	63.2 ± 4.53	0.63
1 hr.	10	3.5 ± 0.47	7.0 ± 1.10	70.9 ± 3.80	81.4 ± 2.90	4.29
2 hr.	10	13.4 ± 2.23	9.3 ± 1.36	43.8 ± 3.84	66.5 ± 4.42	20.15
4 hr.	10	19.6 ± 1.07	4.7 ± 0.34	61.4 ± 2.87	85.7 ± 3.57	22.87
6 hr.	10	27.5 ± 1.77	4.9 ± 0.41	48.3 ± 3.55	80.7 ± 3.81	34.07

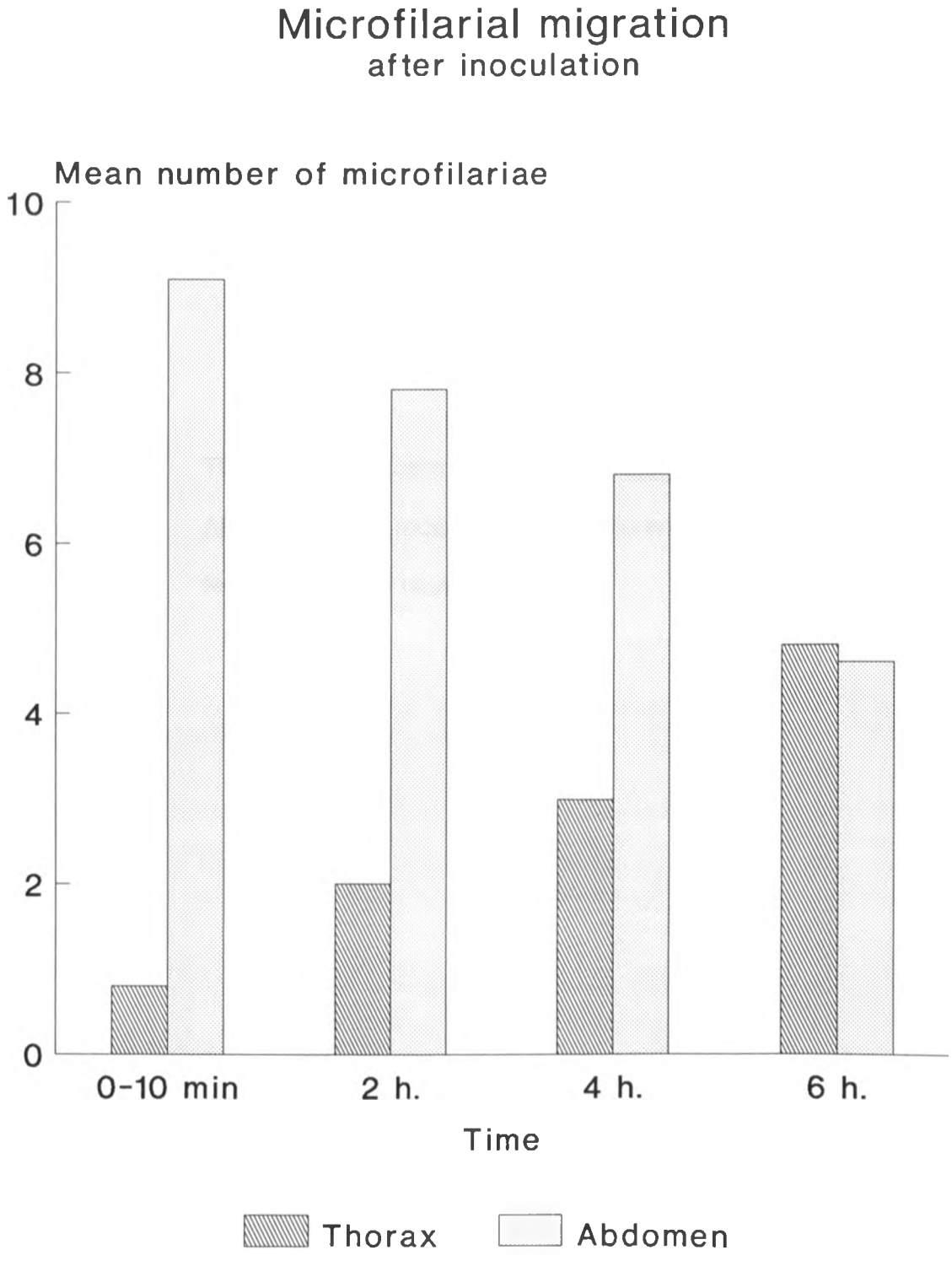
TABLE 16. THE MIGRATION OF B. PAHANGI MICROFILARIAE AND PERCENT OF MELANIZED MICROFILARIAE IN AE. AEGYPTI MOSQUITOES AT VARIOUS TIME INTERVALS FOLLOWING TRANSFER OF HAEMOLYMPH FROM INFECTED DONORS.

Times	No.diss.	Thorax		Abdomen			Total	% Mel.
		Mff	%Mel	%Mig	Mff	%Mel	Mff.	
0-30 min	10	2.2±0.3	0	25	6.6±0.3	1.5(1/66)	8.8±0.4	1.1(1/88)
1 hr.	10	4.1±0.3	4.8(2/41)	44	5.2±0.3	9.6(5/52)	9.3±0.2	7.5(7/99)
2 hr.	10	4.8±0.3	4.2(2/48)	50	4.7±0.4	15(7/47)	9.5±0.2	9.5(9/95)
4 hr.	10	6.1±0.2	4.9(3/61)	66	3.2±0.3	30(10/32)	9.3±0.2	14(13/99)

TABLE 17. THE MEAN NUMBERS OF B. PAHANGI MICROFILARIAE MIGRATING AE. AEGYPTI MOSQUITOES PREVIOUSLY INJECTED WITH LAMINARIN OR TC199, 6 HR AND 4 DAYS AFTER MEMBRANE BLOOD FEEDING.

Treatments	No.diss	6 hrs Mean±SE (Range)			4 Days		
		Thorax	Abdomen	Midgut	Total	No.diss.	Thorax
TC199	10	14.4±0.9	1.9±0.5	5.1±0.9	21.4±1.2	20	18.3±0.8
Laminarin	10	9.9±0.9	1.6±0.2	5.3±0.5	16.8±1.2	19	8.7±0.5

FIG.9. The mean numbers of Brugia pahangi microfilariae migrating in Aedes aegypti mosquitoes at different times after inoculation of microfilariae.



Microfilarial migration Fig.9

FIG.10. The mean numbers of Brugia pahangi microfilare migrating in Aedes aegypti mosquitoes at different times after membrane feeding infected blood.

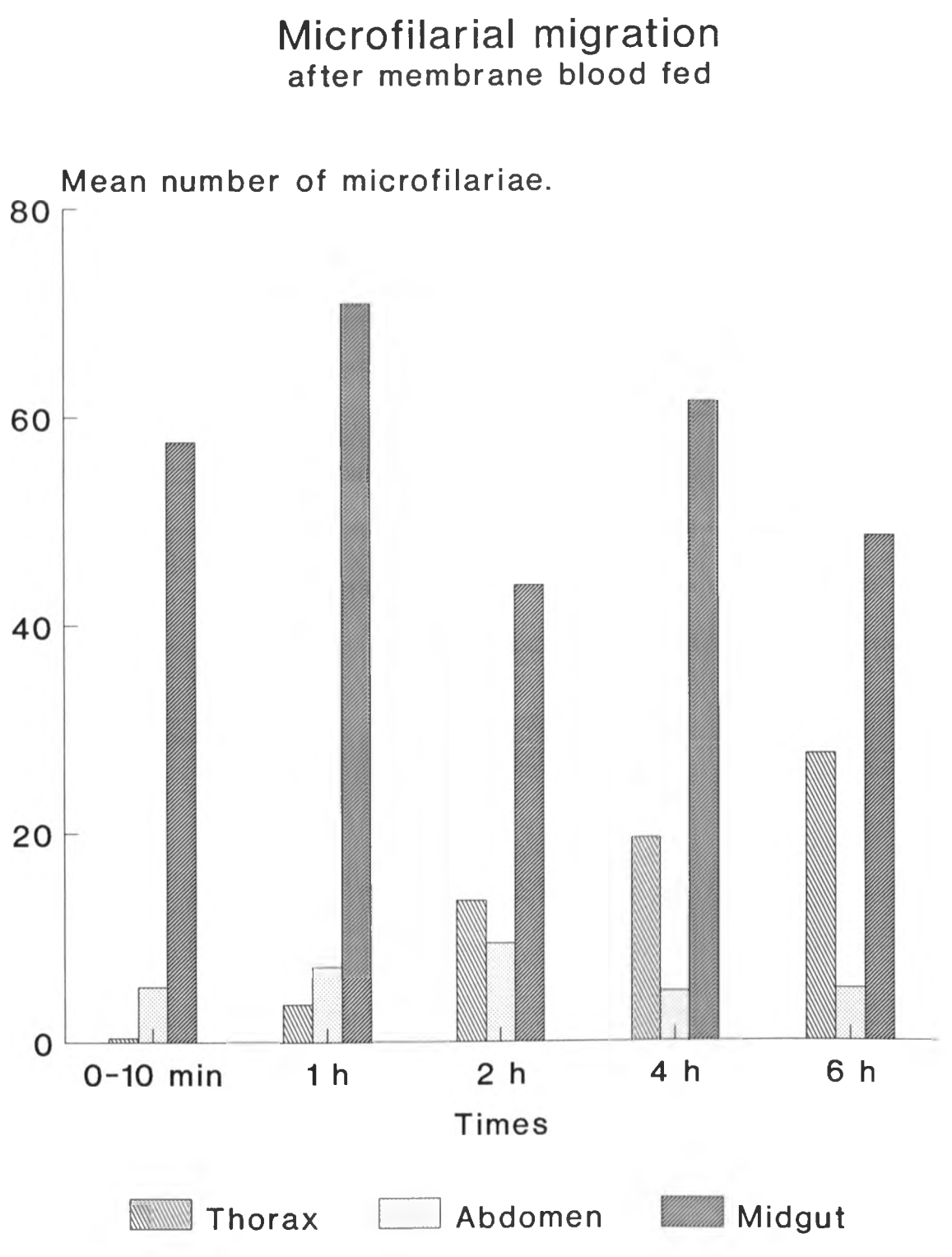
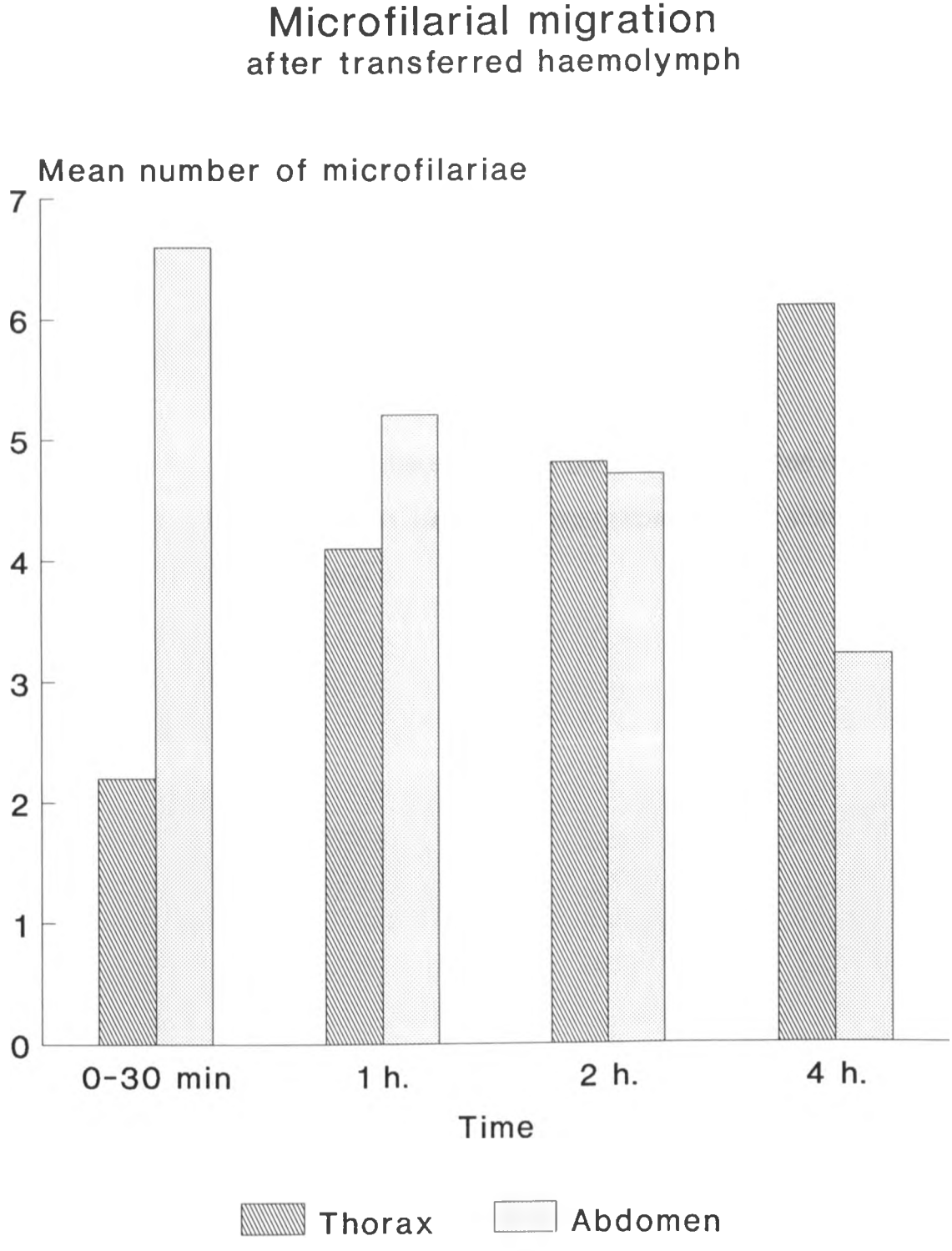

Microfilarial migration
after membrane blood fed
Mean number of microfilariae.
80
60
40
20
0
0-10 min
1 h
2 h
4 h
6 h
Times
Thorax
Abdomen
Midgut

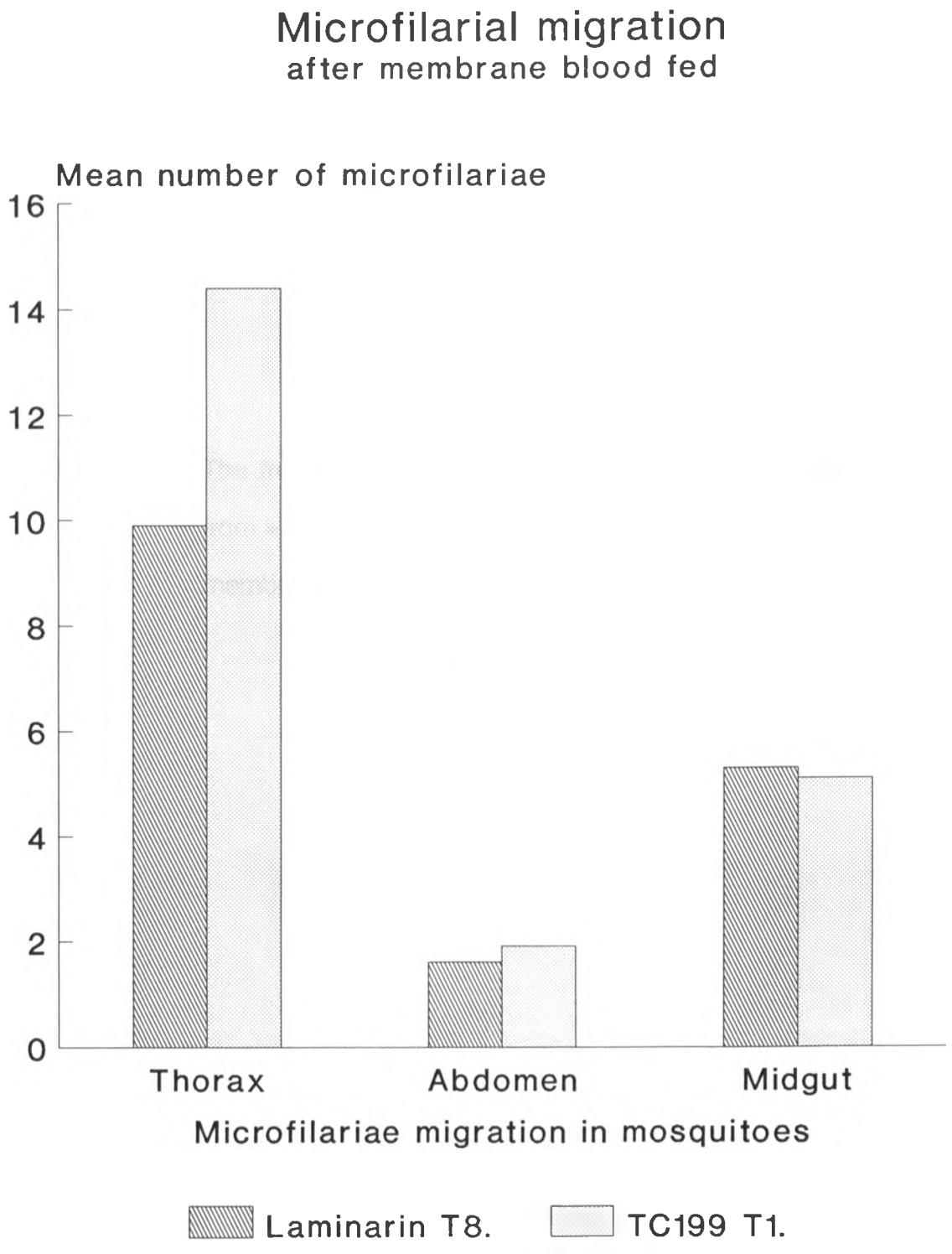
Fig.10

FIG.11. The mean numbers of Brugia pahangi microfilariae migrating at different times in Aedes aegypti recipient mosquitoes following transfer of haemolymph from infected donor mosquitoes.



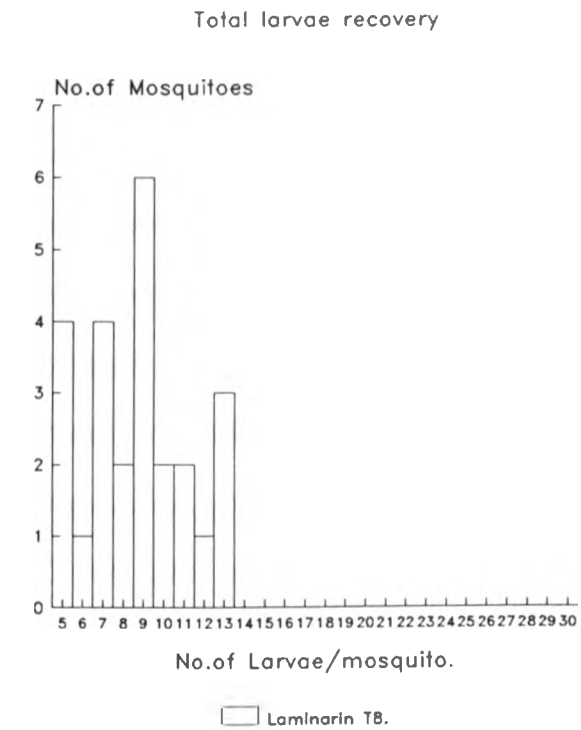
MFF.migration in recipients Fig.11

FIG.12. The mean numbers of Brugia pahangi microfilariae migrating in laminarin and TC199 treated mosquitoes, 6 hours after membrane blood feeding.

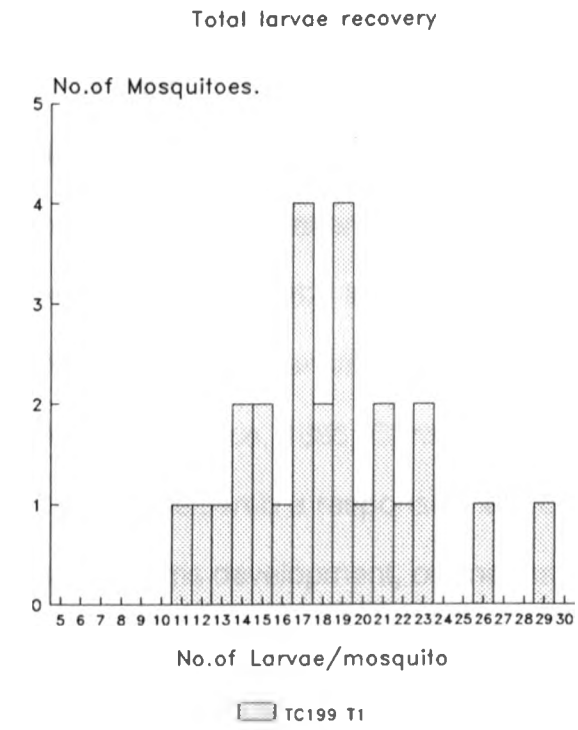


Microfilariae migration 6 h.Fig.12

FIG.13. The frequency distribution of Brugia pahangi larvae recovered from laminarin and sham TC199 treated mosquitoes 4 days after membrane blood feeding.



