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EARLY DETECTION OF LYMPHATIC FILARIASIS AND ATTEMPT AT DEFINING PROTECTIVE ANTIGENS

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WANLOP CHUSATTAYANOND, Ph.D.

PROJECT LEADER

DEPARTMENT OF MICROBIOLOGY
FACULTY OF SCIENCE
MAHIDOL UNIVERSITY

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Project Leader:	Dr. Wanlop Chusattayanond
Organization:	Faculty of Science, Mahidol University Bangkok 10400, Thailand
Co-investigators:	Dr. Prasert Sobhon Dr. Somphong Sahaphong Dr. Chantakorn Shutidamrong Mrs. Pranee Sithisarn Dr. Araya Dharmkrong-at
Project Consultants:	Dr. David A. Denham London School of Hygiene and Tropical Medicine, University of London Dr. Pornchai Matangkasombut Faculty of Science Mahidol University
Authorized Officer:	Dr. Pairote Prempree Dean, Faculty of Science Mahidol University
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1. Introduction

Filariasis is a group of human and animal diseases caused by arthropod-borne nematode parasites of the Order Filarifidea, commonly called 'filariae'. Eight species of these are found to infect human, they are Wuchereria bancrofti, Brugia malayi, B. timori, Onchocerca volvulus, Loa loa, Dipetalonema streptocerca, D. perstans and Mansonella ozzardi. Of these species, O. volvulus transmitted by blackflies of the genus Simulium is the cause of the most serious of filarial diseases. The disease called 'river blindness' affects about 40 million people, mainly in tropical Africa, but als in Central and South America and in foci in the Eastern Mediterranean Region extending to Yemen and the Sudan.

Lymphatic filariasis, the mosquito-transmitted disease is caused by W. bancrofti, B. malayi and B. timori. It affects about 90 million people in Asia, Africa and South America and an estimated 905 million directly exposed to the risk of infection. The infection could lead to disability and disfigurement due to acute adenolymphangitis and chronic lesions like elephantiasis of the extremities and hydrocoeles. While W. bancrofti is found throughout the wet tropical world, B. malayi is confined to Asia and B. timori is only found on the islands of Timor, Flores, Alar and Roti of Indonesia (Wheeling et al., 1975).

In Thailand, W. bancrofti is found in the Western part of the country along the border with Myanmar while the endemic area of B. malayi is in the South. It has been reported that the control programme of filariasis in Southern Thailand during 1961-1979 which covered 6 endemic areas of malayan filariasis with a total

population of about 1.5 million (people at risk) was successful. Thus, the filariasis infection rates was alleged to decrease from 14.1% in 1964 to 0.30% in Chumporn province, 26.4% in 1963 to 0.24% in Suratthani province, 7.7% in 1961 to 0.02% in Nakhorn-Srithammarat province, 61.7% in 1962 to 0.18% in Patthalung province, 8.0% in 1971 to 0.13% in Pattani Province, 11.1% in 1963 to 0.89% in Narathiwat province (Harinasuta and Samahan, 1981).

Unfortunately, this optimism was rather shortlived and reflected only the limited information then (in 1981) available. A more recent report (1982-1985) compiled by the Filariasis Division, Ministry of Public Health including areas previously inaccessible to governmental personnels revealed new areas of high endemicity ranging from 1.16% to 4.6%. As is often the case epidemiological data reflects the extent and limitation of the survey carried out and usually represent underestimation of the problems. In Thailand and most developing countries, this underestimation is found all the time due to under-reporting and relative limitation of personnels and inavailability of proper diagnostic tools for field surveys. For a problem like lymphatic filariasis, in fact special efforts have been needed to survey and monitor the extent and spread of the disease above and beyond the regularly stationed health personnels in the periphery. This is simply because special efforts are required to go into villages, no matter how remote they are, to obtain blood samples at night in order to detect microfilaraemia. Regular personnels are already overtaxed by their heavy health service duties. No

wonder, the more the Filariasis Division intensified its efforts in case detection and started to expand its field activities into new areas, the higher prevalence is reported and more endemic areas are uncovered. Particularly noteworthy and in addition to the high endemicity and new endemic areas for Malayan filariasis uncovered in the south, new areas of endemicity were also uncovered for Bancroftian filariasis, eg., in the Tak and Mae Hong Sorn provinces previously thought to be filariae free, where the prevalence in one area was found to be as high as 14.29%!