Larvae recovered T8.Fig.13



Larvae recovered T1

3.2.4 Discussion

Various observations on the migration of microfilariae in mosquitoes have been reported by Laurence and Pester (1961), Esslinger (1962), Ramachandran (1966), Coluzzi and Trabucchi (1968), Bryan et al. (1974), Christensen and Sutherland (1984), and Sutherland et al. (1984). Esslinger (1962) stated that the migration of B. pahangi microfilariae to the thorax of An. quadrimaculatus occurred 15-30 min after blood feeding. Ewert (1965) reported that B. pahangi microfilariae penetrated through the midgut of Ae. aegypti (Hooper and New Orlean strains) 20 and 25%, and 14 and 20% to thorax within 60 min after ingest blood meal. In the present study some microfilariae had reached the thorax after 10 min, and after 60 min the microfilariae reached the thorax approximately 13%, but even after 2 hrs only 20% of the microfilariae had reached the thorax in membrane blood-fed mosquitoes. The microfilarial migration to the thorax after 6 hrs of inoculation group (51%) seem better than the microfilariae migration in membrane fed group (34%).

Numerous reported to melanization of filarial worms by the mosquitoes have been published (Esslinger, 1962; Intermill, 1973; Oothuman et al., 1974; Nayar and Sauerman, 1975; Christensen. 1981; Ho et al., 1982; Bradley and Nayar, 1985; Chen and Laurence, 1985; Christensen and Forton, 1986). The reaction has long been considered a response that occurs in mosquitoes that are refractory to filarial worms development, or one that occurs so seldom that it is of no consequence to the vector-parasite system. The results reported in this study show that following the transfer of haemolymph from an infected donor, the melanized microfilariae were found both in the abdomen and in the

thorax of the recipients within an hour or two. This melanization is therefore the result of the immune response factor(s) in the immunized haemolymph.

The observations reported support recent investigations which showed that the enhanced haemocyte activity is associated with melanization (Ho et al., 1982; Forton et al., 1985; Chen and Laurence, 1985; Christensen and Forton, 1986).

The reduction in the number of larvae recovered in the mosquitoes previously injected with laminarin is also an effect of immunized haemolymph. The melanized microfilariae were not found in the laminarin treated mosquitoes. This may be because during penetration of the midgut the microfilariae acquire host materials on their surface and are therefore not recognized as foreign invaders by mosquitoes (Sutherland et al.,1984). Nevertheless, the reduction in the number of larvae recovered from the laminarin treated mosquitoes was significantly different from the TC199 control. This is an effect of the immune response factor(s) induced by a ß-1,3-glucan laminarin.

3.3 HAEMOCYTE NUMBERS IN AEDES AEGYPTI FOLLOWING INJECTION OF BRUGIA PAHANGI MICROFILARIAE, ESCHERICHIA COLI, LAMINARIN, LPS AND DEXTRAN

3.3.1 Introduction

The melanization of filariae in mosquitoes has frequently been considered a humoral response, an immune response involving only non-cellular components of the haemolymph (Poinar and Leutenegger, 1971). Esslinger (1962) suggested that the melanization of B. pahangi in An. quadrimaculatus was not directly associated with any haemocyte type. However, Bartlett (1984) reported that haemocytes were present in close association with the microfilariae melanized in the haemocoele. Ho et al. (1982) suggested that the initial melanization reaction of Ae. aegypti to B. booliati was followed by encapsulation by haemocytes. Forton et al. (1985) also reported that haemocytes of adult Ae. trivittatus were involved in melanization of intrathoracically inoculated Di. immitis microfilariae. Christensen and Forton (1986) reported a humoral immune response, a membrane-like structure being formed around the melanized Di. immitis microfilariae in Ae. aegypti, followed by deposition of cellular debris in the later stages of the reaction. Chen and Laurence (1987) demonstrated the encapsulation of the microfilariae of B. pahangi in the haemocoele of the mosquito An. quadrimaculatus combining both humoral and cellular encapsulation. Recently Christensen et al. (1989) has reported that the haemocyte numbers in mosquitoes with Di. immitis increases during infection, and that this is a result of division of circulating blood cells.

Gunnarsson and Lackie (1985) demonstrated haemocytic aggregation (nodule formation) induced in the locust Sch. gregaria and the cockroach P. americana by injecting a suspension of ß-1,3-glucan laminarin and LPS (from E.coli and Serratia marcescens). Gunnarsson (1988) reported the possible role of ß-1,3-glucan in the induction of a cellular defence reaction in the locust Sch. gregaria against fungal infection.

In the case of parasitic infections, the activation of a humoral and cellular immune response depends only on the presence of the elicitor. This elicitors can be supplied by such foreign bodies as laminarin, LPS, bacteria and the parasites. The following study was designed to assess the haemocyte population and determine how the numbers might change during an active immune response to microfilariae, bacteria and cell walls components.

3.3.2 Materials and Methods

Virgin female mosquitoes 5-7 days old were anesthetized with carbon dioxide and kept inactivated on ice in a petri dish. The individual mosquitoes were injected with either 10 microfilariae of B. pahangi, 1μl of $1x10^4$cells/μl E. coli, 1 μl of 1 mg/ml laminarin, 1 μl of 100 μg/ml of LPS, 1 μl of 100 μg/ml of dextran or 1 μl TC199.

Haemolymph was withdrawn from group of mosquitoes daily from 1 to 5 days postinjection. The mosquitoes were injected with 1 μl TC199, then after 30 sec using the same needle without having withdrawn it, approximately the same volume of fluid was removed from the thorax by suction. The haemolymph/TC199 mixture was allowed to settle on a drop of 5 μl of TC199 on a clean glass slide for 10 min to air dry. The fixation method was modified from that Arnold and Hinks (1979). After drying, the slides were heat-fixed at approximately 60°C, then the slides were refixed with 2 drops of ice cold acetone (Christensen et al., 1989). After drying, the slides were stained with Giemsa stain, PBS buffer pH 7.8, and all haemocytes were counted at x400 under a light microscope.

3.3.3 Results

The mean numbers of haemocytes in the mosquitoes following injection with microfilariae, bacteria, laminarin, LPS, dextran, TC199 control and in untreated mosquitoes are presented in Table 18. The haemocyte counts in microfilariae inoculated mosquitoes increased from day 1 to day 3 and decreased on days 4 and 5. The haemocyte counts from all 5 days in these mosquitoes were significantly higher than the counts in mosquitoes injected with the other substances, higher than the untreated mosquitoes (Table 19). Although the haemocyte count had decreased by day 5, the level of the count was still higher than in the other treatments.

The haemocyte counts in the bacteria infected mosquitoes increased on day 1 and day 2, and decreased from day 3 to day 5. The counts from all 5 days were significantly higher compared with the TC199 ($P<0.05$), untreated mosquitoes ($P<0.001$), and dextran ($P<0.001$).

The haemocyte counts in the mosquitoes injected with laminarin and LPS decreased on day 1, but both increased on day 2 to day 3 and decreased on day 4 and day 5. The haemocyte counts from all 5 days in the laminarin and LPS treatments were not significantly different from the TC199 and the dextran treatments or the untreated controls (Table 19).

The haemocyte counts from all 5 days in the mosquitoes injected with dextran were significantly different only compared with the TC199 treatment ($P<0.05$) and the untreated mosquitoes ($P<0.01$). The haemocyte counts in untreated mosquitoes were slightly depressed on day 1 to day 5.

The results demonstrated that the microfilariae induced an increased haemocyte population during their survival and development in the mosquitoes.

The bacteria also induced an increased haemocyte population for 2 days PI before a steady fall during the following 3 days. In laminarin and LPS treatments the haemocyte population was low on day 1 and increased on days 2 and 3 before falling to a level on day 5 which was still higher than that on day 1. The treatment with dextran led to a steady fall in haemocytes from day 1 to day 5 (Fig. 14).

TABLE 18. THE HAEMOCYTE COUNT IN 1 μl SAMPLES WITHDRAWN FROM AE. AEGYPTI MOSQUITOES AT DIFFERENT TIME INTERVALS FOLLOWING INJECTION WITH B. PAHANGI MICROFILARIAE, E. COLI, LAMINARIN, LPS, DEXTRAN AND TC199.

Treatments	No.M. d/d.	Mean ± SE (Range) Day 1	Day 2	Day 3	Day 4	Day 5
TC199 Cont	10	391.8± 7.1	350.7± 6.5	347.0± 8.2	317.0±12.8	259.8±10.3
		(363-439)	(319-378)	(309-384)	(242-376)	(211-317)
Untreated	10	303.9±10.8	309.4± 9.4	252.4±13.7	257.5± 9.8	203.1± 9.1
		(248-360)	(263-359)	(181-313)	(199-297)	(167-249)
Mff	10	485.3± 9.6	498.1±6.9	540.3±11.2	492.2±18.7	307.9± 6.9
		(432-527)	(463-529)	(479-593)	(353-549)	(273-341)
Bacteria	10	389.9±17.5	426.8± 9.7	391.7±14.5	369.1± 9.2	297.7± 8.4
		(349-423)	(378-473)	(328-481)	(319-411)	(249-332)
Laminarin	10	252.7±11.5	299.1± 6.8	391.0±13.5	375.1± 8.3	290.6±11.0
		(198-301)	(264-327)	(325-446)	(335-411)	(243-347)
LPS	10	246.6± 9.0	285.6± 7.6	378.9±11.9	315.7±10.6	284.2±11.6
		(191-291)	(249-317)	(324-436)	(273-363)	(227-338)
Dextran	10	347.7± 7.2	343.8± 7.7	331.5)±5.6	306.0±12.1	226.4± 6.7
		(307-378)	(301-383)	(305-363)	(248-378)	(198-260)

No.M.d/d. = No. mosquitoes dissected/day

Footnote : With the haemolymph sampling method employed, the 1 μl samples comprised with 75% haemolymph: 25% TC199 (see section 3.1.2.1), to give haemocyte counts per μl , the figure in the table should be multiplied by 1.333.

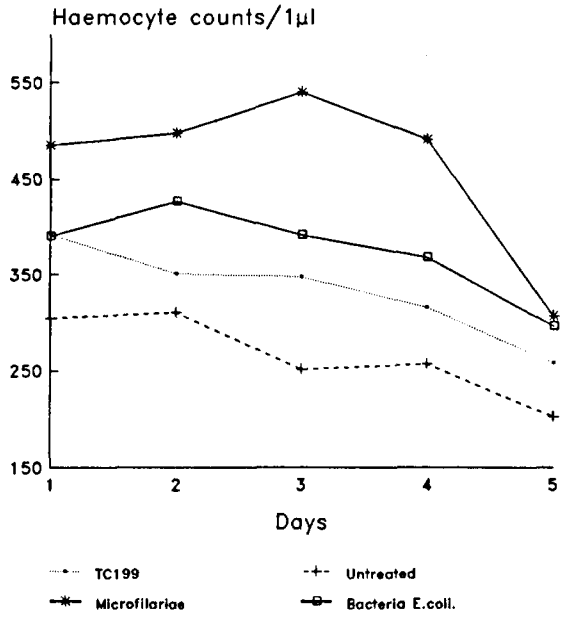
TABLE 19. STATISTICAL ANALYSIS OF THE HAEMOCYTE COUNT IN 1 µl HAEMOLYMPH WITHDRAWN FROM MOSQUITOES INJECTED WITH MICROFILARIAE, BACTERIA, LAMINARIN, LPS, DEXTRAN AND TC199 FROM ALL 5 DAYS (DAY 1 TO DAY 5) POSTINJECTION (ANOVA TWOWAY TEST).

Mean number of haemocyte count						
Mff.	Bacteria	TC199	Laminarin	LPS	Dextran	Untreated
459.2	375.2	333.4	321.8	310.6	302.2	265.0
				<_____>		
		<_____>				
	<_____>					

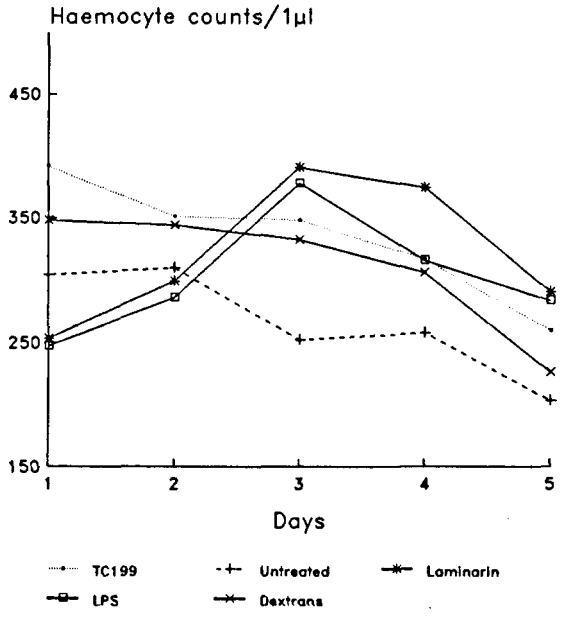
Treatments	Untr.	Mff	Bacteria	Treatments Laminarin	LPS	Dextran
TC199	P<0.05	P<0.001	NS	NS	NS	NS
Untr.	-	P<0.001	P<0.001	P<0.05	NS	NS
Mff	-	-	P<0.01	P<0.001	P<0.001	P<0.001
Bact.	-	-	-	NS	P<0.05	P<0.05
Lamin.	-	-	-	-	NS	NS
LPS	-	-	-	-	-	NS

Untr. = Untreated
TC199 = TC199-inoculated control
Lamin. = Laminarin injection
Bact. = bacteria inoculation
NS = Nonsignificant
Footnote; The groups are not significant at P=0.05 indicated by underlining

FIG.14. The haemocyte counts in 1 μl haemolymph withdrawn on day 1-5 following inoculation of microfilariae, bacteria, laminarin, LPS, dextran, TC199 injection and untreated mosquitoes.



Haemocyte counts



Haemocyte counts

3.3.4 Discussion

These results clearly showed a significant increase in the number of haemocytes in Ae. aegypti infected with B. pahangi microfilariae and E. coli bacteria. The haemocyte count increased up to day 3 PI and declined after day 3 to day 5 PI.

The immune response of adult mosquitoes against filarial worms has been studied most extensively in Ae. aegypti (refractory), Ae. trivittatus, An. quadrimaculatus and Am. subalbatus (Christensen, 1986; Christensen and Tracy, 1989; Beerntsen et al., 1989). Chen and Laurence (1985) suggested that the encapsulation of microfilariae in the haemocoel of mosquitoes combines both humoral and cellular reaction ; humoral encapsulation occurs first and cellular encapsulation take place later and Forton et al. (1985) demonstrated that the haemocytes lyse on the surface of microfilariae in the mosquitoes before the deposition of melanotic substances. Ogura (1987) showed that haemolymph obtained from Am. subalbatus required the inclusion of haemocytes for melanization of living B. pahangi and B. malayi. It has also been demonstrated with Ae. aegypti that the haemocyte surface characteristics are altered when an immune response is initiated by the inoculation of Di. immitis microfilariae (Nappi and Christensen, 1986), and that a large number of haemocytes are incorporated in the melanotic capsule (Christensen and Forton 1986).

In G. mellonella injected with B. cereus the total haemocyte count rapidly decreased for at least 6 hrs. (Gagen and Ratcliffe, 1976). The observations reported in this study showed an increase in the number of haemocytes in the mosquitoes infected with E. coli bacteria (10^4cells), the number increasing to

day 3 and declining from day 3 to day 5 PI. The haemocyte depletion factor is not therefore a general response to infection with bacterial suspension, and the pathogenicity of the bacteria was not a significant factor (Anderson and Chain, 1986). E. coli may possibly lead to an initial short depletion of haemocytes but after that period the haemocyte numbers increase to the high peak on day 3 PI. The observations reported in this study support recent research showing that changes in the number of circulating haemocytes do occur in insects following injection of foreign material, as reported by Brehelin and Hoffmann (1980) and Takle (1988).

The dramatic decrease in haemocyte counts in the laminarin and LPS treatments on day 1 and the following increase on day 2 to day 3 PI, may be the result of operation of the ' **Adhesion factor'** (Johnasson and Söderhall, 1988), the depletion factor or of nodule formation of haemocytes (Gunnarsson, 1988). The haemocyte counts in the laminarin and LPS treatments were not significantly different from the TC199 control and dextran treatment or untreated mosquitoes, but by day 3 PI the numbers of the haemocyte count had risen than those in the TC199, dextran and untreated mosquitoes (Fig. 14). Microbial products, such as bacteria lipopolysaccharide (LPS) and laminarin ß-1,3-glucan from fungal cell walls, have been shown to cause a decreased in the number of haemocytes in vivo (Smith et al., 1984) and stimulation of aggregation or nodule formation in G. mellonella (Schwalbe and Bousch, 1971), in crayfish (Smith et al., 1984), and in Sc. gregaria and P. americana (Lackie et al., 1985).

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CHAPTER 4

IN VITRO STUDIES OF THE ATTENUATION OF MICROFILARIAE MOTILITY BY IMMUNE HAEMOLYMPH

4.1 INTRODUCTION

The pioneer work with laboratory selection clearly demonstrated that resistance to filarial infections is a genetically determined trait (Macdonald, 1962a,1962b; Macdonald and Ramachandran, 1965), but very little research has been concerned with the immune response of adult mosquitoes to filarial worms. The little information available on defence mechanisms of mosquitoes is striking when one considers the public health importance of these insects. In general, the dipteran vectors are more than just transport organisms that transfer parasites from infected to non-infected animals: most of them are also intermediate hosts of the parasites transmitted, and part of the life cycle of these parasites takes place within them. The parasites must therefore be able to develop and live in two different host organisms and consequently to deal with two different defence systems, one in the definitive host and one in the intermediate host.

Defence reactions in Diptera against parasites have been reported by Oothuman et al. (1974); Lehane (1978); Hammerberg et al. (1984); Sutherland et al. (1984); Bradley and Nayar (1985); Götz and Boman (1985). The segregation of foreign organisms from the haemocoele may occur as a non-cellular reaction, which is named humoral encapsulation (Götz, 1969; Chen and Laurence, 1985), melanotic encapsulation (Pionar and Leutenegger, 1971) or melanization response (Christensen et al., 1984; Sutherland et al., 1984), all these authors working with the mosquito response to a filarial nematode. Nevertheless, scant attention has been paid to other immune mechanisms and still less to those of an acquired nature.

Ham et al. (1988) demonstrated the in vitro effect of haemolymph on the motility of fresh O. lienalis and B. pahangi microfilariae incubated with the haemolymph from individual infected simuliids. The motility of both species of parasites was found to be significantly attenuated when compared to microfilariae incubated in control haemolymph. They also reported that the apparent absence of melanization and encapsulation of simuliids may have at least 2 humoral haemolymph components available to them for parasite regulation but the role of the defence mechanism of simuliids to the filarial parasites is as yet unclear.

Humoral immunity can be induced in insects by an injection of either live, non-pathogenic bacteria or heat-killed pathogens (Götz and Boman, 1985). The insect responds to live bacteria by the production of a potent antibacterial activity which is due to synthesis of immune proteins. To this group of proteins belong a lysozyme (Stephen et al., 1962; Mohrig and Messner, 1968; Kinoshita and Inove, 1977; Jolles et al., 1979; Hultmark et al., 1980; Boman, 1982; Huges et al., 1983; Spies et al., 1986a,1986b; Dunn et al., 1987), another novel class of antibacterial proteins, the cecropins (Hultmark et al., 1980; Steiner et al.,1981; Boman and Hultmark, 1981; Qu et al., 1982; Boman et al., 1985; Chadwick and Dunphy, 1986), the attacins (Pye and Boman, 1977; Hultmark et al., 1983; Engström et al., 1984a,b; Kaaya et al., 1987), the cecropin-like sarcotoxin I (Natori, 1977; Okada and Natori, 1983,1984,1985), the attacin-like sarcotoxin II (Okada and Natori, 1985; Ando et al., 1987), diptericin (Keppi et al., 1986; Dimarcq et al., 1988), and apidaecin (Casteels et al., 1989).

Recent studies have shown that the bacteria can induce an immune response reaction leading to a reduction of growth and development of

microfilariae parasites. The present study was designed to develop micro in vitro assays for studying the effect on living microfilariae of immune haemolymph induced by microfilariae, bacteria and the cell walls components laminarin, LPS and dextran.

4.2 MATERIALS AND METHODS

4.2.1 Experiment I. Standard assay for protein contents

Several dilutions of Bio-rad Protein Standard containing from 4 μg/ml to 35 μg/ml are prepared. A plot is made of optical density at 595 nm(OD.595) versus the concentrations of the standard (Bradford, 1976). Haemolymph from mosquitoes previously injected with 10 living or heat-killed microfilariae, 1 μl of living or heat-killed bacteria E. coli $1x10^4$cells/μl, 1 μl of 1 mg/ml laminarin, 100 μg/ml LPS, 1 mg/ml dextran, sham TC199 and from untreated mosquitoes were withdrawn daily from day 0 to day 7 (Trial A, 3 treatments) or day 0 to day 5 (Trial B, 8 treatments). The pooled haemolymph from each treatment making up 15 μl in haematocrit capillary glass was centrifuged for 5 min. Duplicate pools of haemolymph cell free samples, 5 μl for each day, were added to 795 μl phosphate buffer pH 7.2 to make the final volume 800 μl in clean test tubes. 200 μl of dye reagent is added to the test haemolymph samples, to the protein standard solutions and to the blank solution. The contents were mixed several times by gentle inversion of each test tube. After periods of times from 5 min to 1 hr, OD.595 was measured against the blank reagent with a CECIL CE 594 spectrophotometer. The unknown samples were estimated from the standard curve.

4.2.2 Experiment II. Motility assay of micro in vitro haemolymph attenuation of microfilariae.

Haemolymph was withdrawn from mosquitoes 4 days after they were injected with 10 living microfilariae or TC199, or 24 hr after they were injected

with bacteria E. coli $1x10^4$cells/μl, laminarin, LPS, dextran or TC199 using the techniques described earlier. 1.5 μl of TC199 was injected into the thorax of the mosquitoes and approximately the same amount was withdrawn by suction.

25 μg/ml lysozyme solution (Chicken egg white) obtained from Sigma was prepared in PBS pH 6.4.

In vitro haemolymph attenuation of microfilaria motility and of haemolymph toxicity to microfilariae were assessed in 72-well Teresaki microtitration plates (well capacity = 10 μl maximum). Haemolymph from individual mosquitoes was placed in each well and an equal volume of living microfilariae in a suspension of TC199 and Hepes was added to each well, giving a final volume of approximately 3 μl. The plates were placed in a humid chamber and incubated at 27°C (Ham and Garms, 1988).

The numbers of motile microfilariae in each well were counted and expressed percentage normally motile was derived from all of the wells (all mosquitoes in each treatment). Motility was assessed prior to the addition of haemolymph, and at subsequent time intervals during incubation.

Trial A was designed for 6 groups as follows:

i. TC199 and Hepes medium;

ii. Untreated haemolymph;

iii. TC199 control haemolymph withdrawn on day 4 PI;

iv. 10 microfilariae immunized haemolymph withdrawn on day 4 PI;

v. $1x10^4$cells/μl E. coli immunized haemolymph withdrawn on day 1 PI;

vi. 25 μg/ml lysozyme solution.

Trial A.I involved the incubation of approximately 100 microfilariae in 1 μl of TC199 per well and there were two replicates. Trial A.II involved incubation of approximately 50 microfilariae in 1 μl of TC199 per well and there were three replicates.

Trial B was designed for 6 groups as follows:

i. TC199 control haemolymph withdrawn on day 1 PI;

ii. Untreated haemolymph;

iii. 10 microfilariae immunized haemolymph;

iv. Laminarin injected haemolymph;

v. LPS injected haemolymph;

vi. Dextran injected haemolymph.

4.2.3 Experiment III. Motility assay attenuation of toxicity test of laminarin, LPS or dextran solution to microfilariae.

Experiment III was designed to test the toxicity of laminarin, LPS and dextran solution and their attenuation of microfilaria motility. The trial was designed as follows:

Trial A

i. TC199 and Hepes medium;

ii. Laminarin solution;

iii. LPS solution;

iv. Dextran solution.

Trial B

i. Hayes saline;

ii. Laminarin solution;

iii. LPS solution;

iv. Dextran solution.

In Trial A. 2 μl of 1 mg/ml laminarin, 100 μg/ml LPS, 1 mg/ml dextran solution or TC199 medium were added to each well and an equal volume of living microfilariae in a suspension of TC199 and Hepes was added to each well giving a final volume of approximately 4 μl. In Trial B. 3 μl of laminarin, LPS, dextran or Hayes saline were added to each well and 1 μl of living microfilariae in a suspension of TC199 and Hepes was added. The plates were then placed in a humid chamber and incubated at 27°C.

4.3 RESULTS

PROTEIN ASSAY

The data presented in Table 20 and Figs. 15-16 show the results of protein assays of haemolymph cell-free proteins in the treated and untreated mosquitoes. The haemolymph protein concentration in untreated control mosquitoes rose steadily to reach a value some 64-106 % higher by day 5. The protein level in the group inoculated with live microfilariae rose by almost 3 fold over the first day and remained at that level until day 5, but thereafter decreased to the level found in the untreated control. The response to heat-killed microfilariae was broadly similar. The protein level in the bacteria inoculated group showed a 4 fold increase over the first 3 days but thereafter declined to a value some 2 fold higher than that of the untreated control. The response to heat-killed bacteria inoculated was roughly similar. The TC199 control group produced an immediate increase of around 3 fold on day 1 but thereafter declined to reach the some level as untreated control by day 7. The result with dextran was essentially similar to that of the TC199 control group up to day 5. The laminarin and LPS treated groups produced very similar results, with a steady rise over 4 days to a value 5 fold greater than in untreated control, although subsequently declining.

The results demonstrated that although both TC199 and dextran can induce increased haemolymph proteins in the mosquitoes especially on day 1 PI, treatment with microfilariae, bacteria laminarin and LPS induces a much higher level of protein. Furthermore, the response is similar whether living or dead organisms are use.

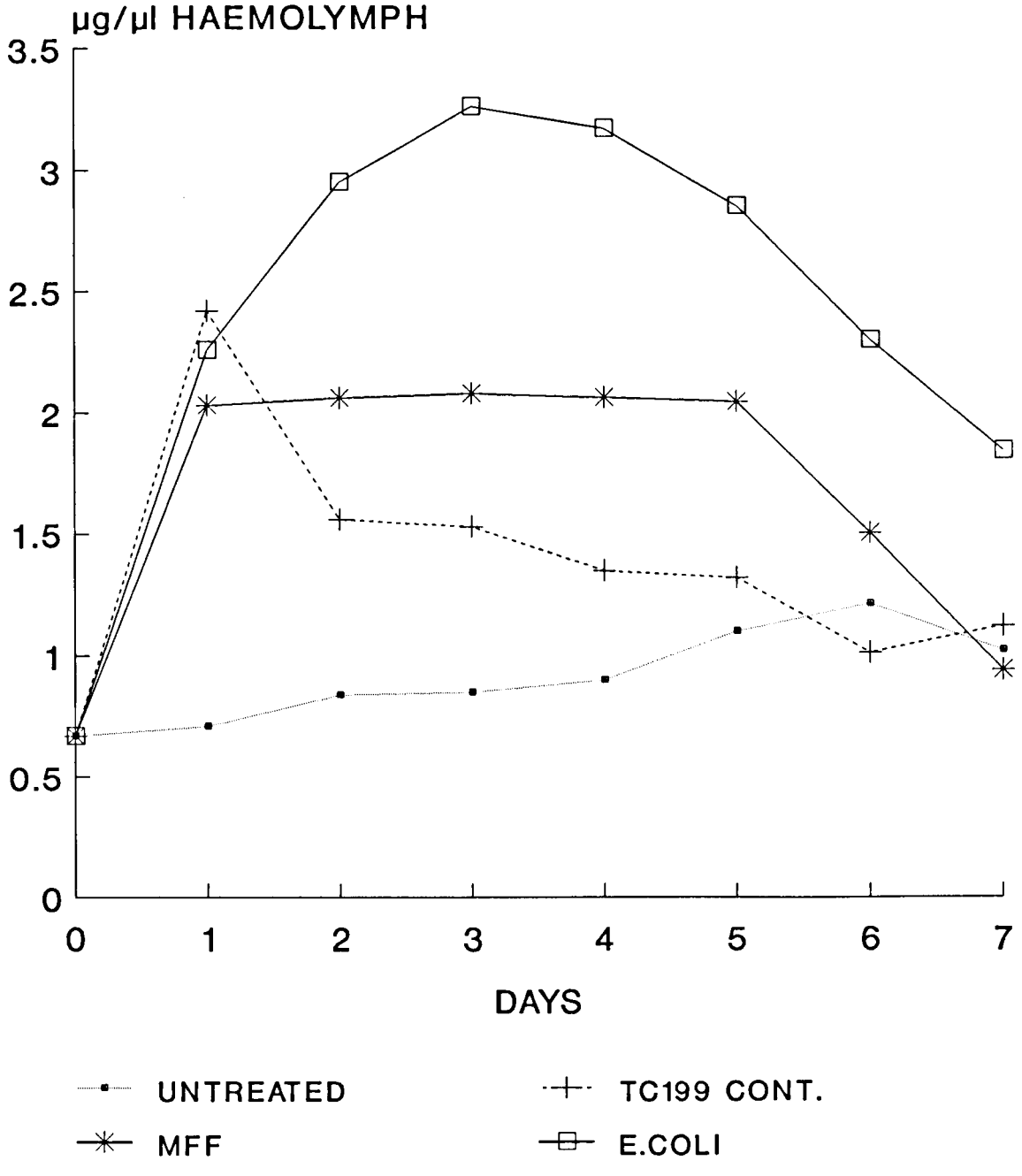
TABLE 20. PROTEIN ASSAY OF HAEMOLYMPH CELL FREE PROTEIN FROM MICROFILARIAE, BACTERIA, LAMINARIN, LPS, DEXTRAN AND SHAM TC199 TREATED MOSQUITOES AND FROM UNTREATED MOSQUITOES AT VARIOUS TIMES POSTINJECTION.

Treatments	Protein concentration ($\mu g/\mu l$)							
	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
Trial A								
Untreated	0.67	0.71	0.84	0.85	0.90	1.10	1.21	1.02
TC199	0.67	2.42	1.56	1.53	1.35	1.32	1.01	1.12
Mff.	0.67	2.03	2.06	2.08	2.06	2.04	1.50	0.94
Bacteria	0.67	2.26	2.95	3.26	3.17	2.85	2.30	1.84
Trial B								
Untreated	0.57	0.67	0.90	1.02	1.07	1.17	-	-
TC199	0.57	2.56	1.96	2.19	1.76	1.63	-	-
Mff	0.57	2.13	2.18	2.41	2.35	2.22	-	-
HK.Mff	0.57	2.24	2.29	2.30	2.33	2.27	-	-
L.E. coli	0.57	2.74	3.31	3.40	3.28	3.03	-	-
HK.E. coli	0.57	2.77	3.19	3.37	3.20	2.88	-	-
Laminarin	0.57	2.36	2.67	2.92	2.83	2.41	-	-
LPS	0.57	3.23	2.38	2.71	2.88	2.35	-	-
Dextran	0.57	2.75	2.34	2.05	1.81	1.55	-	-

Mff = 10 living microfilariae inoculated haemolymph
HK.Mff = 10 Heat-killed microfilariae inoculated haemolymph
L.E. coli = $1x10^4$cells/μl E.coli injected haemolymph
HK.E. coli = Heat-killed $1x10^4$cells/μl E. coli injected haemolymph

FIG.15. Haemolymph protein assay of mosquitoes inoculated with microfilariae, bacteria and TC199 treated mosquitoes and from untreated mosquitoes at various times postinoculation in Trial A.

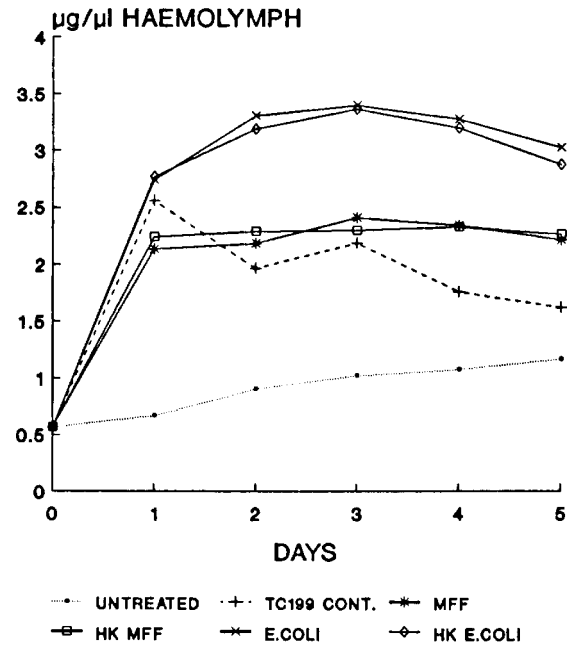
PROTEIN ASSAY



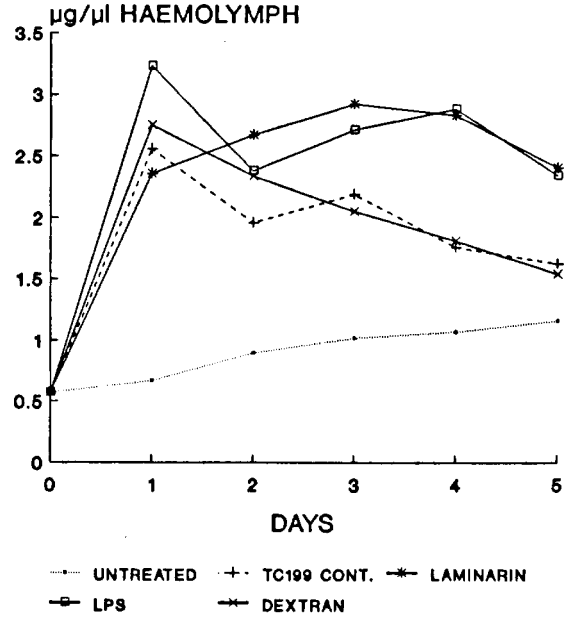
TRIAL A

FIG.16. Haemolymph protein assay of mosquitoes inoculated with living and heat-killed microfilariae, living and heat-killed bacteria, laminarin, LPS, dextran, TC199 and from untreated mosquitoes at various times postinoculation in Trial B.

PROTEIN ASSAY



TRIAL B



TRIAL B

MOTILITY ASSAY

The results of Trial A.I (2 replicates) and Trial A.II (3 replicates) are given in Figs. 17-21 , in which the motility of fresh microfilariae incubated with haemolymph withdrawn from mosquitoes inoculated with microfilariae, bacteria and TC199 control is compared with that of microfilariae incubated in TC199 medium, haemolymph from untreated mosquitoes and lysozyme solution. The results of a two way analysis of variance on the data from Trial A.I are given in the Table 21. It was not possible to account for the non-linear regression of motility against time because the significance levels quoted under estimate the significance difference between groups. However they do demonstrate comparison differences at the 5 percent significant levels. The results demonstrate that the addition of TC199 to haemolymph from treated mosquitoes usually produces a slightly enhanced motility of the exposed microfilariae. The single exception to this (Trial A.II, Fig.18) may be the result of undiagnosed infection in the mosquitoes from which the haemolymph was derived. However, the motility level of microfilariae exposed to the haemolymph from sham TC199-inoculated control groups is consistently less than that of microfilariae exposed to the haemolymph of untreated mosquitoes. The motility levels of microfilaria exposed to the haemolymph of microfilariae-inoculated mosquitoes and bacteria-inoculated mosquitoes are very similar to each other, being less than those of microfilariae exposed to the haemolymph from sham TC199-inoculated controls. The reduction in motility of microfilariae exposed to haemolymph microfilariae- or bacteria-inoculated mosquitoes compared to those exposed to the haemolymph from sham TC199-inoculated controls was range of 31-39% by 48 hrs and 21-72% by 72 hrs (Trial A.II,Rep I,II,III), after

which all the microfilariae were dead or showed decreased activity. Furthermore, the motility levels of microfilariae exposed to lysozyme solution are less than that of microfilariae exposed to haemolymph from sham TC199-inoculated controls, untreated mosquitoes control, microfilariae inoculated mosquitoes, bacteria inoculated mosquitoes and TC199 medium without haemolymph. The reduction in motility level of microfilariae exposed to the lysozyme solution steadily declined to zero by 24 hrs (Trial A.II, Fig. 20), 48 hrs (Trial A.I, Fig. 18; Trial A.II, Figs 19 and 21), and by 72 hrs (Trial A.I, Fig 17). A preliminary test of attenuation of microfilariae motility at different concentration of lysozyme (results not shown) showed highly variable results. Similarly the 25 μg/ml of lysozyme solution used in Trial A (Fig. 17) had a smaller effect on microfilariae mortality than the same concentration in other trials. However, the results clearly demonstrate that at 25 μg/ml(final concentration = 0.0063 μg/μl) lysozyme has an effect on microfilariae <u>in vitro</u> , the lowest concentration at which an effect was detected was 15 μg/ml (final concentration = 0.005 μg/μl)(results not shown).

Trial B, is presented in Fig. 22. The motility level of microfilariae exposed to the haemolymph of sham TC199-inoculated control groups is similar to those of dextran injected mosquitoes and was consistently less than that of microfilariae exposed to the haemolymph from untreated mosquitoes. The motility level of microfilariae exposed to the haemolymph from microfilariae inoculated mosquitoes, are similar to those of the motility of microfilariae exposed to the haemolymph of laminarin and LPS injected mosquitoes, being significantly less than those of microfilariae exposed to the haemolymph from sham TC199-inoculated control and dextran-injected mosquitoes. The reduction

in motility level of microfilariae exposed to the haemolymph of microfilariae inoculated mosquitoes, laminarin or LPS injected mosquitoes compared to the microfilariae exposed to the haemolymph from sham TC199-inoculated control and dextran injected mosquitoes was 35% by 24 hrs to 32- 58% by 72 hrs, after which all microfilariae were dead.

The slight differences between the results for Trial A and Trial B may be due to haemolymph in Trial A, the TC199 controls was withdrawn on day 4 PI, when the protein level had decreased to a value close to that of untreated controls. On the other hand the haemolymph of Trial B was withdrawn on day 1 PI when the haemolymph protein level was much higher than that of untreated mosquitoes. The protein level induced by the bacteria were higher than those induced by microfilariae, but the effect of the immunized haemolymph of bacteria inoculated mosquitoes on the motility rates was similar to that of microfilariae inoculated mosquitoes. Also the motility level of microfilariae exposed to the haemolymph from laminarin and LPS injected mosquitoes are similar; this result may be compared to the protein levels in the haemolymph of laminarin and LPS injected mosquitoes which rose in a same manner. Furthermore, the motility of microfilariae exposed to the haemolymph of sham TC199-inoculated controls and dextran injected groups both showed the similiar pattern.

The results of Trial C are presented in Fig. 23. The motility level of microfilariae exposed to TC199 medium is similar to the motility level of microfilariae exposed to the laminarin, LPS and dextran solutions by 48 hrs. Subsequently, the motility level of microfilariae exposed to TC199 medium rapidly declined but remained higher than that of microfilariae exposed to

laminarin, LPS and dextran solution 53% at 96 hrs. Furthermore, the motility level of microfilariae exposed to Hayes saline solution is similar to the laminarin, LPS and dextran solutions at 24 hrs.

These results demonstrate that the laminarin, LPS and dextran solutions have no adverse effect on the motility rates of microfilariae in the cultures. They show the same motility levels in TC199 medium and Hayes saline by 48 hrs. The motility level of microfilariae in TC199 and Hayes saline show higher levels from those in laminarin, LPS and dextran by 72 to 96 hrs. However these may relate to the nutritional effects on microfilariae survival rather than directly to toxic effects . Furthermore, the different concentrations of laminarin, LPS and dextran solutions in the cultures (50% in Experiment C.1 and 75% in Experiment C.2, Trial C) showed no corresponding effect on the microfilariae motility.

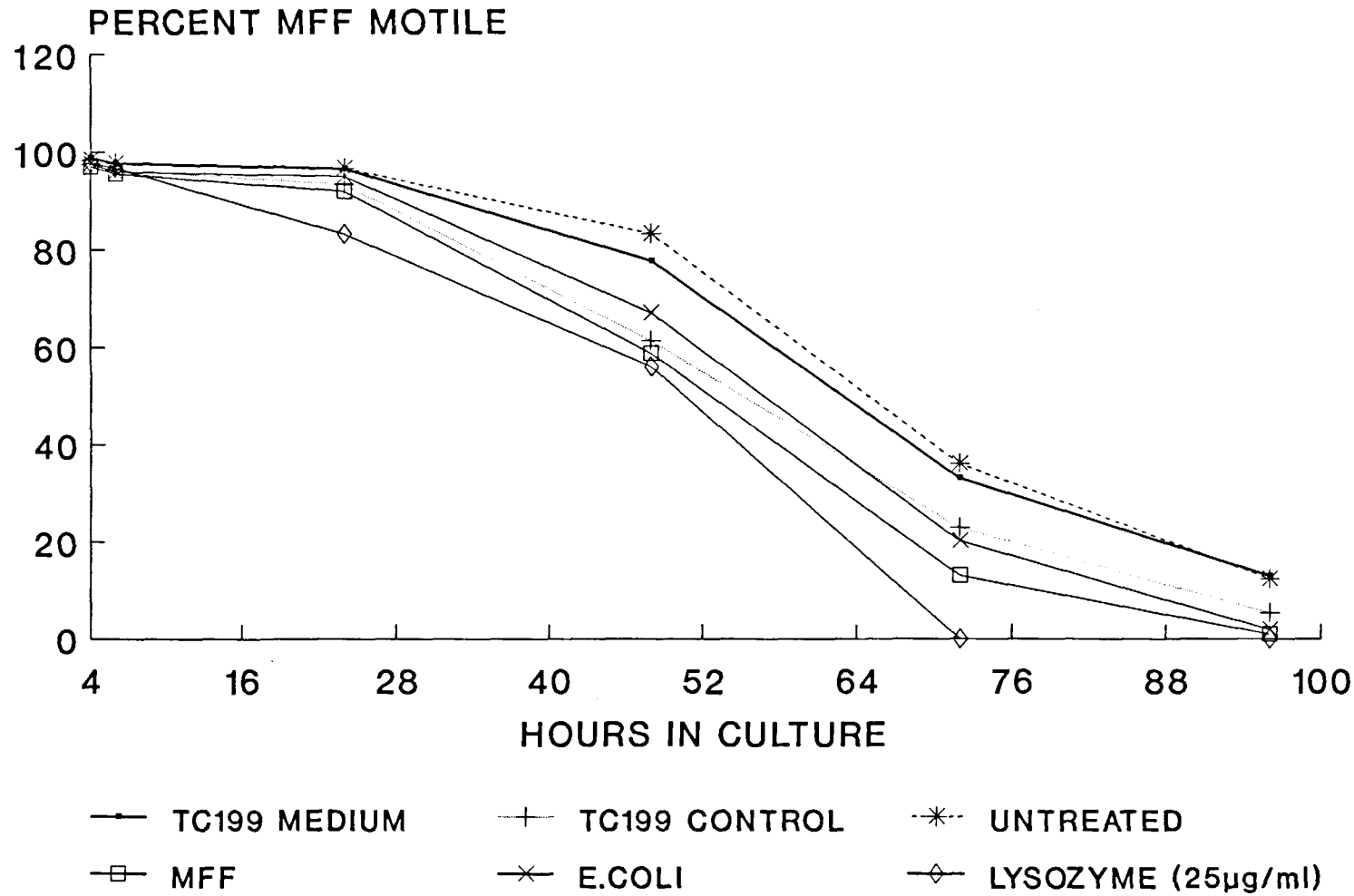
TABLE 21. STATISTICAL ANALYSIS OF VARIANCE (TWO WAY) OF THE MEAN PERCENT MOTILITY OF BRUGIA PAHANGI MICROFILARIAE DURING MAINTENANCE IN INDIVIDUAL WELLS (12 PER GROUP) OF TERISAKI PLATES, EACH CONTAINING HAEMOLYMPH FROM MICROFILARIAE, BACTERIA AND SHAM TC199 TREATED MOSQUITOES COMPARED WITH TC199 MEDIUM, UNTREATD HAEMOLYMPH AND 25 µg/ml LYSOZYME SOLUTION (2 REPLICATES).