It can not be overemphasized that the recent efforts of the Filariasis Division in intensifying case detection in known areas and in expanding into adjacent areas were carried out with a great deal of difficulties, otherwise it could have gone ahead and present a more true picture of filariasis in Thailand. The same difficulties in the assessment of real prevalence are likely met with in any countries of the developing world. Among the difficulties, limited financial resources asides, is the technical limitation of the currently available diagnostic techniques in the field which has remained the detection of microfilariae in thick blood film at least for now. The fact that blood samples need to be taken at night when microfilariae appear in the blood circulation is a major hindrance in the case detection and thus in the control programme. Attempts at serological test for epidemiological monitoring have met with the problem of specificity and sensitivities since available tests thus far depends on the use of crude antigen preparations. Furthermore, available tests are aimed at antibodies detection which appeared late and do not allow detection of early

(premicrofilaraemic stage) infection when chemotherapy can be simpler and more effective and when transmission can be interrupted.

Improved immunodiagnostic tests, particularly those aiming at detection of early stages of parasite antigens with high specificity and sensitivity, are required. Previous attempts have met with the problem of complexity of the antigenic structure of the various developmental stages of the parasite and the want of clinical and parasitic materials. The present proposed studies will employ an armament of recently available methodologies, eg, hybridoma technology, immunoblotting etc. to dissect the antigenic structure of human lymphatic filariae and related species to derive species-and stage-specific immunodiagnostic reagents taking advantage of an established life cycle in experimental animals and the readily available clinical materials in endemic areas in Thailand. Furthermore, the reagents derived and assay systems devised will be field tested, modified if needed and eventually provide for local production of immunodiagnostic assays for large scale use.

These systems and information gained on antigenic structure of these parasites can be adapted for the development of similar tests for other filariae prevalent elsewhere in the developing world.

2. Background

2.1 Life cycle and mode of transmission

Filariae are ovoviviparous nematodes producing microfilariae, the first stage-larvae, into blood circulation or

tissue. Microfilariae have to be ingested by specific blood sucking arthropods in which they migrate to certain site or organ to complete two moultings and become infective third-stage larvae. The intermediate hosts of all human filariae are blood-sucking insects of the Order Diptera.

Lymphatic filariasis is transmitted by insects of the Family Culicidae, the mosquitoes. The infective larvae deposited on human skin during the process of feeding, enter the host through the wound which remains after the infected mosquito has completed its blood meal. In human body the larvae enter the lymph node, moult twice and become adults in lymphatic vessel, microfilariae will migrate through the thoracic lymph duct to the blood stream. The pre-patent period in man is approximately 3.5 months for B. malayi (Edeson et al, 1960) and 11 months for W. bancrofti (Cowell et al, 1970). Microfilariae have a unique periodicity and are found in the peripheral blood circulation during certain period of the day. The mosquito vectors also appear to have a circadian rhythm in which they bite during specific period of the day. The correlation between peak peripheral microfilaraemia and peak biting time suggests that filarial nematodes have adapted their periodicity patterns to those of vector to facilitate their transmission by mosquitoes (Wharton, 1973). In W. bancrofti, 3 strains differing in the microfilarial periodicity have been recognized: a nocturnally periodic strain distributed widely throughout the tropical and subtropical zones of the world; a diurnally subperiodic strain restricted to the Polynesian subregion and a nocturnally

subperiodic strain reported in West Thailand (Sasa, 1976). The mosquito vectors of W. bancrofti are certain species of Aedes, Culex and Anopheles. In B. malayi, nocturnally periodic and nocturnally subperioidic strains are commonly found. The two strains of B. malayi in human were first described by Turner and Edeson (1957). The periodic strain is endemic in open rice fields or swamp area and is transmitted mainly by the genus Anopheles; the subperiodic strain is endemic among inhabitants of forest areas and is transmitted by certian Mansonia mosquitoes. Laing et al (1960) showed that the subperiodic form is zoonotic which also occurs in wild and domestic animals. A peculiar form, termed diurnally subperiodic, showing a peak hour in the day time (around 1600 hours), has been described from Perak, Malaysia (Dondero et al, 1971). The diurnally subperiodic strain has also been recently found in Southern Thailand with a peak hour around 1200 hours (Filariasis Division, personal communication) in addition to the nocturnally subperiodic strain which is commonly found. A naturally Brugia infected cat was found in Surattani Province, South of Thailand and the parasite was identified as B. malayi (Phantana et al, 1987) and this is the first confirmed report of the parasite found in animals in Thailand. The finding suggests the possibility of zoonotic transmission of the parasite in the country. In previous reports in Thailand, the parasites found in domestic cats were merely identified as B. malayi - like (Harinasuta et al, 1970 (a and b); Guptavanij et al, 1971; Guptavanij et al, 1971) no further species identification had been made. Unlike B. malayi, the only natural mammalian host for W. bancrofti is human. Many transmission experiments carried out

in the search for a suitable laboratory host were not very successful. Patient infections were found only in immunosuppressed leaf-monkeys (Presbytis sp) (Cross et al, 1979, Harinsuta et al, 1981).