Treayments	untreated	Test against TC199 Medium	Mff	Bacteria	Lysozyme
TC199 Cont.	NS	P<0.025	P<0.010	P<0.010	P<0.010
Untreated	-	P<0.025	P<0.005	P<0.005	P<0.010
TC199 Medium	-	-	P<0.010	P<0.025	P<0.010
Mff	-	-	-	P<0.050	P<0.025
Bacteria	-	-	-	-	P<0.025

Mff = 10 living microfilariae immunized haemolymph
Bacteria = $1x10^4$ cells/µl E. coli immunized haemolymph
NS = Non significant

FIG.17. Experiment II, Trial A.I(Rep.I): The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in the haemolymph from mosquitoes inoculated with microfilariae, bacteria and TC199 4 days previously, compared with those for microfilariae incubated in haemolymph from untreated mosquitoes, or in TC199 medium or in 25 μg/ml lysozyme solution.

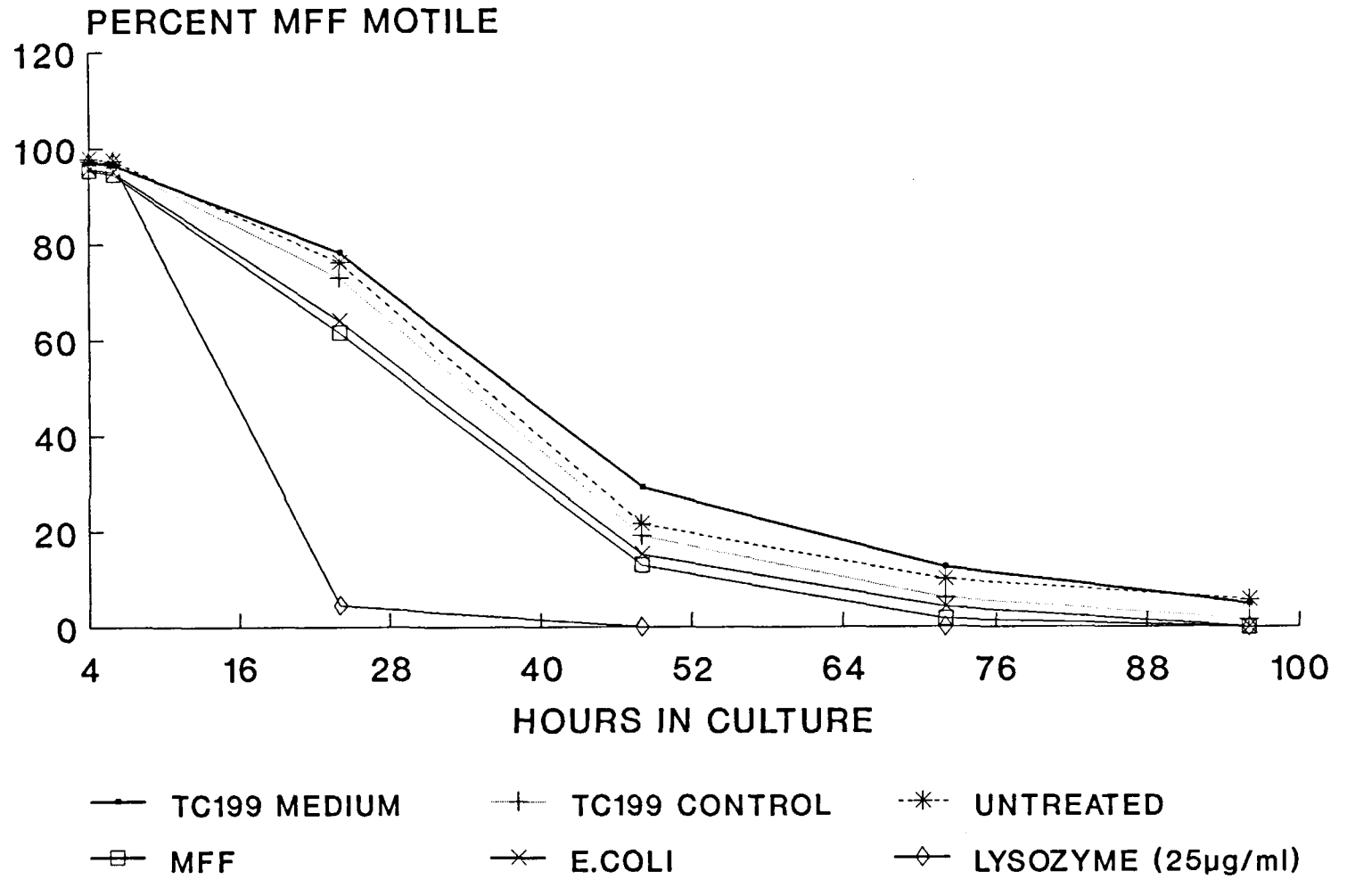
MOTILITY ASSAY



Trial A.I.R.I

FIG.18. Experiment II, Trial A.I (Rep.II): The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in the haemolymph from mosquitoes inoculated with microfilariae, bacteria and TC199 4 days previously, compared with those for microfilariae incubated in haemolymph from untreated mosquitoes, or in TC199 medium or in 25 μg/ml lysozyme solution.

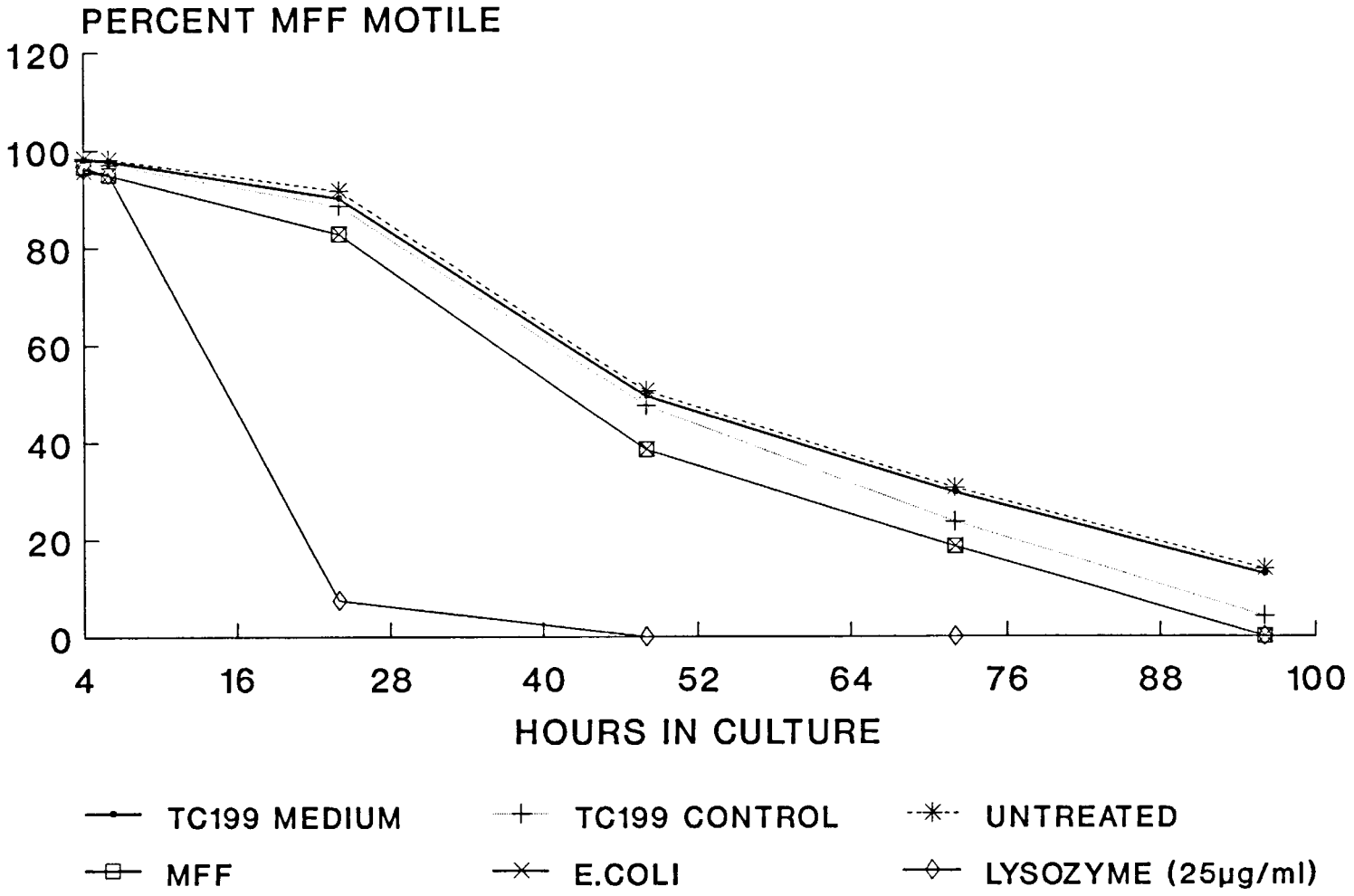
MOTILITY ASSAY



TRIAL A.I.R.II

FIG.19. Experiment II,Trial A.II (Rep.I) : The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in the haemolymph from mosquitoes inoculated with microfilariae, bacteria 4 days previously and TC199 24 hours earlier, compared with those for microfilariae incubated in haemolymph from untreated mosquitoes or in TC199 medium or in 25 μg/ml lysozyme solution.

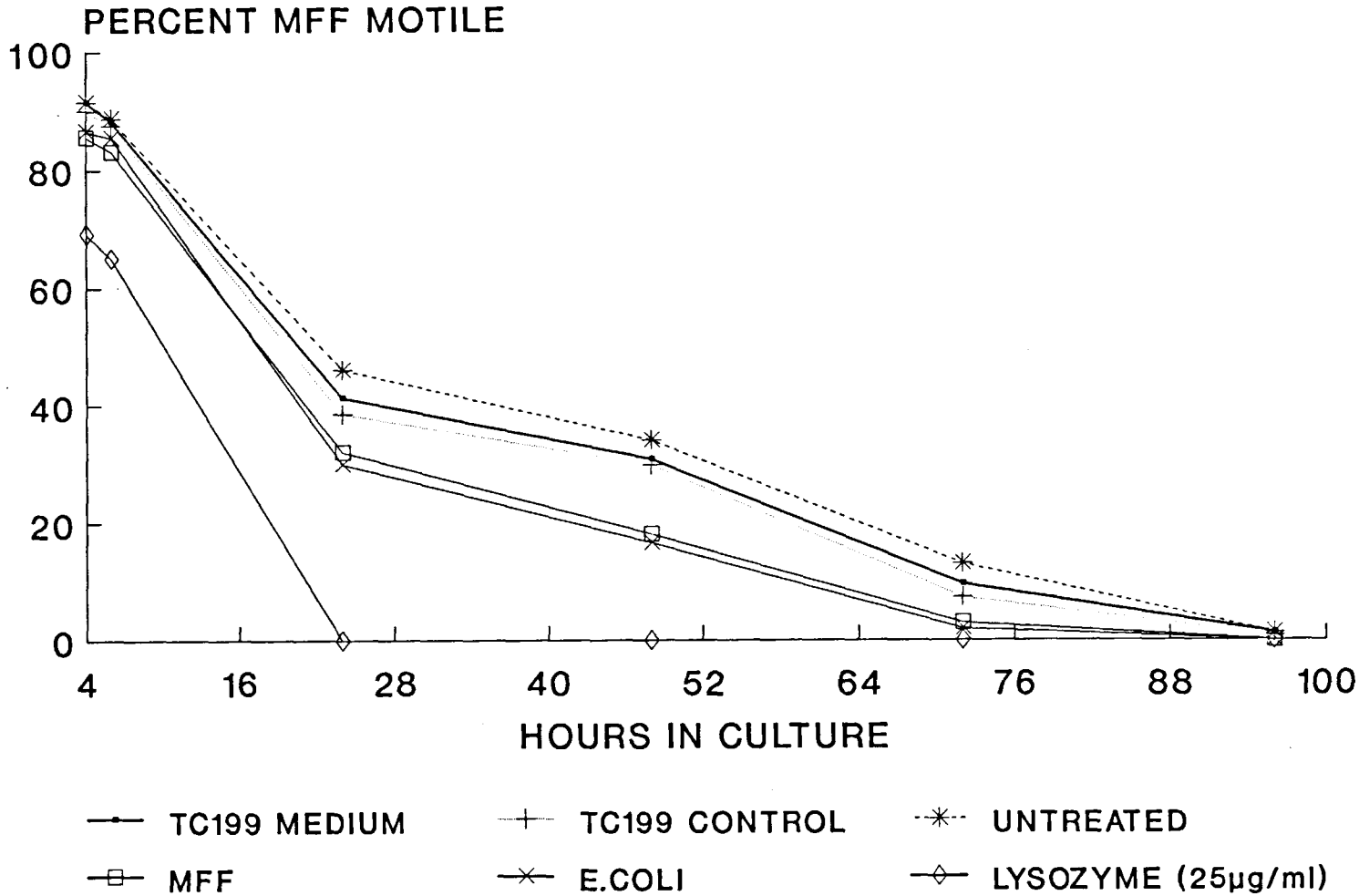
MOTILITY ASSAY



TRIAL A.II.R.I

FIG.20. Experiment II, Trial A.II (Rep.II) : The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in the haemolymph from mosquitoes inoculated with microfilariae, bacteria 4 days previously and TC199 24 hours earlier, compared with those for microfilariae incubated in haemolymph from untreated mosquitoes or in TC199 medium or in 25 μg/ml lysozyme solution.

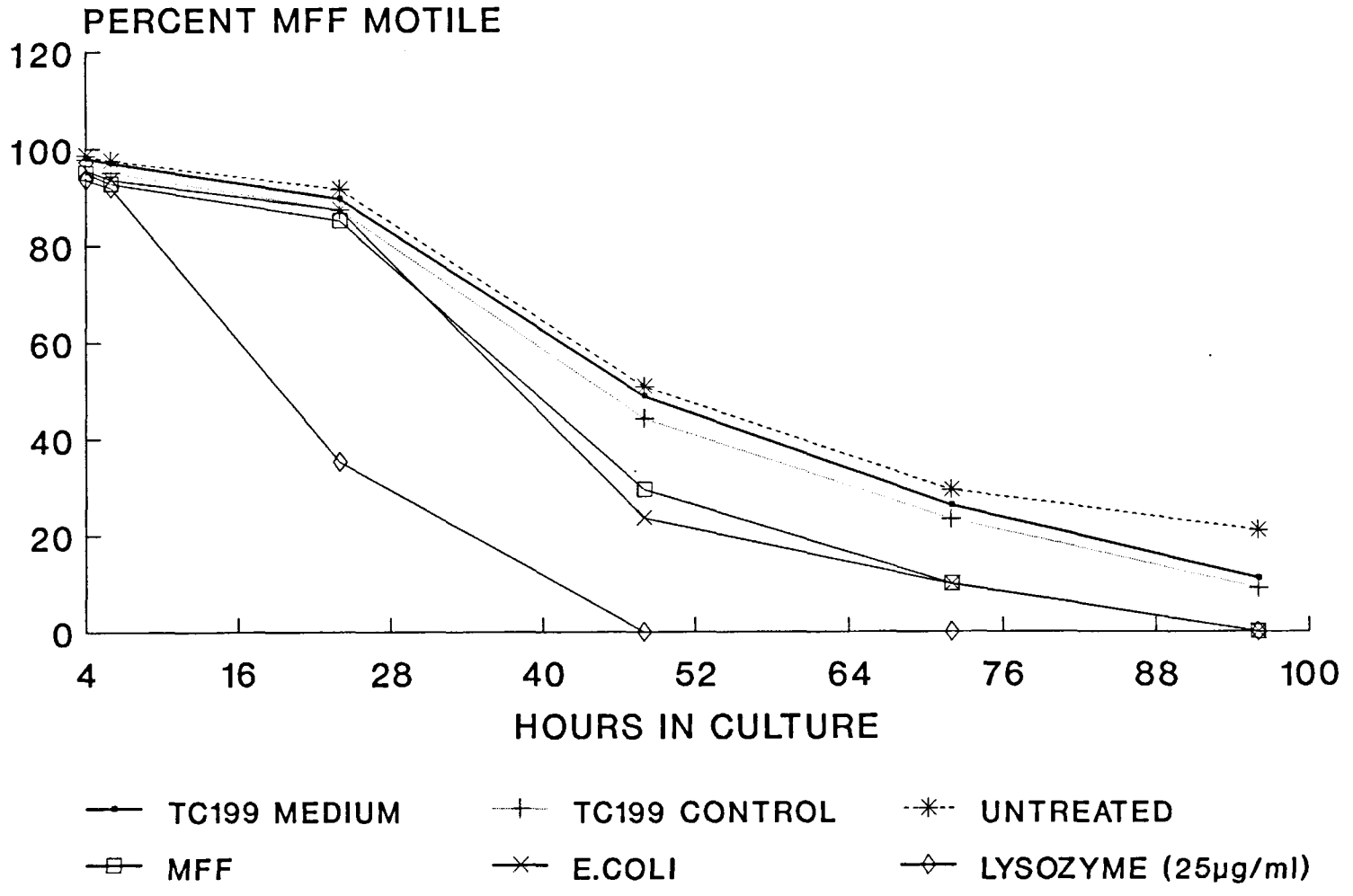
MOTILITY ASSAY



TRIAL A.II.Rep.II Fig.20

FIG.21. Experiment II, Trial A.II (Rep.III) : The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in the haemolymph from mosquitoes inoculated with microfilariae, bacteria 4 days previously and TC199 24 hours earlier, compared with those for microfilariae incubated in haemolymph from untreated mosquitoes, or in TC199 medium or in 25 μg/ml lysozyme solution.

MOTILITY ASSAY



TRIAL A.II.R.III Fig.21

FIG.22. Experiment II, Trial B : The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in the haemolymph from mosquitoes inoculated with microfilariae 4 days previously, and from laminarin, LPS, dextran and TC199 injected mosquitoes 24 hours beforehand, compared with microfilariae incubated in haemolymph from untreated mosquitoes.

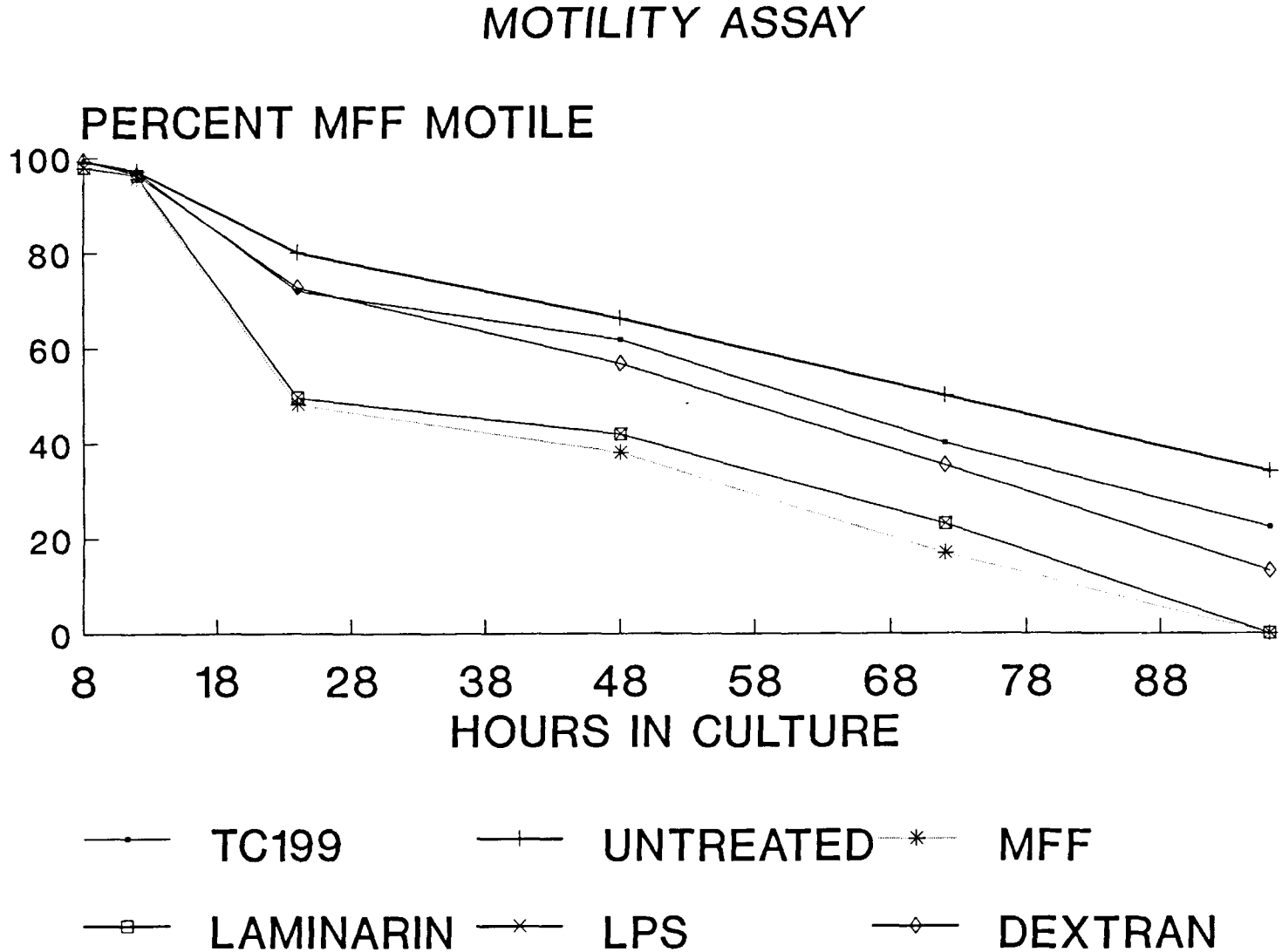
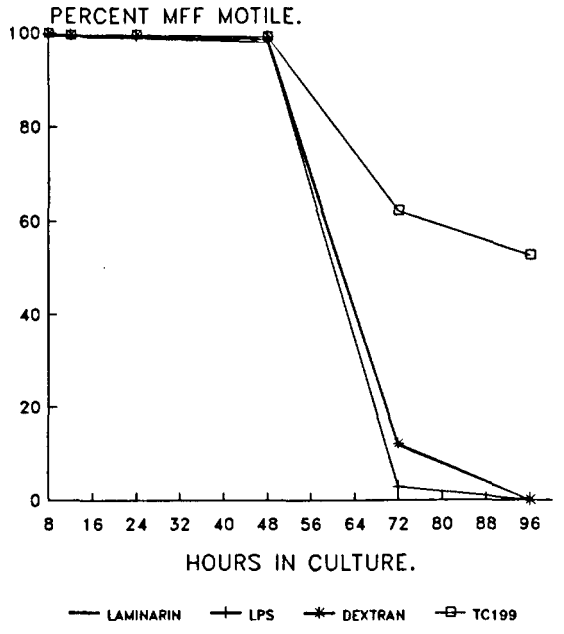
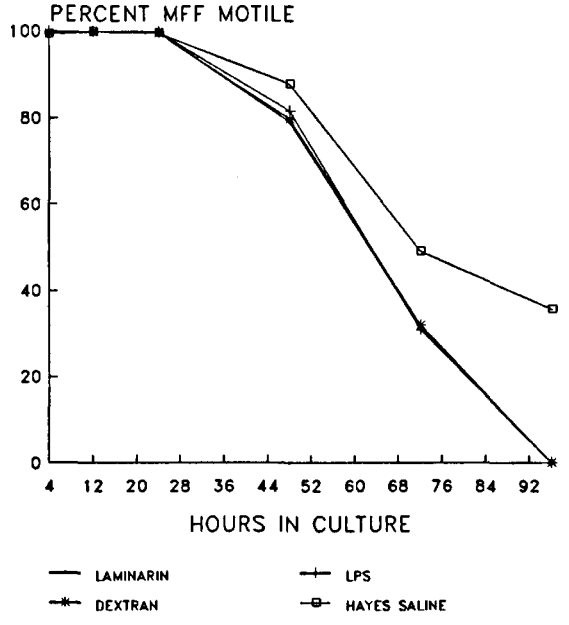

MOTILITY ASSAY
PERCENT MFF MOTILE
100
80
60
40
20
0
8
18
28
38
48
58
68
78
88
HOURS IN CULTURE
TC199
UNTREATED
MFF
LAMINARIN
LPS
DEXTRAN

FIG.22

FIG.23. Experiment III, Trial A and B : The motility rates of fresh Brugia pahangi microfilariae during in vitro culture in 1 mg/ml laminarin, 100 μg/ml LPS, 100 μg/ml dextran solution and TC199 medium or Hayes saline solution.



Toxicity Test Experiment C.1



Toxicity Test Experiment C.2

4.4 DISCUSSION

The results of this study show that not only does immune haemolymph have a filaricidal effect in vivo but it also can kill microfilariae in vitro. In related work Ham et al., (1988) observed that haemolymph factors in simuliids infected with O. lienalis are sufficiently non-specific to kill B. pahangi microfilariae in vitro.

The slight differences between the results of Experiment II, Trial A and B may be correlated with the high level of proteins in the haemolymph 24 hrs PI(Trial B) and lower level on day 4 (Trial A). These results are supported by Komano et al. (1980) who demonstrated respectively that lectins induced in the haemolymph of S. peregrina larvae on injury and sham injection with sterile salt solution led to a related injury response with nearly similar total protein synthesis but with only low bactericidal activity. Boman et al.(1981) demonstrated that lysozyme induced in the haemolymph of H. cecropia on injury and sham injection response with nearly similar to total protein synthesis and has low bactericidal activity.

The identity of the factor in the haemolymph which leads to lower microfilarial motility is uncertain. It may be lysozyme (Powning and Davidson, 1973; Jolles et al., 1979; Dunn et al., 1987), which is known to have many sites that can bind with antibody and act as a proteolytic enzyme (Boman and Hultmark, 1987).

Although, the protein levels induced by the bacteria were higher than those induced by the sham control and by microfilariae, the effect of the bacteria-injected haemolymph on the motility rate was similar to that of

haemolymph from microfilariae inoculated mosquitoes. There can be little doubt that the death of microfilariae, both in vivo and in vitro, was the result of induced immunity factors, but the mode of action of the unknown factors is not yet understood. In field conditions bacterial cells may reach the mosquito haemocoele through the epithelium of the midgut when the microfilariae migrate through the gut wall to the haemocoele. Since the mosquitoes produce an immune response to the bacterial invaders, this may have an effect on the nematode parasites. Such a phenomenon may assist the control of the intensity of parasite infection in the vector.

The immunity induced by laminarin and LPS had a partial effect on killing microfilariae in vitro. The bacteria cell wall (LPS) has been reported to activate prophenoloxidase in plasma of M. sexta (Saul and Sugumaran, 1987). Several authors have reported that prophenoloxidase is variably activated by microbial components, so that whilst laminarin, a ß-1,3-glucan is a strong elicitor (Ashida, 1981; Ratcliffe et al., 1984; Dularay and Lackie, 1985; Leonard et al., 1985a,b.), glucose polymers with an α-1,6- linkage glucan dextran are not (Ratcliffe et al., 1984). Whereas laminarin and LPS produced an immune response in this study, the non-reactivity towards dextran indicated that there is some degree of specificity in the immune response mechanisms.

Untreated haemolymph is a rich medium for microfilaria survival and development, and it showed a higher motility level compared with TC199 medium or TC199-inoculated controls. Horohov and Dunn (1982) reported that the haemolymph of non-immunized insects is not bactericidal and is a rich medium for growth of bacteria.

Insect haemolymph contains lectins, lysins and complement-like substances (Lackie, 1980) and displays opsonic activity against natural pathogens (Ratcliffe and Rowley, 1984). Lectins are widely reported in insects and they appear to constitute part of the insect defence mechanism (Komano et al., 1980,1981, 1983; Ibrahim et al., 1984; Ingram et al., 1984; Minnick et al., 1986; Castro et al., 1987; Stiles et al., 1988; Shiraishi and Natori, 1989; Richards et al., 1989). Ingram et al. (1984) demonstrated the action of lectins against trypanosomatid flagellates in the presence of cell free haemolymph of the locust, Sch. gregaria, and the cockroach, P. americana, in vitro. In the same insects lectins were induced by LPS and laminarin (Gunnarsson and Lackie, 1985). Ibrahim et al. (1984) also reported the presence of lectins in the haemolymph of G. austeni. Ham et al. (1988) showed that lectins may act on the developing stages of filarial parasites in simuliid fly haemolymph in vitro.

It seems likely from the results obtained in the present study that at least 2 humoral mechanisms are effective in mosquitoes. First, there may be a non-specific de novo synthesis of lytic protein (Faye et al.,1975; Jolles et al.,1979; Dunn et al., 1987), which could be fast acting, and second, an antibacterial response, which may act on the developing stage of filariae and be more specific in nature.

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CHAPTER 5

SDS-PAGE STUDIES OF THE HAEMOLYMPH PROTEIN OF AEDES AEGYPTI MOSQUITOES INDUCED BY INOCULATION OF BRUGIA PAHANGI MICROFILARIAE, ESCHERICHIA COLI AND THE CELL WALL COMPONENTS LAMINARIN, LPS AND DEXTRAN

5.1 INTRODUCTION

Several insects have been shown to have immune response to protect themselves from the invasion of various pathogenic microorganisms (Boman et al., 1974) and parasites (Hurd and Arme, 1984). The humoral system is particularly interesting because it can be induced by inoculation of certain dead or live organisms (Boman et al., 1981) and by helminthic parasites (Hurd and Arme, 1984). This reaction is thought to be a defence reaction to prevent invasion of various bacteria and parasites.

The haemolymph bactericidal factors that killed gram-negative bacteria were first discovered by Stephens (1962) and Hink & Briggs (1968) in larvae of G. mellonella. The haemolymph immune protein isolated and characterized from larvae of G. mellonella and the silkmoth B. mori was lysozyme (Powning and Davidson, 1973, 1976). Lysozyme has also been reported in other insect species (Mohrig and Messner, 1968; Hoffmann and Brehelin, 1976). The best defined antibacterial activity comes from work on the pupae of H. cecropia (Fay et al., 1975; Boman and Steiner, 1981; Boman and Hultmark, 1981). This activity was described as due to cecropins (Boman and Hultmark, 1981) and attacins (Hultmark et al., 1983) which have molecular weights around 4,000 and 20,000-23,000 respectively.

Natori (1977) reported that the bactericidal activity induced by injury in the haemolymph of S. peregrina larvae was due to a protein with a molecular weight less than 10,000 KDa. Okada and Natori (1983; 1984) described this bactericidal protein and referred to it as sarcotoxin I. It was found to have a

molecular weight of approximately 5,000. Subsequently, (Okada and Natori, 1985) they reported that sarcotoxin consists of three structurally related proteins termed sarcotoxin IA, IB and IC. Ando et al. (1987) purified sarcotoxin II, and showed that it consists of three structurally related proteins which they named sarcotoxin IIA, IIB and IIC.

Comparable immune responses have also been found in several flies (Okada and Natori, 1985; Keppi et al., 1986; Flyg et al., 1987; Kaaya et al., 1987) and in beetles (Spies et al., 1986a). An inducible antibacterial protein was detected in and purified from larvae of the fly P. terranovae; it was described as diptericin (Keppi et al., 1986; Dimarcq et al., 1988). Casteels et al. (1989) demonstrated an inducible antibacterial protein of molecular weight 2.1 kDa in the honey bee, which they called apidaecin.

Komano et al. (1980) purified a lectin from the haemolymph of S. peregrina larvae, which was obtained after injury to their body wall. This lectin was found to have molecular weight of 190,000 and to consist of four α-subunits and two ß-subunits, with molecular weights of 32,000 and 30,000 respectively. Minnick et al. (1986) also purified a lectin from M. sexta haemolymph; this lectin was found to have a molecular weight of approximately 70 kDa and was comprised of 2 polypeptides of 36 kDa. Castro et al. (1987) isolated and characterized the lectin from the haemolymph of H. cecropia larvae and pupae; the molecular weight of this lectin was approximately 160,000 and subunits were 41 kDa and 36 kDa.

Södehall et al. (1988) purified the prophenoloxidase plasma protein from B. craniifer induced by laminarin; this protein was found to have a molecular weight around 63 kDa, and 52 kDa under reducing conditions. Tsukamoto et al.

(1986) demonstrated latent phenoloxidase in prepupae of the housefly, M. domestica, and estimated it to be 178 KDa as determined by gel filtration and sucrose density gradient centrifugation. Furthermore, when latent phenoloxidase was activated a higher molecular weight protein of 340 kDa was recorded.

The present study was planned to examine the induction of haemolymph proteins by immunizing Ae. aegypti with B. pahangi microfilariae and E. coli HB.101, and following inoculation of cell wall components laminarin, LPS and dextran.

5.2 MATERIALS AND METHODS

5.2.1 Mosquitoes protein control

The head, thorax and abdomen of 5 virgin female mosquitoes were homogenized with 500 µl phosphate buffer saline at pH 7.2. The homogenized protein solution was centrifuged at 4000g for 10 min at 4°C (Maniatis et al., 1986). The supernatant was separated and the pellet discarded. The protein concentration was determined using the method of Bradford (1976) with bovine serum albumin, Bio-Rad protein standard and dye reagents. The protein samples were diluted to give 3-5 µg/µl (Okada and Natori, 1983). The mosquito protein samples were put in plastic vials and stored at -70°C.

5.2.2 Bacteria protein control

The culture of E. coli was used at the midlog phase of growth, that is when OD.600 = 0.3 5 ml of the culture was centrifuged at 4000g for 10 minutes at 4°C. The supernatant was drained off as much as possible , any remaining medium being removed with a pasteur pipette. Sonication buffer was added, the bacterial culture suspended, and then the suspension centrifuged at 4000g for 10 minutes at 4°C. The supernatant was then discarded, and 1.6 ml of sonication buffer was added to resuspend the pellet thoroughly. The resulting homogeneous suspension was transferred to a small clean plastic tube. Sonication was applied in short bursts at maximum power (150 Watts) using a microtip probe. The tube was immersed in ice-water and the temperature of the sonication buffer was not allowed to exceed 4°C. Sonication was continued

until the solution cleared. The debris was removed by centrifugation at 12,000g for 10 minutes at 4°C. Approximately 1.5 ml of the supernatant was added to an equal volume of cold sonication buffer and 1/6 volume of freshly prepared packing buffer. 15 µl aliquots were dispensed into precooled (4°C), 1.5 ml plastic vials. The caps of the vials were immediately closed and after a brief immersion in liquid nitrogen the vials were transferred to -70°C for long term storage (Mamiatis et al., 1986). The bacterial protein concentration was determined using the method of Bradford (1976) with Bio-Rad standard protein and dye reagents. The bacterial protein concentration was diluted to 3-5 µg/µl with sonicate buffer (Okada and Natori, 1983).

5.2.3. Immunization of mosquitoes with cell wall components

The mosquitoes were anesthetized with carbond dioxide and injected with 1 µl of 1 mg/ml laminarin, 100 µg/ml LPS or 1 mg/ml dextran each solution containing 100 mg/ml streptomycin sulphate.

The mosquitoes were inoculated with living or heat-killed microfilariae, living or heat-killed bacteria have previously described. The haemolymph were withdrawn from the mosquitoes inoculated with living or heat-killed bacteria at 10 hrs to day 4 PI, inoculated with living or heat-killed microfilariae on day 1 to day 4 PI, and injected with laminarin, LPS, dextran or TC199 on day 1 to day 4 PI respectivly

5.2.4 Electrophoresis techniques

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for separating and identifying the proteins in the haemolymph

withdrawn from the treated mosquitoes. SDS-PAGE was performed with 3-12.5% and 5-15% acrylamide slab gels in a discontinuous buffer system according to the method of Laemmli (1970). To the protein samples were added 4 times as much solubilizer cocktail mixture containing 2% SDS and 0.1M dethiothreital (Sigma), and the mixtures were immediately boiled at 100°C for 3-5 minutes in a water bath. The samples were then applied to SDS-PAGE slab gels and electrophoresis was performed at 30 mA., maximum voltage, using pH 8.3 running buffer for approximately 4 hours until the tracking dye bromphenol blue was leaving the gel.

The gel was then fixed in 50% methanol and 12% acetic acid and stained for protein with silver stain (Bio-Rad) (Table 1). The apparent molecular weight of the proteins was determined by comparison with the electrophoretic mobility of known protein standard markers (BDH) in the range 78 to 12.3 kDa (Marker I) and 16.9 to 2.5 kDa (Marker II), using standard curves and correlation coefficients.

5.3 RESULTS

The major proteins, and their molecular weights, in haemolymph withdrawn from the mosquitoes previously inoculated with microfilariae, bacteria, laminarin, LPS dextran and TC199 control, are presented in Tables 22, 23 and 24. The haemolymph protein-banding patterns of treated and untreated mosquitoes are shown in Figs. 24 to 31. A total of 25 major proteins are easily detected in the photographs of the gels and these have been designated and their molecular weight mobility determined for discussion in this study.

The five protein bands which were detected only in the microfilariae-infected haemolymph, the bacteria-infected, and the laminarin and LPS injected haemolymph are presented in Table 22. The molecular weights of four proteins presented following injection in all treatments were 66 kDa, 24 kDa, 8 kDa and 4 kDa respectively. The fifth protein, of 42 kDa, was present only in mosquitoes inoculated with microfilariae and bacteria treatments and not by the others.

Another 15 protein bands which were present only in the haemolymph of mosquitoes infected with microfilariae or bacteria or laminarin, LPS or dextran are shown in Table 23 and Figs. 24 to 31. These bands were not present in all individual mosquitoes within any treatment groups.

Of the remaining five bands, three, 28 kDa, 17 kDa and 12 kDa were present in all treatments and in the untreated controls, and two, 22 kDa and 20 kDa, were sometimes present in TC199 control and in the other treatments, but not in the untreated controls (Table 24).

Protein bands of 28 kDa, 17 kDa and 12 kDa were always present at relatively high levels both in TC199 control and in infected haemolymph, but at low levels in untreated haemolymph. The protein bands 22 kDa and 20 kDa were always present at relatively high levels in both TC199 control and infected haemolymph.

Figs. 24, 25 and 26 show the protein bands at different time intervals after inoculation of living microfilariae or heat-killed microfilariae and their estimated molecular weights. Band 12 KDa showed high levels on day 1 PI decreasing to day 4 PI. Fig. 27 shows the protein bands following ingestion of microfilariae from membrane feeding, dextran injection, untreated mosquito haemolymph and TC199 controls.

Figs. 28 and 29 show the protein bands at different time intervals following inoculation of living or heat-killed bacteria. Band 12 kDa showed high levels at 10 hours and on day 1 PI but declined thereafter to day 4 PI. Bands 22 kDa and 20 kDa showed variable levels at the different time intervals.

Figs. 30 and 31 show the protein bands at different time intervals following injection of laminarin and LPS. Once again band 12 kDa showed high levels on day 1 and then a decreased to day 4 PI.

TABLE 22. SUMMARY THE CHARACTERISTICS OF MAJOR HAEMOLYMPH PROTEIN BANDS FROM THE INDIVIDUAL MOSQUITOES, PRESENT ONLY IN MOSQUITOES PREVIOUSLY INFECTED WITH MICROFILARIAE, BACTERIA, LAMINARIN AND LPS BY SDS-PAGE.

Protein bands	M.W. (kDa)	T4	T4HK	T4mb	T5	T5HK	T8	T9	Correlated classification
1.	66	+	+	+	+	+	+	+	PO
2.	42	+	+	+	+	+	-	-	-
3.	24	+	+	+	+	+	+	+	Attacins
4.	8	+	+	+	+	+	+	+	Diptericins
5.	4	+	+	+	+	+	+	+	Cecropins

M.W. = Molecular weights kiloDaltons
T4 = Living microfilariae inoculation
T4HK = Heat-killed microfilariae inoculation
T5 = Living bacteria E. coli infection
T5HK = Heat-killed bacteria E. coli injection
T8 = Laminarin injection
T9 = LPS injection
- = Not present
+ = Present only in infected

TABLE 23. SUMMARY THE CHARACTERISTICS OF MAJOR HAEMOLYMPH PROTEIN BANDS FROM INDIVIDUAL MOSQUITOES, SOMETIMES PRESENT ONLY IN MOSQUITOES PREVIOUSLY INFECTED WITH MICROFILARIAE, BACTERIA, LAMINARIN, LPS AND DEXTRAN BY SDS-PAGE.