The microfilaraemic host serves as the source of infection for the mosquito vector. Microfilariae taken up by the mosquitoes exsheath and within an hour they penetrate the mid-gut to migrate to the thoracic muscles to undergo 2 moults to become infective larvae within 10-14 days. The infective larvae migrate mainly to the proboscis but they can be seen in other parts of the mosquito.

In our laboratory, we have sucessfully maintained the life cycle of B. malayi (isolated from a naturally infected cat in Southern Thailand) and B. pahangi (originally isolated in Malaya by Buckley and Edeson in 1956) in both cats (Felis catus) and jirds (Meriones unguiculatus). Aedes aegypti (homozygous for the fm gene) which are susceptible to infection with many filariae (MacDonald, 1962a, b) are used as the vector.

2.2 Clinical feature and pathology

Brugian filariasis was first recognized as clinically distinct from Bancroftian filariasis by Lichtenstein (1927). Although elephantiasis of the legs is one of the sequelae to both of them, that caused by Brugian filariasis is confined to that part of the legs from the knee downward while Bancroftian filariasis involves the entire leg. It is noticeable that elephantiasis of the scrotum, hydrocoele and chyluria caused by Bancroftain filariasis are absent from the Brugian filariasis.

When the adult worms are healthy and intact, few inflammatory cells are present and lymph vessel dilatation is minimal. With the death and disintegration of the parasites either due to treatment or other causes, inflammatory reactions with granuloma formation occur. The dead and dying parasites are engulfed in a cellular tissue mass consisting of large numbers of eosinophils, lymphocytes and other mononuclear cells. Subsequent healing with fibrosis of granulomatous lesions may cause complete loss of the histo- architecture and obliteration of the lymph node and lymphatic vessels (Connor, 1932). Elephantiasis has been postulated to result from the pathological reactions to the death of the adult worms in the lymphatic system (Jordan, 1955).

In most microfilaraemic patients, microfilariae do not normally cause lesions. However, in heavy microfilaraemia, there is destruction of microfilariae in the spleen giving rise to acute and chronic inflammatory reactions. In some cases, microfilariae cannot be found circulating in the blood which are attributed to a hypersensitive response to microfilarial antigens. It was first described by Meyers and Kouwenaar (1939) as tropical pulmonary eosinophilia (TPE), a form of occult filariasis. There is a patchy inflammatory reaction to dead and degenerating microfilariae giving rise to both exudative and granulomatous lesions in the lungs. In the exudate lesions, there are amorphous eosinophilic materials, eosinophils and chronic inflammatory cells while in granulomatous lesion, foreign body, giant cells and epitheloid cells are present (Danaraj *et al* 1966).

2.3 Currently available diagnosis

Although it is easy to recognize classical obstructive filariasis, many people who are infected do not show signs or symptoms. Laboratory diagnosis is therefore necessary. Parasitological, immunological, histopathological and DNA probing techniques are available for this purpose.

Parasitological techniques involve the demonstration and identification of circulating microfilariae. Normally 60 ul thick blood films are prepared from blood taken by finger pricking. They are allowed to dry, preferably overnight, for at least an hour and stained with diluted Giemsa stain. However, a parasitological diagnosis frequently fails to detect microfilariae in several conditions as followed:

1. asymptomatic non-patent infection which could be attributed to unisex infection
2. pre-patent infection
3. tropical pulmonary eosinophilia
4. late obstructive filariasis

often introduces difficulties in making the parasitological diagnosis. Furthurmore, when 20-100 ul blood films are examined in the conventional method, there is often a failure to detect infections with low level of microfilaraemia unless concentration techniques are employed. These techniques require large volume of blood which can be obtained only by venepuncture and this is less well accepted by the patients when compared to the finger pricking method. DNA probing technique, on the other hand, is actually unapplicable when no microfilaria is present in blood circulation. In the situations mentioned above immunological

diagnostic methods could have much to offer since they would be able to detect early, late or even chronic obstructive cases.