Protein bands	M.W. (kDa)	T4	T4HK	T4mb	T5	T5HK	T8	T9	T10
1.	58	-	-	-	-	#	-	-	-
2.	48	-	-	-	#	#	-	-	-
3.	45	#	#	-	-	-	-	-	-
4.	36	#	-	-	#	#	-	-	-
5.	33	-	-	-	-	#	-	-	-
6.	19	#	#	#	#	#	#	#	#
7.	16	#	-	#	#	#	#	#	-
8.	14	-	-	-	#	-	#	#	#
9.	13	-	-	-	#	-	#	#	#
10.	10	-	-	-	#	-	-	#	-
11.	9.5	-	-	-	#	-	-	#	-
12.	7.5	#	#	-	#	-	-	-	-
13.	6	#	-	-	#	#	-	-	-
14.	3	#	#	#	#	-	-	-	-
15.	2.5	#	#	#	#	#	#	#	-

M.W. = Molecular weights Kilodaltons
T4 = Living microfilariae inoculation
T4HK = Heat-killed microfilariae inoculation
T4mb = Microfilariae membrane feeding
T5 = Living bacteria E. coli infection
T5HK = Heat-killed bacteria E. coli injection
T8 = Laminarin injection
T9 = LPS injection
T10 = Dextran injection
- = Not present
= Sometimes present but only in inoculated groups

TABLE 24. SUMMARY THE CHARACTERISTICS OF ALL MAJOR HAEMOLYMPH PROTEIN BANDS FROM THE INDIVIDUAL MOSQUITOES PREVIOUSLY INJECTED WITH MICROFILARIAE, BACTERIA, LAMINARIN, LPS, DEXTRAN AND SHAM TC199 BY SDS-PAGE.

Prot. bands	M.W. (kDa)	T1	T2	T4	T4HK	T4mb	T5	T5HK	T8	T9	T10	Cor.Cl
1.	66	-	-	+	+	+	+	+	+	+	+	PO
2.	58	-	-	-	-	-	-	#	-	-	-	-
3.	48	-	-	-	-	-	#	#	-	-	-	-
4.	45	-	-	#	#	-	-	-	-	-	-	-
5.	42	-	-	+	+	+	+	+	-	-	-	-
6.	36	-	-	#	-	-	#	#	-	-	-	-
7.	33	-	-	-	-	-	-	#	-	-	-	-
8.	28	±	±	±	±	±	±	±	±	±	±	-
9.	24	-	-	+	+	+	+	+	+	+	+	Attacins
10.	22	♦	-	♦	♦	♦	♦	♦	-	-	-	-
11.	20	♦	-	♦	♦	-	♦	♦	♦	♦	-	-
12.	19	-	-	#	#	#	#	#	#	#	#	-
13.	17	±	±	±	±	±	±	±	±	±	±	-
14.	16	-	-	#	-	#	#	#	#	#	-	-
15.	14	-	-	#	-	-	#	-	#	#	#	-
16.	13	-	-	-	-	-	#	-	#	#	-	-
17.	12	±	±	±	±	±	±	±	±	±	±	Lysozyme
18.	10	-	-	-	-	-	#	-	-	#	-	-
19.	9.5	-	-	-	-	-	#	#	-	#	-	-
20.	8	-	-	+	+	+	+	+	+	+	+	Diptericin
21.	7.5	-	-	#	#	-	#	-	-	-	-	
22.	6	-	-	#	-	-	#	#	-	-	-	
23.	4	-	-	+	+	+	+	+	+	+	-	Cecropins
24.	3	-	-	#	#	#	#	-	-	-	-	
25.	2.5	-	-	#	#	#	#	#	#	#	-	Apidaecin?

T1 = Sham TC199 control
T2 = Untreated control
T4 = Living microfilariae inoculation
T4HK = Heat-killed microfilariae inoculation
T4mb = Microfilariae membrane feeding
T5 = Living bacteria E. coli infection
T5HK = Heat-killed bacteria E. coli inoculation
T8 = Laminarin injection
T9 = LPS injection
T10 = Dextran injection
- = Not present
+ = Present only inoculation
± = Increased levels in inoculated groups
= Sometimes present but only in inoculated groups
♦ = Sometimes present but in TC199 control and inoculated groups
Cr.Cl = corresponding classification

FIG.24. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph day 4 PI, after inoculation with 10 living microfilariae(T4), $1x10^4$cells/μl E. coli bacteria(T5), TC199 control (T1), and of untreated mosquito haemolymph (T2), mosquito protein(Tm) and bacteria protein(Tb). 1.5 μl of individual mosquito haemolymph/PBS mixture was applied to 1.5 mm thick, 5-12.5% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on Marker 1 (Kilodaltons) and weight of the protein bands are indicated.

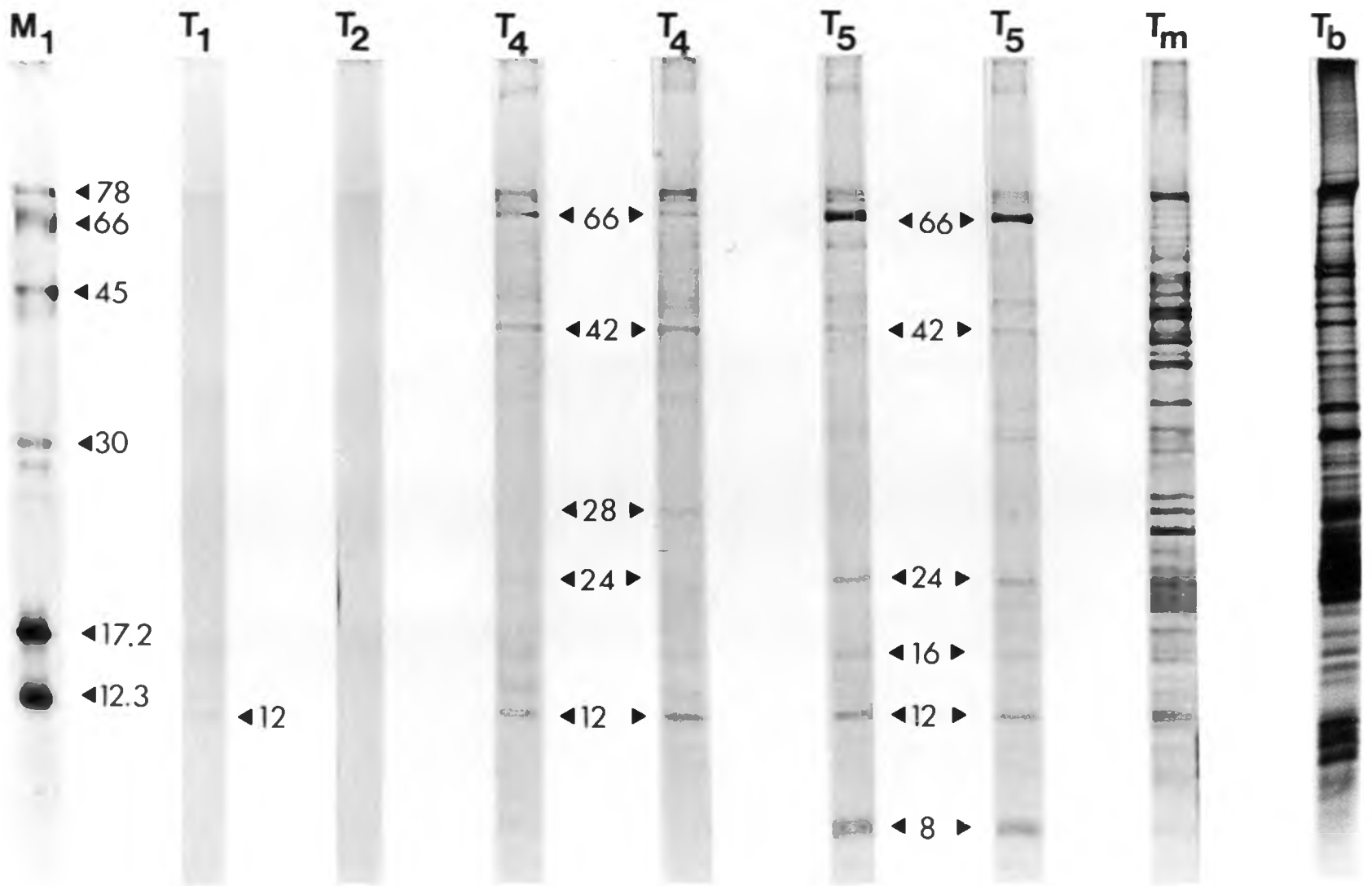

M1
T1
T2
T4
T4
T5
T5
Tm
Tb
78
66
45
30
17.2
12.3
12
66
42
28
24
12
66
42
24
16
12
8

FIG.25. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph day 1 to day 4 PI(D1-D4), after inoculation with 10 living microfilariae(T4), 1.5 µl of individual mosquito haemolymph/PBS mixture of each was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph, Molecular weight standards are noted on Marker 1 and 2 (Kilodaltons) and weight of the protein bands are indicated.

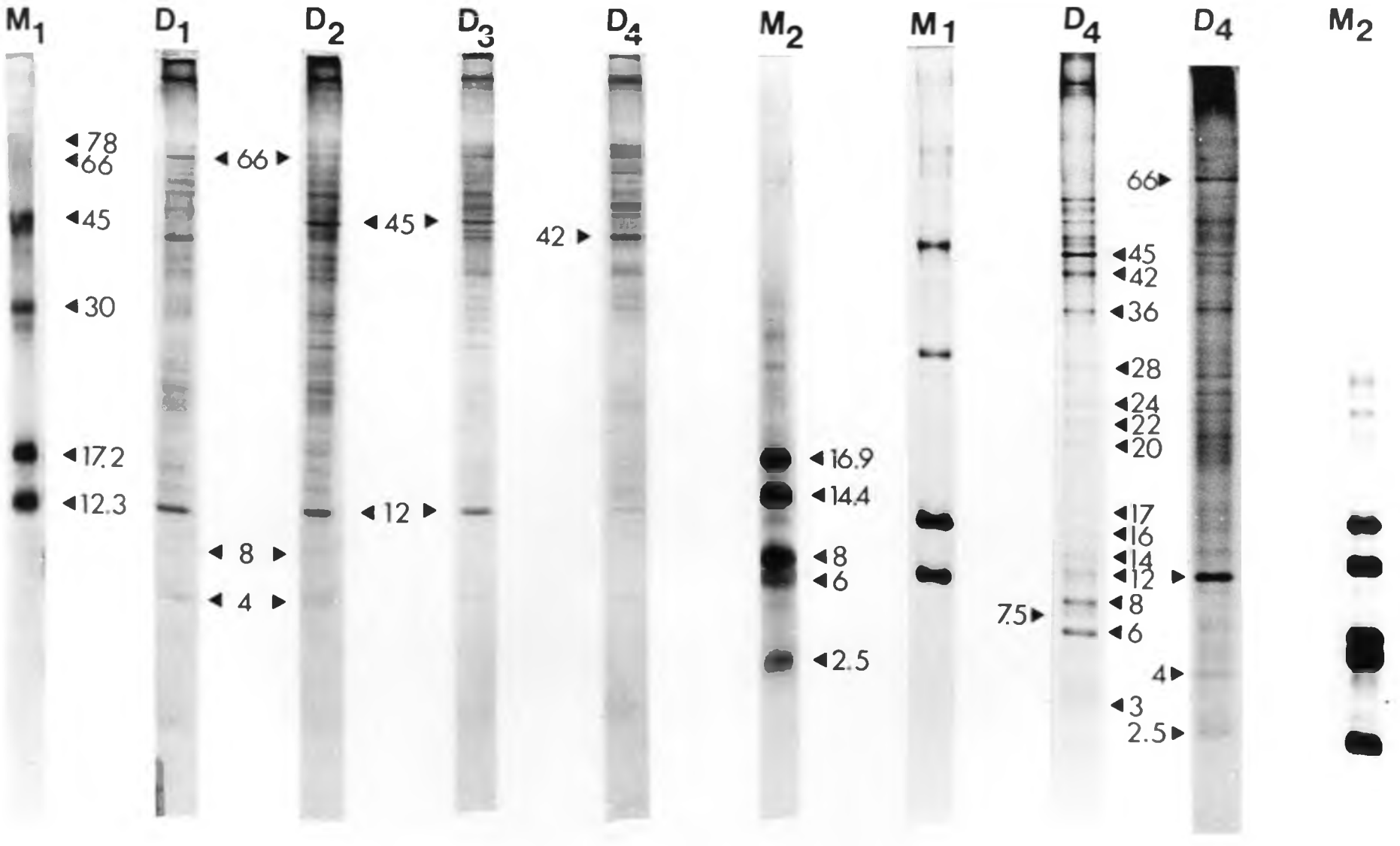

M1
78
66
45
30
17.2
12.3
D1
66
8
4
D2
45
12
D3
D4
42
M2
16.9
14.4
8
6
2.5
M1
D4
7.5
66
45
42
36
28
24
22
20
17
16
14
12
8
6
4
3
2.5
D4
M2

FIG.26. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph day 1 to day 4 PI(D1-D4), after inoculation with 10 heat-killed microfilariae(T4HK), 1.5 µl of individual mosquito haemolymph/PBS mixture of each was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on Marker 1 and 2 (Kilodaltons) and weight of the protein bands are indicated.

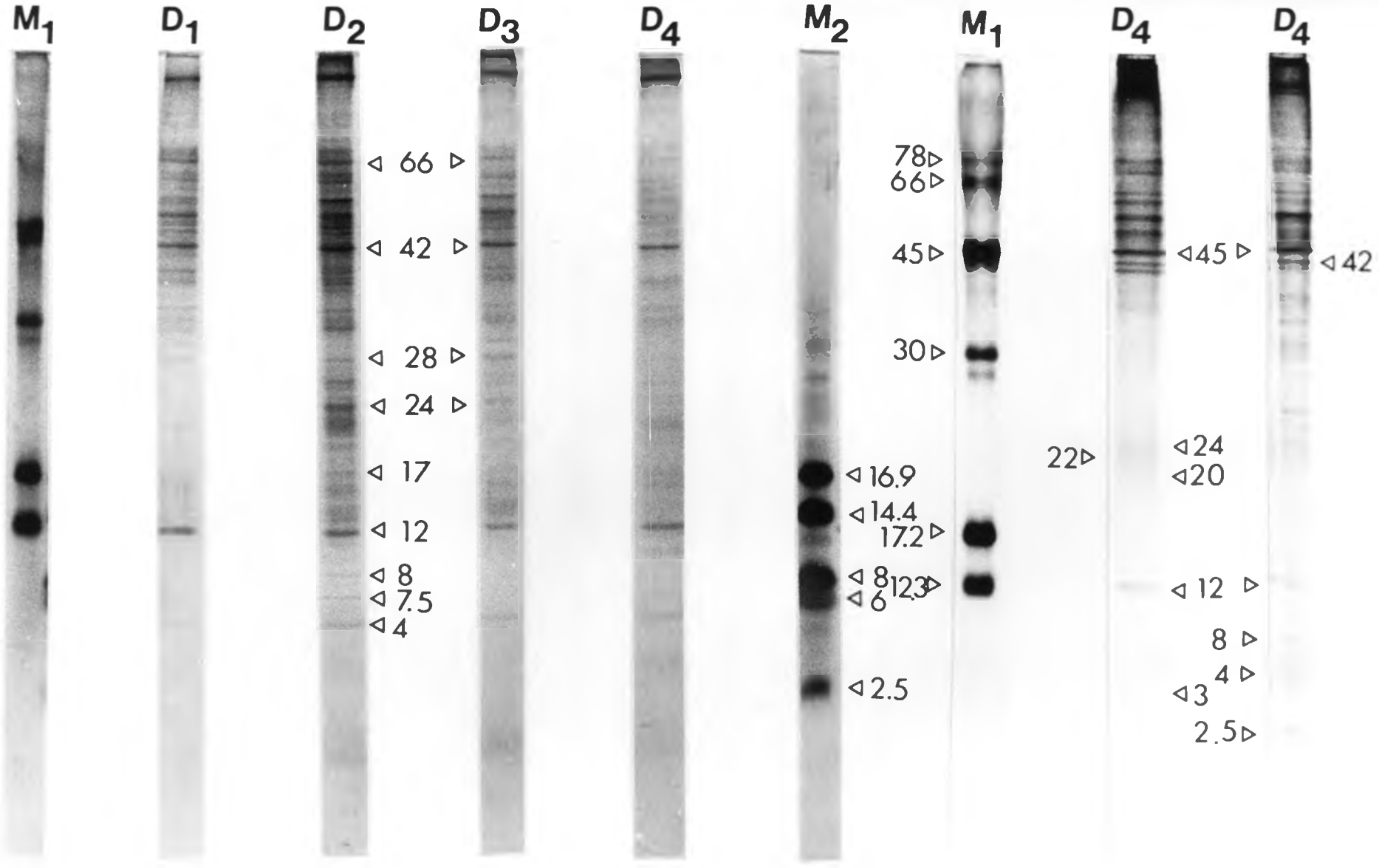

M1
D1
D2
D3
D4
M2
M1
D4
D4
66
42
28
24
17
12
8
7.5
4
78
66
45
30
22
16.9
14.4
17.2
8
12.3
6
2.5
45
42
24
20
12
8
4
3
2.5

FIG.27. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph day 4 PI (D4), after membrane feeding(Tmb), dextran injection(T10), TC199 control(T1) and of untreated mosquito haemolymph(T2). 1.5 µl of individual mosquito haemolymph/PBS mixture of each was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on Marker 1 and 2 (Kilodaltons) and weight of the protein bands are indicated.

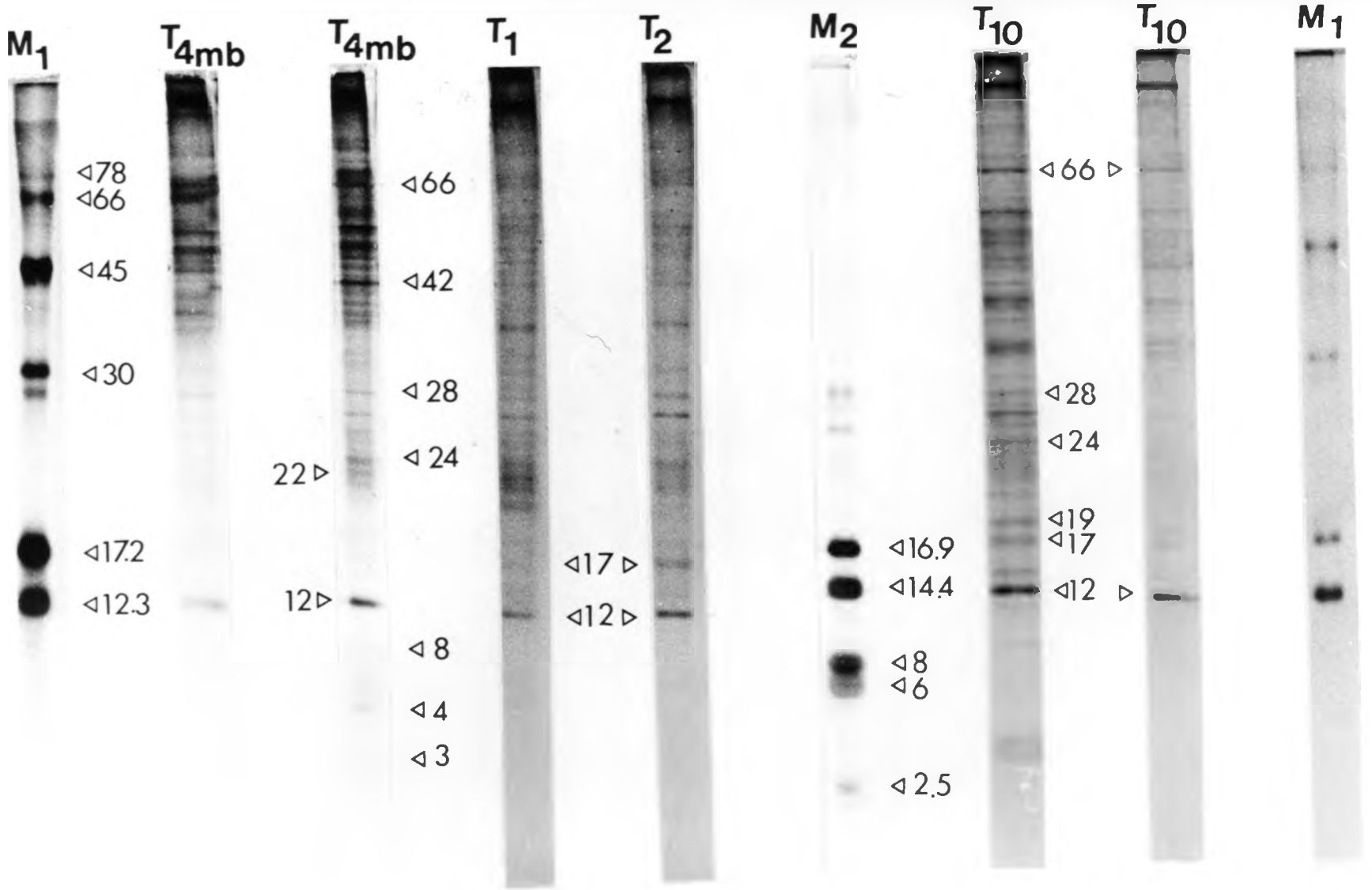

M1
T4mb
T4mb
T1
T2
M2
T10
T10
M1
78
66
45
30
17.2
12.3
66
42
28
24
22
12
8
4
3
17
12
16.9
14.4
8
6
2.5
66
28
24
19
17
12

FIG.28. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph 10 hours to day 4 PI (10h-D4), after inoculation with $1x10^4$cells/μl living E. coli (T5). 1.5 μl of individual mosquito haemolymph/PBS mixture of each was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on Marker 1 and 2 (Kilodaltons) and weight of the protein bands are indicated.

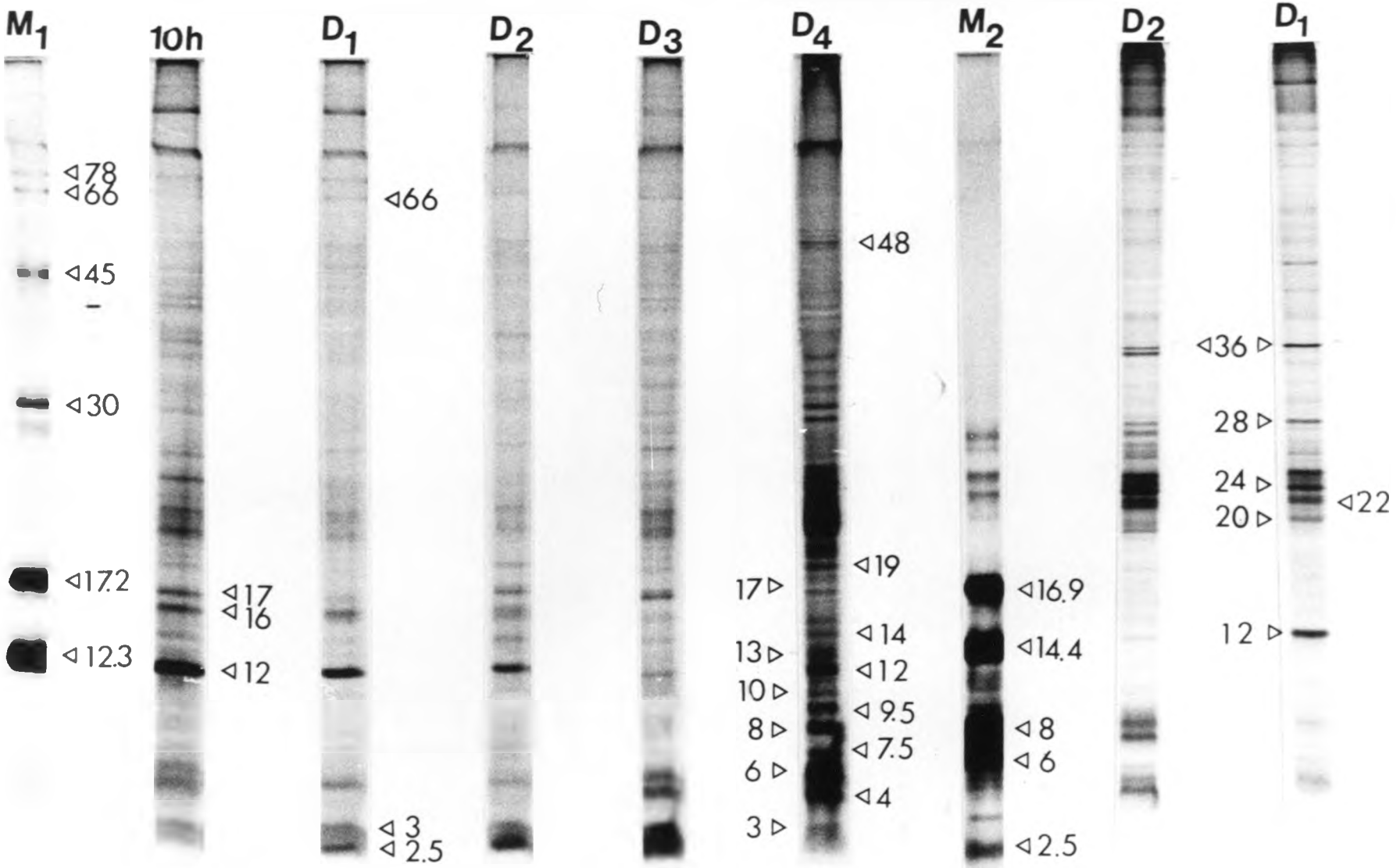

M1
78
66
45
30
17.2
12.3
10h
17
16
12
D1
66
3
2.5
D2
D3
D4
48
19
17
14
13
12
10
9.5
8
7.5
6
4
3
M2
16.9
14.4
8
6
2.5
D2
D1
36
28
24
20
22
12

FIG.29. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph 10 hours to day 4 PI(10h-D4), after inoculation with $1x10^4$cells/μl heat-killed E. coli (T5HK). 1.5 μl of individual mosquito haemolymph/PBS mixture of each was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on M1 and M2 (Kilodaltons) and protein bands are indicated.

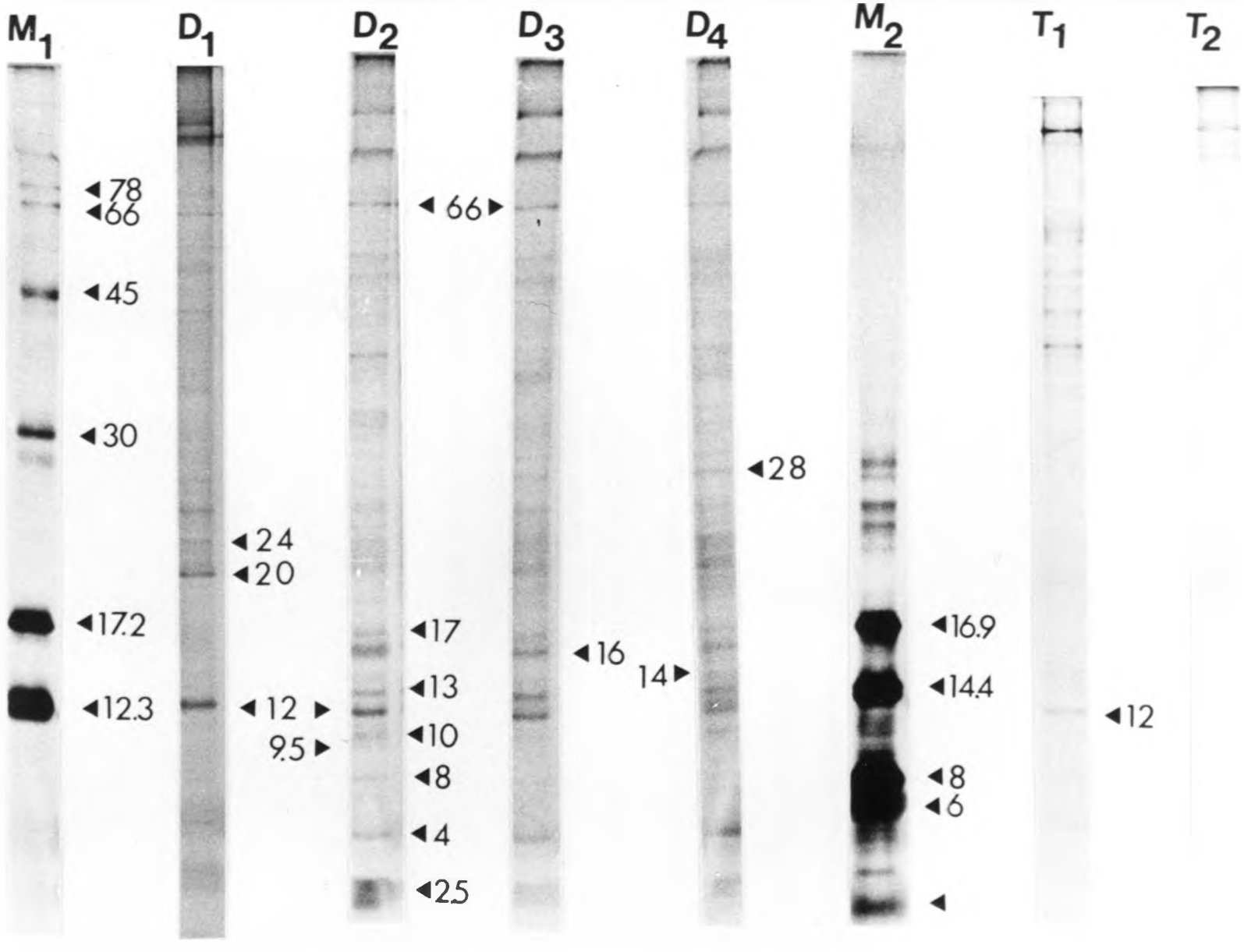

M1
D1
D2
D3
D4
M2
T1
T2
78
66
45
30
17.2
12.3
24
20
12
9.5
66
17
13
10
8
4
2.5
16
14
28
16.9
14.4
8
6
12

FIG.30. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph day 1 to day 4 PI(D1-D4), after injection with laminarin(T8), TC199 control(T1) and of untreated mosquito haemolymph (T2). 1.5 µl of individual mosquito haemolymph/PBS mixture of each was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on Marker 1 and 2 (Kilodaltons) and weight of the protein bands are indicated.

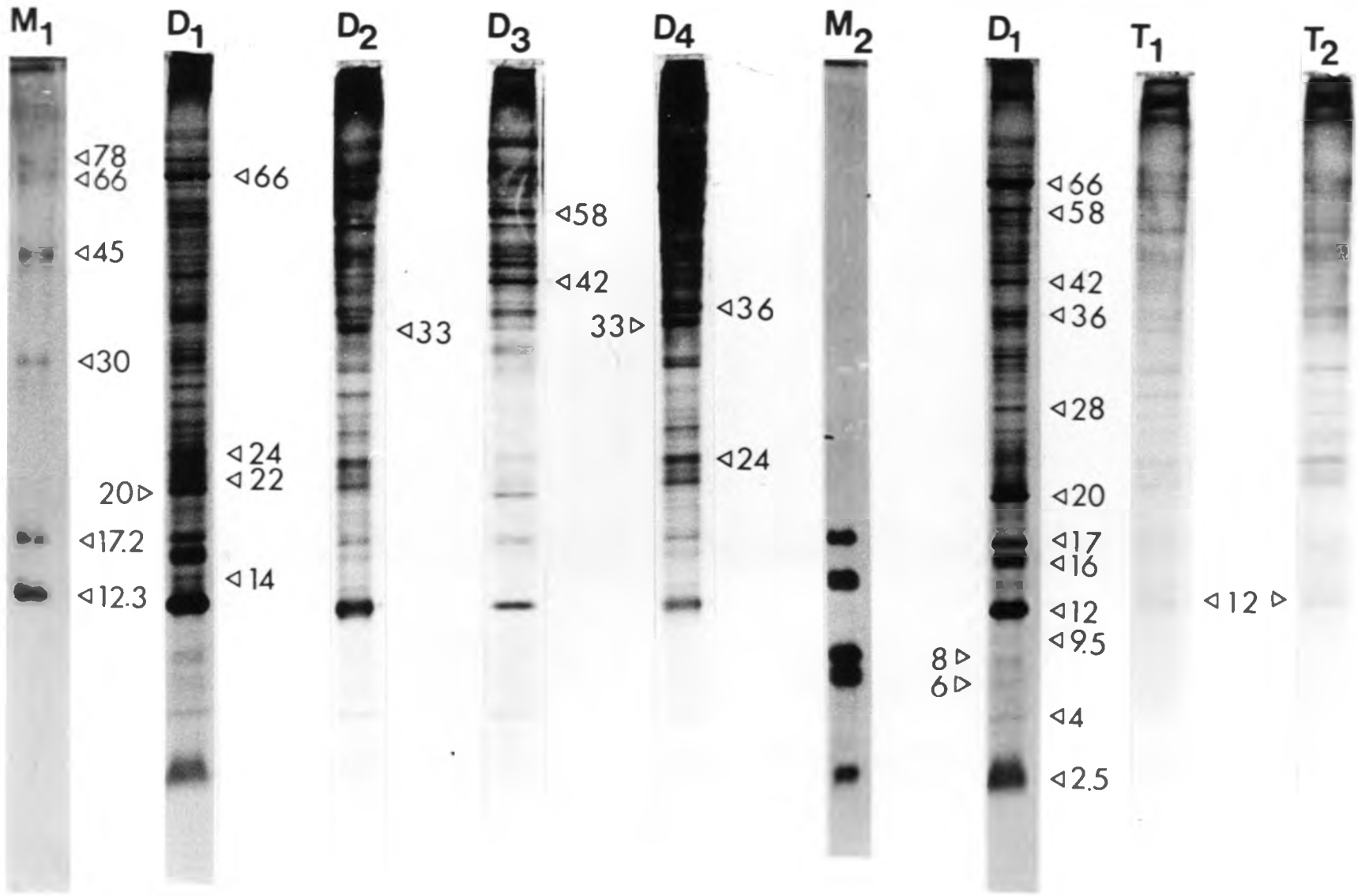
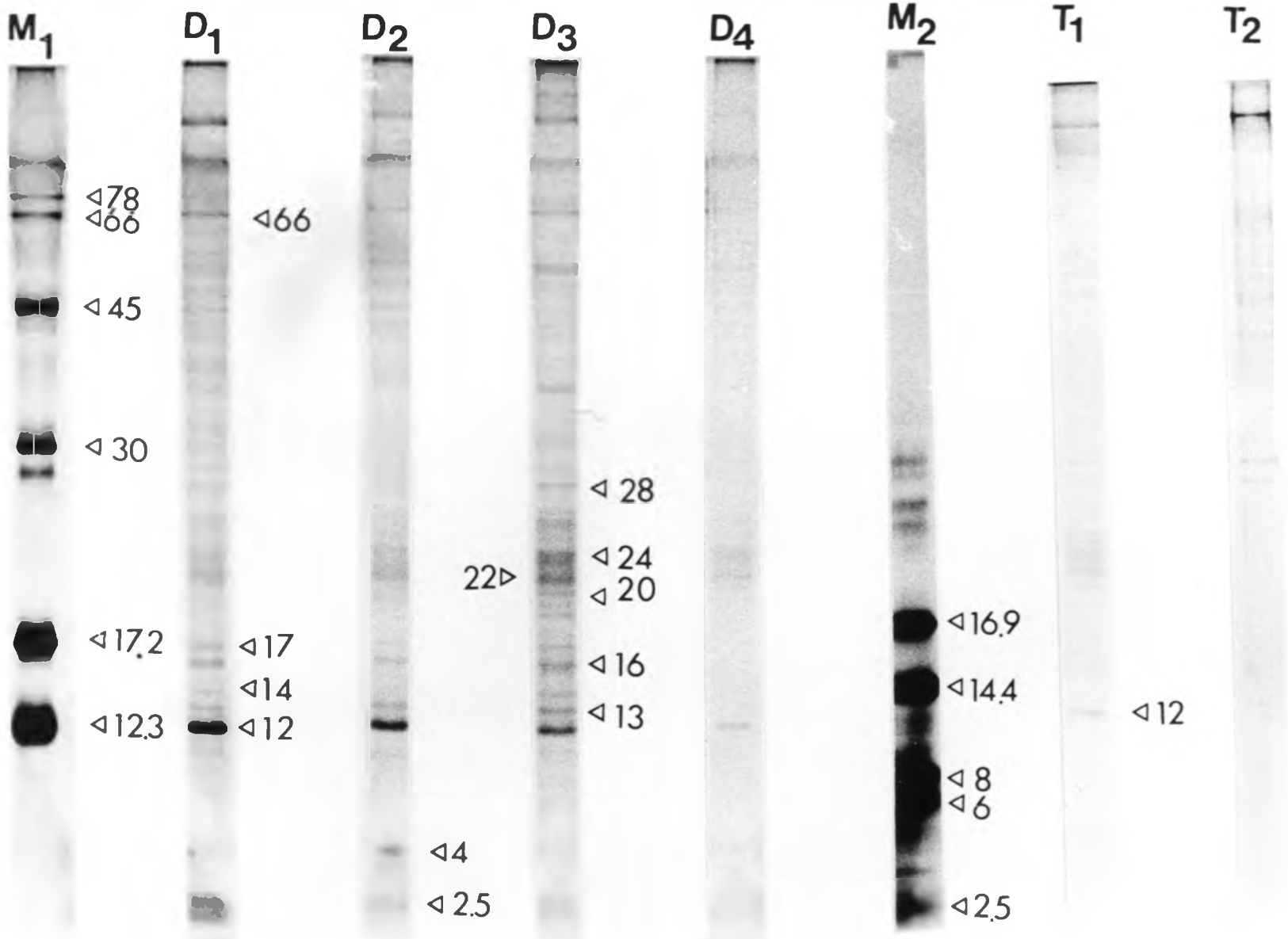

M1
D1
D2
D3
D4
M2
D1
T1
T2
78
66
45
30
20
17.2
12.3
66
24
22
14
33
58
42
33
36
24
66
58
42
36
28
20
17
16
12
9.5
8
6
4
2.5
12

FIG.31. SDS-PAGE protein-banding pattern of Ae. aegypti haemolymph day 1 to day 4 PI(D1-4), after injection with LPS lipopolysaccharide (T9), TC199 control(T1) and of untreated mosquito haemolymph(T2). 1.5 µl of individual mosquito haemolymph/PBS mixture was applied to 1.5 mm thick, 5-15% acrylamide concentration gel. Samples are mercaptoethanol treated haemolymph. Molecular weight standards are noted on M1 and M2 (Kilodaltons) and weight of the protein bands are indicated.


M1
D1
D2
D3
D4
M2
T1
T2
78
66
45
30
17.2
12.3
66
17
14
12
4
2.5
22
28
24
20
16
13
16.9
14.4
8
6
2.5
12

5.4 DISCUSSION

In this study 25 haemolymph proteins of Ae. aegypti have been characterized with respect to their apparent molecular weight. Nineteen were induced by inoculation of microfilariae. Protein band 45 kDa is interesting since it was induced by inoculated microfilariae but not by microfilariae which were membrane-fed. Since it can not be induced by microfilariae which penetrated through the gut, the microfilariae are not recognized as foreign by the mosquitoes (Christensen and Forton, 1984). Protien band 42 kDa is induced both in microfilariae and bacteria inoculated mosquitoes. Twenty-four proteins were clearly induced by bacteria in the mosquitoes; the antigenicity of living bacteria appears to be better than that of heat-killed bacteria. Only 15 proteins were induced by LPS while heat-killed bacteria induced 19. Laminarin (ß-1,3-glucan) induced 13 proteins, whilst glucan polymer with α-1,6-linkage dextran, induced only 8 proteins.

Molecular weight comparisons with the results of other workers suggest that protein 66 kDa may correspond to the 63 kDa (prophenoloxidase) of B. craniifer plasma protein induced by laminarin (Söderhall et al., 1988). Similar comparisons with the data of other workers suggest that protein 12 kDa may correspond to lysozyme (Fay et al., 1975; Pye and Boman, 1977; Rasmuson and Boman, 1979; Hultmark et al., 1980), protein 24 kDa to attacins (Pye and Boman, 1977; Hultmark et al., 1983; Engström et al., 1984a; 1984b; Kockum et al., 1984), protein 8 kDa to diptericin (Keppi et al., 1986; Dimarcq et al., 1988) , protein 4 kDa to cecropins Boman et al., 1978; Hultmark et al., 1980; Steiner

et al., 1981; Hultmark et al., 1982; Qu et al., 1982; Merrifeild, and Boman, 1985), and while protein 2.5 kDa may correspond to apidaecin (Casteels et al., 1989).

Furthermore, the molecular weight comparisons suggest that protein 48 kDa may correspond to 48 kDa protein (P4), which has not yet been named, of Rasmuson and Boman (1979) and Lee et al. (1983), which has been isolated from immunized H. cecropia pupal haemolymph. Proteins of 42 kDa, 36 kDa may correspond to 41 kDa and 38 kDa of Castro et al. (1986), and 36 kDa and 33 kDa from bacteria-induced coagulation protein of M. sexta (Minnick et al., 1986; Minnick and Spence, 1988), and to 32 kDa, a lectin induced in the haemolymph of S. peregrina larvae on injury (Komano et al., 1980;1981), and to 32 kDa, the Thuringiensis-induced protein in M. sexta (Rupp and Spence, 1985). The protein 7.5 kDa may correspond to 7 kDa, the inducible antibacterial factor in the haemolymph of R. prolixus (Azambuja et al., 1986).

Hughes et al. (1983) and Spies et al. (1985b) demonstrated a bacteria-induced haemolymph protein of 20 kDa appeared strong band in M. sexta pupae and faint band in untreated pupae, but not found in bacteria induced haemolymph of the larvae. This may correspond to proteins 20 kDa and 22 kDa which were sometimes apparent in TC199 control and treated haemolymph but not in untreated haemolymph. The protein 17 kDa may correspond to the 17 kDa of M. sexta which was apparent in untreated and increased in bacteria-induced haemolymph of both larvae and pupae (Spies et al., 1986b).