2.4 Chemotherapy

Following the discovery that diethylcarbamazine (DEC), a derivative of piperazine, is an effective filaricidal agent (Hewitt et al, 1947a, b, c) this compound has been extensively employed in the treatment of filarial disease and it remains the drug of choice for the treatment of lymphatic filariasis. This compound, is most effective against microfilarial stage of the parasite but evidence obtained on the basis of experimental work and on experience with the use of the drug in humans show that DEC if given in sufficient doses, is not only a potent microfilaricidal drug but also has a deleterious if not lethal effect on the adults of W. bancrofti and B. malayi (Mak, 1983). However, the usefulness of the drug has been marked by the severe side reactions commonly in the form of fever, gastrointestinal upsets and other allergic like manifestations attributed to the release of products following the rapid destruction of microfilariae by the drug and these pose serious problems to the drug's acceptability in filariasis control programmes. An ideal drug would be a purely macrofilaricide without affecting the microfilariae. Such a drug would achieve the same purpose of curing the patient without the servere side reactions. The microfilariae would then be cleared on its own after a few months (Ponnudurai et al, 1975) or slowly got rid off with small doses of microfilaricidal drug. Many drugs which show promising results have been reported and it is suggested that the search for nontoxic macrofilaricidal and embryostatic agents

should be continued (TDR, Seventh Programme Report, 1985).

As an alternative to chemotherapy, chemoprophylaxis could be another practical and effective control measure. DEC-C was shown to prevent infective larvae of D. immitis from developing in dogs (Kune et al, 1964), that of B. malayi in cats (Ew ·t and Emerson, 1975) and leaf monkeys (Presbytis melalophos) (Mak and Lim, 1983). Flubendazole was shown to have a 100% chemoprophylactic activity against adult B. pahangi transplanted into the paritoneal cavity of jirds (Meriones unguiculatus) (Denham and Brandt, 1980). Court (1982) found that flubendazole killed 96% of the infective larvae of B. pahangi confined within diffusion chambers in the peritoneal cavity of jirds. One injection of flubendazole protects jirds against infection with B. pahangi infective larvae (Chusattayanond and Denham, 1984) and the larvae inoculated during the period of drug protection are immunogenic which can induce a 40% protection against subsequent challenge when the injected drug is no longer reactive (Chusattayanond and Denham, 1984). Flubendazole was found to be very effective against onchocerciasis without an immediate microfilaricidal effect following weekly intramuscular injections for five weeks, unfortunately the drug induced local pain and inflammation (Dominguez-Vazquez et al, 1983). The drug is now being reformulated.

2.5 Previous attempts at the development of immunodiagnosis

Serological tests are traditionally used for the confirmation of a filarial aethiology as in the diagnosis of lymphoedema or elephantiasis and possibly as a

seroepidemiological tool. A positive serological test is necessary for the diagnosis of occult filariasis as in tropical pulmonary eosinophilia (TPE) for the clinical feature of the disease is similar to symptoms caused by other known parasitic infections and it is further complicated by similar symptoms presented in allergic conditions. Danaraj et al (1957) developed the filarial complement fixation test (FCFT) using alcoholic extract of dried Dirofilaria immitis powder as antigen. It was found that TPE patients could be discriminated from patients with other causes of hypereosiophilia (Danaraj et al, 1959). Pacheco and Danaraj (1966) used the indirect haemagglutination test (IHA) with extracts of various helminths in the diagnosis of TPE. They found that using D. immitis as antigen the FCFT and IHA were similar in sensitivity and the low degree of specificity, ie., neither test appeared to identify specific parasite as aetiology.

Many attempts have been made to use the immunodiagnosis for epidemiological purposes, not just a supperative to a clinical diagnosis. Various techniques have been tried for the detection of antibodies developed in experimental animals and in human sera from the endemic areas. The techniques involve the indirect fluorescent antibody test (IFAT) (Wong and Guest, 1969; Yong, 1973; Santos et al, 1976; Singh et al, 1979; Singh et al, 1980a, b; Au et al, 1982; Lim et al, 1984), enzyme-linked immunosorbent assay (ELISA) (Barlett et al, 1975; Spencer et al, 1981; Ottesen et al, 1982; Au et al, 1982, Tomisato et al, 1983; Crandall et al, 1983; Lim et al, 1984), counter immunoelectrophoresis (CIE) (Desowitz and Una, 1976; Au et al, 1982), radioallergosorbent test (RAST), staphylococcus protein A radioimmuno assay (Staph A-

RIA) (Weiss et al, 1982; Kaushal et al, 1984) and the indirect haemagglutination test (Singh et al, 1980). However, the resutls often showed no correlation with the clinical status of the filarial patients.

For indication of active infection and the degree of infection, methods based on detecting parasite antigens in the sera and urine would be more beneficial. In recent years, the focus of immunodiagnosis of human filariasis has changed from antibody detection to the detection of excreted, secreted and shed antigens of the parasites. Antibodies used in the test are either human filarial serum collected from patients with microfilaraemia and clinical filariasis (Kaliraj et al, 1981; Reddy et al, 1984) or monoclonal antibodies (Forsyth et al, 1985; Maizels et al, 1985).