The initial 10^4 bacteria cells injected contained approx 0.0036 $\mu g/\mu l$ protein, LPS 0.01 $\mu g/\mu l$ protein, 0.1 $\mu g/\mu l$ laminarin protein. It is apparent from the data (Fig. 20) that the protein components of the inoculated bacteria and microbial components were not recorded in the haemolymph at the time of protein

analysis. It should also be noted that in most TC199 controls faint bands at 20 and 22 kDa, which corresponded to those found in the treated haemolymph samples, were detected, whereas such bands were never detected in the untreated controls. Hence these bands are likely to be derived from immune response mechanisms in the mosquitoes.

Several differences between the response of mosquitoes to microfilariae, bacteria, and the microbial components laminarin, LPS and dextran were detected. First, the protein at 45 kDa is specifically inducible by living and heat-killed microfilariae inoculation, but not by microfilariae ingested by membrane feeding. Second, the protein 42 kDa is induced by microfilariae and bacteria. Third, the proteins 58 kDa and 33 kDa are inducible only by heat-killed bacteria injection, whereas 48 kDa is induced by both living and heat-killed bacteria injection. Furthermore, protein 9.5 kDa is specifically inducible by living and heat-killed bacteria and by the bacteria component LPS, whereas protein 10 kDa is induced by living bacteria and LPS, and not by heat-killed bacteria. Protein 12 kDa is always induced at higher levels in treated mosquitoes than in untreated controls.

Several lines of evidence suggest that the protein 66 kDa may be related to 12 kDa. At 10 hours and day 1 PI, both proteins showed the highest levels, falling after that to low levels, giving only faint bands on day 2 to day 4 PI. However, the protein band 66 kDa has decreased faster than the protein 12 kDa which still shows apparently faint band on day 4 PI whilst the protein 66 kDa sometimes can not detect on day 4 PI.

It is clear from these observations that the induced response of mosquitoes to foreign materials are distinguishable in several respects. The specific

proteins induced are often quite different, according to whether the inducing agents are microfilariae, living or heat-killed bacteria, or cell walls components.

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CHAPTER 6

GENERAL DISCUSSION

DISCUSSION

Of all insect vectors of disease, mosquitoes have received the most attention, especially with regard to studies of their immune systems against the filarioid nematodes (see reviews of Christensen, 1986; Stoffolano, 1986; Nappi and Christensen, 1986; Christensen and Tracy, 1989). Studies have also been made on mosquito-Plasmodium relationships (Collins et al., 1986; Rudin and Hecker, 1989), insect-Hymenolepis relationships (Heyneman and Voge, 1971; Hurd and Arme, 1984; Lackie, 1981, 1986a), beetles and Raillietina (Gordon and Whitfield, 1985), tsetse and trypanosomes (Bitkowska et al., 1982), Simulium and Onchocerca (Ham, 1986; Ham and Garms, 1988) and insect-trypanosome models (Kaaya et al.,1985, 1986, 1987; Molyneux et al., 1986). Few of the above studies have been designed to assess the basic mechanism of arthropod immune responses to parasites, but some recent work has provided evidence regarding immune activation and effector processes in insects, as well as the ability of parasites to avoid and/or inhibit the immune response.

There is a clear reduction in the rate of infection and numbers of parasites recovered from mosquitoes receiving haemolymph of donors previously inoculated with microfilariae. Similarly, in mosquitoes previously immunized with microfilariae, there is a reduced capacity for survival and development of a challenge infection. Under in vitro conditions, the motility rates of microfilariae in culture medium containing haemolymph from mosquitoes previously inoculated with microfilariae are significantly lower than those in medium

containing haemolymph from sham (TC199) inoculated or untreated mosquitoes.

It is quite clear from the present studies that increased resistance to B. pahangi infection was found in Ae. aegypti given haemolymph from previously infected mosquitoes and that some factor(s) can be transferred in the haemolymph of the infected mosquitoes that will protect recipient mosquitoes from subsequent infection. The experiments involved inoculating recipient mosquitoes with microfilariae first and transferring haemolymph from the infected donor 2 hrs later. The results of the microfilaria migration study show that approximately 20% of microfilariae have migrated to the thorax by 2 hrs. Had the recipient mosquitoes received immune haemolymph first and inoculated microfilariae 2 hrs later or at the same time, then it is possible that an even more accurate suppression of development would have been detectable, since more microfilariae would have been exposed to the immune haemolymph before migration to the thorax. To confirm this possibility would require further study.

The present data relating to the immune response in double infections of B. pahangi show that the immune response suppresses development of further multiple infections in the same mosquito. Ham (1986) also reported that the immune suppression of further multiple infections in the same individual blackfly S. ornatum with O. lienalis is the result of an immune response to the parasite and that this is due to components of the haemolymph. The present data show that haemolymph factor(s) in the mosquitoes are inducible by microfilariae, bacteria, and cell wall components laminarin and LPS, but not by dextran.

Christensen and LaFond (1986) have demonstrated a parasite-induced suppression of immune response in Ae. aegypti by B. pahangi. They suggest that the parasites interfere with the ability of circulating haemocytes to recognize the parasites as foreign or with their ability to be activated in response to the parasite. In the present studies, the possibility of parasite-mediated suppression of the immune response has not been specifically investigated and there is little evidence of such an effect. However it has been observed that laminarin and LPS have the potential to depress the haemocyte numbers and this could conceivably impede the immune response.

J.L. Huang and H. Townson (unpublished) also demonstrated that prophenoloxidase increased during the immune response to inoculated microfilariae in Ae. aegypti strains showing a genetic difference in susceptibility to filariae.

There is evidence that microfilariae acquire protection from immune recognition during their exposure to the environment of the mosquitoes midgut (Sutherland et al., 1984; LaFond et al., 1985), and that this allows microfilariae to migrate unmolested through the haemolymph to the thoracic muscle. Subsequently, developing larvae suppress the host immune mechanisms, thereby ensuring their continued development to the infective stage.

Microfilariae derived from the peritoneal fluid of the jird include some young and non- infective microfilariae. The microfilariae used in the microfilaria migration study, both in inoculated mosquitoes and membrane blood-fed mosquitoes, are of the same origin, that is they originate from jird peritoneal washings. Both the inoculated microfilariae and the microfilariae derived through the midgut penetration are sheathed. It is now believed that

exsheathed microfilariae rarely occur within the midgut, and the available data suggest that the sheath is likely to be weakened or broken at the anterior end of the microfilariae during midgut penetration and that they subsequently free themselves of the sheath in the haemocoele (Christensen and Sutherland, 1984; Chen and Laurence, 1985; Silva and Spielman, 1985). Hence, both the microfilariae used in the inoculation of mosquitoes and those in the membrane blood-fed mosquitoes will have exsheathed in the haemocoele. However, the microfilariae in the blood-fed mosquitoes which penetrated through the midgut would be active; in contrast any young or inactive microfilariae would remain in the midgut. The young or inactive microfilariae of inoculated mosquitoes would remain in the haemocoele exposed to haemolymph and this could alter the nature of the induced the immune response. It is possible that the young or inactive microfilariae exposed to the immunized haemolymph are those subsequently found melanized or encapsulated.

It is possible that the death of microfilariae, both in vivo and in vitro, was due to the presence of factor(s) in the haemolymph, acting directly on the surface of the microfilariae. In the in vitro studies the haemolymph from inoculated mosquitoes produced dramatic increases in B. pahangi mortality. Many of the worms coiled up tightly immediately on exposure to the haemolymph and this was especially true of exposure to lysozyme solution. However, within an hour or so motility was back to normal in all groups except the lysozyme solution. The precise nature of the haemolymph factor(s) responsible for the mortality of microfilariae in in vitro studies is not known.

Lysozyme has for long been considered to be one of the substances produced by insects in response to invasion by foreign bodies and recently other antimicrobial proteins have been isolated (cercopin A and B) (Hultmark et al., 1980). Chadwick (1970) demonstrated that the lysozyme concentration peak at 36 hrs postinfection in G. mellonella infected with Ps. aeruginosa is still maintained after 72 hrs. Powning and Davidson (1973) described lysozyme in G. mellonella and B. mori which had specific properties similar to egg white lysozyme but was about six times more active than that lysozyme. Dunn et al. (1987) demonstrated that RNA synthesis required for increased lysozyme activity occurred within 4 hrs after infection of larvae of M. sexta with bacteria. Lysozyme is also considered to be digestive rather than cidal (Lackie, 1986b).

On the basis of many reports, it is also clear that phenoloxidases play an important role in the response of insects against foreign invaders (Christensen and Tracy, 1989). These enzymes occur as two forms, monophenoloxidase and diphenoloxidase (Söderhall, 1982; Nappi et al., 1987). The activation of phenoloxidase can be induced by bacteria lipopolysaccharide (LPS) (Pye, 1978), laminarin a ß-1,3-glucan (Söderhall, 1982; Ratcliffe et al.,1984; Leonard et al., 1985a, 1985b) and live or heat-killed bacteria (Pye, 1974; Yoshida and Ashida, 1986). Nappi et al. (1987) demonstrated that the monophenoloxidase activity in Ae. aegypti inoculated with Di. immitis microfilariae increased approximately two fold, compared with untreated or saline injected mosquitoes. Li et al. (1989) also reported that haemocyte monophenoloxidase activity in microfilariae inoculated mosquitoes was significantly increased at 24 hrs PI in Ae. aegypti infect with Di. immitis. J.L. Huang and H. Townson (unpublished) also demonstrated that phenoloxidase significantly increased at 24 hrs

postinoculation in various genetically different strains of Ae. aegypti inoculated with B. pahangi.

Komano et al. (1980, 1981, 1983) suggested that lectins induced in the haemolymph of S. peregrina larvae or pupae following injury of the body wall may be involved in the defence mechanisms of insects. Ingram et al. (1984) demonstrated that lectins increased significantly in the locust Sc. gregaria and the cockroach P. americana immunized with Trypanosoma brucei and Leishmania hertigi. Ibrahim et al. (1984) suggested that the natural lectins in the haemolymph and gut of G. austeni may play a role in the tsetse defence mechanisms. Minnick et al. (1986) demonstrated bacteria induced lectins activity and haemocyte coagulating activity in M. sexta haemolymph.

From the experiments reported in this thesis it is clear that some factor(s) in the haemolymph of mosquitoes injected with laminarin or LPS have a partial effect on the development and growth of microfilariae. However, until recently it had been thought that the immune proteins induced by the bacteria, laminarin and LPS were specific for the prokaryotic cell membrane. Nevertheless there remains a possiblility that immune response effect is on the eukaryotic cell wall. Siden and Boman (1983) and Andreu et al. (1985) suggested that the induced amphipathic α-helix at the C-terminus of cecropin A may be required for activity against the bacteria cell wall. The C-terminal half of cecropin A is involved in activity against bacteria. However, the N-terminal half of cecropin A does clearly play a role in the interaction with bacteria. The cecropin makes the surface layer of the bacteria cell wall more hydrophobic. The membrane surface of the eukaryotic cell comprises lipids and proteins. These lipids have amphipathic molecules, with polar hydrophilic and

hydrophobic terminals (Walter, 1989). The antibacterial proteins may therefore play a role on the eukaryotic membrane surface, making the surface layer membrane more hydrophobic and hence leaving the cells open to immune attacks.

The present study with SDS-PAGE of haemolymph from individual mosquitoes inoculated with living or heat-killed microfilariae revealed up to 19 protein bands ranging from approximately 2.5 kDa to 66 kDa. It is quite clear from observations both in vivo and in vitro that the immune response is of parasite origin. The protein 45 kDa may possibly be a specific immune response to the filarial parasite B. pahangi.

Most parasites transmitted by mosquitoes must penetrate the intestinal tract to reach their site of development. This mechanical barrier is a means of defence for mosquitoes and undoubtedly provides protection from infection by certain parasites. A review of the role that the peritrophic membrane might play in limiting infection in insects that serve as biological vectors of parasites concluded that it is not a barrier of any consequence (Orihel, 1975). Physiological differences in the structure of the midgut, however, do appear to limit parasite infection in certain species (Sutherland et al., 1984). A protein of 45 kDa was induced in mosquitoes inoculated with either living or heat-killed microfilariae, but not in mosquitoes fed microfilariae through the membrane, suggesting that this is a specific immune response to microfilariae which have **not** penetrated through the midgut. This protein may be responsible for immune response to the filarial parasite. Protein 42 kDa was induced in mosquitoes both inoculated with microfilariae and bacteria, this protein may correspond to lectins and may have a role in nonself recognition (Castro et

al.1986). However, other immune proteins, such as 24 kDa, 8 kDa, 4 kDa and 2.5 kDa, may also have a role in the defence mechanisms of mosquitoes. The protein of 66 kDa may possibly play a role in melanization and encapsulation, being a prophenoloxidase, and protein 12 kDa is possibly a lysozyme with a role in digestion of microfilariae.

Humoral and cellular immune responses can be induced in arthropods by an injection of either live or heat-killed non-pathogenic bacteria or heat-killed pathogens (Stephens and Marshall, 1962; Ratcliffe and Gagen, 1977; Gagen and Ratcliffe, 1977; Kinoshita and Inove, 1977; Chain and Anderson, 1982; Dunn and Drake, 1983; Ratcliffe and Walters, 1983; Walters and Ratcliffe, 1983; Yoshida and Ashida, 1986; Keppi et al., 1986; Spies et al., 1986a,1986b; Azambuja et al., 1986; Götz et al., 1987; Berg et al., 1988), and by injection of ß-1,3-glucan laminarin (Söderhall, 1982, 1983; Söderhall et al., 1983; Ashida et al., 1983; Leonard et al., 1985a,1985b; Gunnarsson and Lackie, 1985; Yoshida et al., 1986; Gunnarsson, 1988; Söderhall et al., 1988; Brehelin et al., 1989; Brookman et al., 1989) and by injection of lipopolysaccharide, LPS (Pye, 1978; Chadwick, 1970; DeVerno et al., 1984; Söderhall and Hall, 1984; Gunnarsson and Lackie, 1985). The present studies clearly show that either live or heat-killed E. coli can induce humoral and cellular immune response in the mosquitoes and that these have a partial effect on the development and growth of microfilariae both in vivo and in vitro.

The protein bands detected on SDS-PAGE of living or heat-killed bacteria-infected haemolymph revealed up to 24 bands ranging from 2.5 kDa to 66 kDa. Protein band 66 kDa may correspond to prophenoloxidase (Söderhall et al., 1988), 24 kDa to attacins (Hultmark et al., 1983; Engström et al., 1984a,1984b;

Kockum et al., 1984), 12 kDa to lysozyme (Mohrig and Messner, 1968; Jolles et al., 1979; Powning and Davidson, 1973; Hultmark et al., 1980; Boman 1982), 8 kDa to diptericins (Keppi et al., 1986; Dimarcq et al., 1988), 4 kDa to cecropins (Boman et al., 1978; Hultmark et al., 1980; Steiner et al., 1981; Hultmark et al., 1982; Merrifeild et al., 1982; Qu et al., 1982; Siden and Boman, 1983; Andreu and Merrifeild, 1985), and 2.5 kDa to apidaecins Casteels et al., 1989). Some of these attributions must be regarded as tentative until further characterisation is carried out. Protein bands of 58 kDa and 33 kDa are specifically induced only by heat-killed bacteria, whereas 48 kDa is induced by living and heat-killed bacteria inoculation. Furthermore, protein 9.5 kDa is specifically induced by living and heat-killed bacteria and the bacterial component LPS, whereas protein 10 kDa is induced only by living bacteria and LPS, and not by heat-killed bacteria.

Both laminarin and LPS induce an immune response in mosquitoes and there is a partial effect on development and growth of microfilariae both in vivo and in vitro. The protein bands detected on SDS-PAGE of laminarin injected haemolymph revealed up to 13 protein bands and LPS up to 15 protein bands, ranging from 2.5 kDa to 66 kDa. The protein band 66 kDa may correspond to prophenoloxidase. Both laminarin and LPS are known to be strong elicitors of the prophenoloxidase activity system Brehelin et al., 1989).

Haemolymph from dextran inoculated mosquitoes showed no effect on microfilariae in vitro, but the protein bands detected on SDS-PAGE of dextran injection haemolymph revealed up to 8 protein bands ranging from 2.5 kDa to 66 kDa. The protein band 66 kDa may correspond to prophenoloxidase, 24 kDa to attacins, 12 kDa to lysozyme, and 8 kDa to diptericins. Dextran, which

is produced by L. mesenteroides, induces some immune proteins but they have a low immune response effect.

Prophenoloxidase has been the most intensively investigated component of arthropod immune effector mechanisms but its mode of action and the regulation of its activity are unclear. Prophenoloxidase has been detected both in haemocyte and haemolymph plasma as a catalytically inactive proenzyme (Ashida, 1971; Iwama and Ashida, 1986), and the enzyme can be activated by microbial components such as laminarin (Söderhall, 1982; Ashida et al., 1982) and LPS (Söderhall, 1981; Saul and Sugumaran, 1987). Activation is correlated with the immune response and it has been suggested by Söderhall (1982) that the activation process itself may be the basis of non-self recognition. Parasites or pathogens could elicit a response on at least two different levels to avoid recognition; firstly on a humoral level, secondly on the cellular level(Chen and Laurence, 1985,1987).

In the present studies, LPS may have activated prophenoloxidase (protein band 66 kDa) in the mosquitoes, which would support Saul and Sugumaran (1987), who observed the activation of prophenoloxidase by LPS in M. sexta plasma, and Brehelin et al. (1989) who observed prophenoloxidase in haemocyte lysate and in serum of L. migratoria. On the other hand, the studies of Ratcliffe et al. (1984) with G. mellonella, Dularay and Lackie (1985) with S. gregaria, and Yoshida and Ashida (1986) with B. mori, revealed no prophenoloxidase activity using LPS as an activator.

The present study suggested that protein of 66 kDa may be correlated with 12 kDa. Observation of SDS-PAGE shows that protein bands 66 kDa and 12 kDa were detected as heavy bands on day 1 PI, subsequently decreasing to

day 4 PI, but on day 4 the protein band 12 kDa was still clearly detected while the protein 66 kDa was seen as a very faint band. It seems possible that prophenoloxidase activation is a consequence of release of lysozyme.

Unfortunately, in this experimental work, it has been found very difficult to investigate the standard assay for antibacterial activity used widely with Lepidoptera (Boman et al,.1981). The main reason is that the quantity of haemolymph available from mosquitoes is very much less than that from Lepidopteran pupae. The volume of haemolymph in the 1 µl of haemolymph/TC199 or haemolymph/PBS mixture was of the order of 0.75 µl and hence not suitable for quantitative and qualitative assay. Furthermore the strain of E. coli HB 101 used is less suitable for such tests than the D31 used by Boman's group(Boman et al., 1974). Furthermore, pooled untreated haemolymph produces highly variable results when compared with the treated haemolymph because of the impossibility of protecting from infection untreated mosquitoes with organisms in the insectary environment. We have so far not been able to separate the killing and the lytic activity of the immune proteins of microfilariae-immunized haemolymph and bacteria-immunized haemolymph or for the immunized haemolymph injected with cell wall components laminarin and LPS against strain E. coli HB 101. The source of the antibacterial activity of the immune haemolymph in microfilariae-immunized haemolymph mosquitoes remains unclear and the possible involvement of humoral and cellular cooperation requires further study.

Jaynes (1989) has recently published a popular review of the therapeutic potential of antimicrobial peptides. He and his collaborators have chemically synthesized one of a family of lytic peptides, cecropin B, and several of its

derivatives, with minor changes in their amino acid sequence. These peptides have been found to kill every type of bacteria tested. The peptides disrupt the bacteria cell membrane, leading to lysis as the cells lose their ability to control their fluid balance. Electron micrographs showed large pores in the membrane. He also demonstrated that the peptides eliminated the malaria parasite P. falciparum from infected red blood cells and T. cruzi from infected cells in vitro. By designing a new peptide which altered slightly more than half of the amino acids in the cecropin B's peptide chain, the new peptide showed greater lytic activity than the natural cecropin B and was named Shiva I. The new peptide, Shiva I, is similar to natural cecropin in that the altered peptides retained certain physical properties of the origin cecropin molecule. Jaynes argues that this explains why other natural proteins are lytic, even though their sequence of amino acids bears no resemblance to that of cecropins. Examples include sarcotoxins from flesh fly S. perigrena (Okada and Natori, 1983), diptericins from P. terranovae (Dimarcq et al,. 1988) and magainins, from the skin of frogs, Xenopus laevis (Zasloff, 1987). At low doses, lytic peptide also makes cells divide more rapidly, both skin cells and cells of the immune system seem to respond to peptides in this manner (Jaynes, 1989). There is also evidence that these lytic peptides show structural similarities with other amino acids and with certain signal peptides. The work of Jaynes and his associates raises the possibility of commercial scale synthesis of such lytic peptides for therapeutic use against microbial and parasitic infections of man. The availability of synthetic peptides should also assist in the determination of the structure-function relationships of antibacterial peptides.

The response reported in this thesis of Ae. aegypti mosquitoes to B. pahangi microfilariae, bacteria E. coli and the cell wall components laminarin, LPS and dextran, and the demonstration of a stimulation of immune systems, represents an important advance in our understanding of the vector-parasite association. However, it is apparent from the data presented here, and from the other recent reviews (Lackie, 1986a; Stoffolano, 1986; Nappi and Christensen, 1987; Christensen and Tracy, 1989), that our understanding of the immune processes in insects is inadequate for us to be able to define the mechanisms whereby parasites can, to their advantage, circumvent the immune response.

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