In a workshop organized by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases in Little, France in November 1983 and reported subsequently (WHO, 1984) two noncompetitive radiolalelled monoclonal antibody immunoassays, ie, the radioimmunoprecipitation polyethylene glycol assay (RIPEGA) and the immunoradiometric assay (IRMA) were compared for sensitivities and specificity. Both were found to have reasonable sensitivities while still having "background noises" and false positive results in sera from patients with connective tissue diseases. The sensitivity of both assays were further depressed when the test sera contained antibodies. Furthermore the assays were not quantitative and were based on radioisotope thus not suitable for areas where the required

facilities do not exist as are usually the case where these tests are needed the most, ie, in remote areas in the developing tropics. It was recommended by the participants that improved assays be developed which are more specific, sensitive and which do not require radioisotope. Ultimately, it would be preferred if a quantitative assay could be devised.

During the same workshop, the specificity of 65 monoclonal antibodies then available was analysed by ELISA and immunoblotting against 37 crude filarial antigen preparations and 10 crude non-filarial antigens. Wide spread cross reactivities were observed among various filarial species though two monoclonal antibodies showed some promise for lymphatic filaria. One noteworthy observation from the immunoblot studies during the workshop was that monoclonal antibodies raised by different investigators often are directed to the same bands and that single monoclonal antibodies often to several bands on the gels. These suggested to us that most monoclonal antibodies tested were "first generation" MAb's resulted from immunizing mice with crude preparations therefore tend to be directed at the more potent immunogens and were not primarily selected on the criteria of species or stage specificities. That single monoclonal antibodies were often found to bind to several bands on the same gels also suggested to us that there had been extensive proteolytic degradation and/or dissociation/aggregation during the preparation of the crude antigens and during SDS-PAGE.

The antigenic complexities of filariae were highlighted by the same workshop where over 100 bands were shown on polyacrylamide gel for each of the crude extracts tested. Of

particular relevant to the present proposal is the finding that adult worms from Brugia species showed a higher degree of similarity than among Onchocerca species and that there were certain MW ranges among Brugia antigens that often react to filarial serum pools. These may turn out to be important leads in our proposed studies. However, these leads are more likely of limited value for two main reasons. Firstly, in the antigenic characterization studies carried out during the workshop on polyclonal sera except for the not so well defined human sera were available for testing. In fact, carefully produced polyclonal antibodies usually play an important role in comparing antigenic composition of related parasite species when used in immunoblotting in which common and distinctive bands can be relatively readily defined. These bands then can serve as much better starting materials for the generation of "second generation" monoclonal and monospecific polyclonal antibodies. The use of human sera or serum pools as in the workshop hardly was a substitute for the deliberately produced polyclonal antibodies.

A second limitation of the lead from the WHO Workshop had to do with the antigenic materials included. While they represented antigenic preparations from adult worms and microfilariae which most investigators devoted themselves to, they did not include parasitic stages important in the early infection, ie., infective larvae and subsequent larval stages which are particularly relevant in view of our objectives in the present proposal. Furthermore, the antigen preparations need be and can be more

refined. That these limitations existed was quite understandable since starting parasitic materials have been a problem for the investigators who work in filaria-free countries. Sufficient materials can now be generated from our full life cycle experimental models to provide for systematic analysis of antigenic structure of each stage of B. malayi and B. pahangi.

2.6 Rationale and approaches of the present studies

The main problem in the control of filariasis resides in the difficulty in breaking the life cycle in endemic areas. Existing dignostic techniques allow detection of blood microfilariae by which time the host has already served as a source of transmission. Early detection would not only allow for more satisfactory results of chemotherapy but more importantly also helps in terminating the infection before it reaches a stage of transmitting to others. However, attempts at development of immunodiagnosis of lymphatic filariasis thus far led to confirmations of several expected obstacles:

a) Parasitic antigenic structures are highly complex and the use of crude antigenic preparations and corresponding sera usually led to wide spread cross-reactivities among related parasitic species or even with unrelated parasites.

b) These complexities are further aggravated by variation in antigenic expression among different stages of a parasite. Difficulties encountered in obtaining proper starting parasitic materials have been a major limitation in systematic analysis of stage specific antigenic molecules and/or epitopes. Of particular importance, has been the limitation of relevant materials for the detection of early stage of infection where

infective larvae and subsequent larval stages are needed and not readily available thus far.

c) Monoclonal antibodies described in the literature thus far represent "first generation" Mab's in that they were derived mainly from mice immunized with crude antigenic preparations from adult worms or microfilariae. Advantages in using polyclonal antibodies to help defining relatedness (common and species/stage specific) of the antigenic components have not been taken in combination with monoclonal antibodies development.

d) Armament of current tools for systematic analysis of antigenic structures usually exist where parasitic and clinical materials are not readily available and vice versa.

3. Objectives

The overall aim is to improve disease control by developing better immunodiagnostic tests for lymphatic filariasis as well as new tests for the detection of early, ie., premicrofilaraemic stage of filarial infection based on detection of stage specific antigens in the serum and urine. These tests will not only improve the efficiency of case diagnosis and epidemiological monitoring but will also make it possible to treat infected individuals before they become microfilaraemic and transmit the parasite to others thereby interrupting the spread of infection.

The specific objectives are to

1. analyse antigenic structure for the development of immunological reagents specific for *B. malayi* and *W. bancrofti* and those specific for various stages of the parasites.

2. devise simple, sensitive, specific, accurate and

inexpensive immunoassays for the diagnosis of clinical lymphatic filaria as well as for the detection of early asymptomatic infection.

4. Materials and Methods

4.1 Experimental animal model

4.1.1 Existing "full life cycle" animal model

The existing "full life cycle" animal models have been established with a re-entry grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (WHO/TDR) to the Principal Investigator. The existing "full life cycle" animal models consist of susceptible mosquitoes (*Aedes aegypti*, carrying homozygous *fm* genes); cats and gerbils (*Meriones unguiculatus*).

Cats less than one year old were obtained from houses in central Bangkok that possessed unwanted kittens. They were examined for natural infection of filarial parasites before using for experimental infection. Rabies vaccine and anthelmintic drug were given a few weeks after arriving the Animal Centre, Faculty of Science, Mahidol University. These cats were fed twice a day with appropriately cooked fish mixed with steamed rice. Commercial cat biscuits were also given for supplementation. Gerbils, on the other hand were the offsprings of those kindly given by Dr. D.A. Denham of London School of Hygiene and Tropical Medicine, London, England and Dr. Mak Joon Wah of Institute for Medical Research, Kuala Lumpur, Malaysia. Water and Commercial pellet diet were given *ad libitum*

4.1.2 Rabbits

Adult rabbits weighing about 2 kg were used for the production of hyperimmune sera against B. pahangi and against filarial human sera. The animal were purchased from the Animal Centre, Faculty of Veterinary, Kasetsart University and were kept at the Animal Centre, Faculty of Science, Mahidol University throughout the experimental period. They were fed ad libitum with water and commercial food pellet.

4.1.3 Mice

4.1.3.1 Swiss Albino mice

The animal were used for the production of polyclonal antisera to filarial and non-filarial worm antigens. These mice were obtained from the Animal Centre, Faculty of Science, Mahidol University. They were fed ad libitum with water and commercial pellet diet throughout the experimental period.

4.1.3.2 Balb/c/J mice

Eight week old female inbred strain of Balb/c mice were used for the production of monoclonal antibodies against B. pahangi and B. malayi antigens. The animal were bred under conventional conditions at the Animal Centre, Faculty of Science, Mahidol University.

4.2 Collection of worms

4.2.1 Filarial worms

4.2.1.1 Brugia pahangi and B. malayi

Various developmental stages of the parasites were obtained from the existing experimental animal models by the following techniques.

1) Infective third-stage larvae

As shown in Figure 1, the mosquitoes, Aedes aegypti were allowed to feed on microfilaraemic cats which have been anaesthetized by intraperitoneal injection with sodium pentobarbitone (Sagatal, May and Baker, Ltd.). The cats were previously infected subcutaneously with 70-90 infective third-stage larvae and only those harbouring 3-4 microfilariae per ul of peripheral blood (counted in chambers by the Technique of Denham et al, 1971) were used. Ten to twelve days after feeding, the mosquitoes were stunned by leaving them in a freezer (-20°C) for 2-3 minutes. They were then collected from the cage and sprayed with an insecticide which had been proved to have no effects on filarial larvae. The dead mosquitoes were lightly crushed with a glass test tube in a small volume of phosphate buffered saline (PBS) pH 7.2 on a glass plate (as shown in Figure 2) and washed into the sieve (mesh size 75 um) of a Baermann's apparatus filled with PBS (shown in Figure 3). Third-stage larvae migrated rapidly through the mesh of the sieve and were collected from the bottom of the apparatus (Denham et al, 1984, Chusattayanond and Denham, 1986). Four to five layers of gauze were placed on top of the sieve when clean larval preparations were needed.

2) Adult worms

Adult B. pahangi and B. malayi were obtained from peritoneal cavity of gerbils previously infected with 100-200 third-stage larvae of the parasite for more than 8 weeks (McCall et al, 1973). The worms were washed twice with PBS and kept frozen at -20°C in the presence of small volume of PBS.

3) Microfilariae

Microfilariae were obtained in BME (Basal Medium Eangle, BRL) when adult B. pahangi or B. malayi were maintained in vitro at 37°C in the presence of 5% CO_2. The larvae were separated from the culture medium by gravitational sedimentation at room temperature for a few minutes.

4.2.1.2 Wuchereria bancrofti

Since life cycle of this parasite cannot be maintained in the experimental animals, the only source of infection is the microfilaraemic patients living in the endemic areas of the disease at the border line between Western Thailand and Myanmar. Microfilariae and infective third-stage larvae were obtained as follow.

1) Microfilariae

Fifty ml of heparinized blood was obtained from microfilaraemic patients who carried 300-500 microfilariae/60 ul peripheral blood. A volume of 1 ml of the heparinized blood was mixed with 10 ml of PBS, pH 7.2. The diluted blood was filtered through a nuclepore membrane, 25 mm in diameter, 5 um poresize (Nuclepore Corporation, Pleasanton, CA) and microfilariae were recovered from the membrane by shaking in PBS and centrifuged at 1,000 rpm for 5 min. The larvae were washed once with PBS and the density was estimated under a dissecting microscope. The larval suspension was kept frozen at -20°C until used.

2) Infective third-stage larvae

Infective third-stage larvae of W. bancrofti were obtained from mosquitoes that had been membrane-fed with

microfilaraemic blood obtained from the patients (as shown in Figure 4). Larvae were separated from infected mosquitoes by exactly the same technique already described in paragraph 4.2.1.1. The larvae were washed once with PBS and kept frozen at -20°C in the presence of small volume of PBS.

4.2.1.3 Dirofilaria immitis

Adult male and female dog heart worms were obtained from microfilaraemic stray dogs sacrificed by the Bangkok Municiple Dog Pound. The worms were maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO_2 for 2 wks. Microfilariae were harvested daily from the medium by spontaneous sedimentation for a few minutes. The worm were stored frozen at -20°C until used.

4.2.2 Non-filarial worms

4.2.2.1 Angiostrongylus cantonensis

A complete life cycle of Angiostrongylus cantonensis has been established in the Department of Microbiology, Faculty of Science, Mahidol University (Techasoponmani and Sirisinha, 1980, Dharmkrong-at and Sirisinha, 1983). The Cycle involves the development of the larvae from the first to the second and the third stages in fresh water snails, Biomphalaria glabrata (Australorbis glabratus) and develop to the fourth stage larvae in albino rats.

(1) Infective thrid-stage larvae

The infective third-stage larvae were freed from the tissues of infected fresh water snails by artificial digestion with 1% pepsin, pH 2.0. The snails had previously been infected

with the first-stage larvae from rat faeces for 3 wks. The third-stage larvae were separated from the fluid by gravitational sedimentation and washed in 0.85% NaCl.

(2) Adult worms

Adult male and female worms were harvested from the pulmonary arteries of albino rats that had been infected with 50-60 infective third-stage larvae of *A. cantonensis* for at least 6 wks. The animals were ether-anaesthesized to death before the chests were opened and the arteries dissected.

4.2.2.2 *Gnathostoma spinigerum*

Infective third-stage larvae of this parasite were collected from the liver of naturally infected eels brought from a local market in Bangkok. Liver from several eels were pooled and digested at 37°C overnight with pH 2.0-pepsin solution (Dharmkrong-at *et al*, 1986). Living larvae were identified using morphological criteria described by Daengsvang (1980). The larvae were individually picked and separated from the digested liver tissue.

4.2.2.3 *Toxocara cati*

Female adult *Toxocara cati* were collected from faeces of a kitten receiving anthelmintic drug. The worms were washed several times with PBS before keeping frozen at -20°C in a small volume of PBS.

4.3 Antigen preparation

Crude aqueous somatic extract of the filarial and non-filarial worms was prepared as followed:

Worms of all stages of the parasites collected for somatic antigen preparations were washed 3-4 times with PBS and

kept frozen at -20°C in a small volume of saline until adequate amount was obtained. When preparing the antigens, frozen worms were thawed and homogenized in a ground glass tissue grinder in the presence of protease inhibitors (0.1 mM TPCK, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone and 0.1 mM PMSF, Phenyl methylsulfonyl fluoride, Sigma). The homogenate was broken up further by brief sonication. The sonicate was ready to be used as a whole worm extract or was centrifuged at 10,000 rpm for 30 min and the supernate was obtained as an aqueous extract. All the processes described were performed at 4°C. Protein concentrations of the aqueous extract were determined by a micromodification of Lowry method (1951). The antigens were stored at -20°C until used.

4.4 Collection of human sera

Human sera were collected for characterizing the filarial worm antigens. Venous blood was taken from normal people and filarial patients. Sera were obtained and stored at -20°C. Serum collected from the endemic areas were grouped according to their signs and symptoms as followed:

4.4.1 Normal human sera from non-endemic area

Sera were obtained from 20 ml of venous blood of 17 healthy staffs and graduate students of the Department of Microbiology, Faculty of Science, Mahidol University. These blood donors have never been travelling to any of the known endemic areas of lymphatic filariasis in Thailand. They have no previous history of any tissue parasite infection and no intestinal parasites were detected in any of their fecal

specimens.

4.4.2 Normal human sera from endemic areas of lymhatic filariasis

Sera were obtained from 5 ml of venous blood of healthy normal people living in endemic areas of B. malayi and endemic areas of W. bancrofti. These people have never had experience of any sign or symptom of filariasis. Number of cases and their residential areas were as followed:

Number of cases	Endemic areas of	Provinces
45	B. malayi	Suratthani
50	B. malayi	Nakornsri-thammarat
86	W. bancrofti	Tak

4.4.3 Filarial patient sera

Sera were collected from patients suffering from lymphatic filariasis in endemic areas of B. malayi and endemic areas of W. bancrofti in Thailand by the Filariasis Division, Department of Communicable Diseases Control, Ministry of Public Health.

Serum samples were grouped together according to signs and symptoms of the patients as followed:

Group I Sera from asymptomatic microfilaraemic patients

Group II Sera from microfilaraemic patients having lymphadenitis and lymphangitis

Group III Sera from amicrofilaraemic patients following successive treatment with diethylcarbamazine

Group IV. Sera from amicrofilaraemic patients with elephantiasis and still having lymphadenitis and

lymphangitis

Group V. Sera from amicrofilaraemic patients with elephantiasis and still having lymphadenitis and lymphagitis

Group VI. Sera from amicrofilaraemic patients having lymphadenitis and lymphagitis

Group VII. Sera from amicrofilaraemic patients with elephantiasis

Group VIII Sera from amicrofilaraemic patients with elephantiasis treated with diethylcarbamazine but still having lymphadenitis and lymphangitis

Group IX. Sera from amicrofilaraemic patients with elephantiasis treated with diethylcarbamazine and showed no other symptoms

Group X. Sera from microfilaraemic patients with elephantiasis

Group XI. Sera from a patient who spontaneously turned to amicrofilaraemic condition.

4.5 Production of polyclonal mouse antisera

Polyclonal mouse antisera to antigens of the lymphatic filarial worms as well as antigens of non-filarial nematodes have been produced for characterizing the filarial worm antigens. Ten groups of 3 mice were injected with 10 different somatic antigens as follow:-

1. female adult *Brugia malayi*
2. male adult *B. malayi*
3. female + male adults *B. pahangi*

4. female adult Dirofilaria immitis
5. male adult D. immitis
6. female adult Toxocara cati
7. female adult Angiostrongylus cantonensis
8. male adult A. cantonensis
9. third-stage larvae of A. cantonensis
10. third-stage larvae of Gnathostoma spinigerum

Before immunization, serum was obtained from each mouse and checked for the presence of antibodies against these antigens by ELISA. All mice were found to be non-reactive. Each animal was injected intraperitoneally with 200 ug antigen mixed with Freund's complete adjuvant. After 3 weeks, another dose of 200 ug antigen mixed with Freund's incomplete adjuvant was injected. Blood was collected from tail tip of each animal 2 weeks after this injection. Most of the animals gave high ELISA value and they were sacrificed for blood collection. The animals that gave low ELISA value were injected again with 100 ug antigen mixed with Freund's incomplete adjuvant. Their sera were checked and they were sacrificed to collect blood one week later. All antisera were stored at -20°C.

4.6 Production of monoclonal mouse antibodies.

The experiments were set for two fusions namely fusion no. 93 (F93) and fusion nos. 105, 106 and 107 (F 105, F 106 and F 107). The fusion no. 93 was performed as follow. Eight week old Balb/c/J mice were immunized 3 times with 60 ug/0.1 ml, 30 ug/0.1 ml and 30 ug/0.1 ml of female adult B. malayi extract mixed with Freund's complete adjuvant, incomplete

adjuvant and normal saline respectively. The first and the second immunization was given intraperitoneally, while the third one was given intravenously. Spleen cells from the immunized mouse ($1.1x10^8$ cells) were isolated 3 days after the last injection and fused with X63-Ag8.653 (.653) myeloma cells ($2.46x10^7$ cells), using polyethylene glycol Mw. 3700 (PEG, Sigma). Antibody production was assessed between day 14 to 17 by means of enzyme-link immunosorent assay (ELISA) utilizing B. malayi antigen fixed to ELISA plates (Costar) at concentration 5 ug/ml. Cultures secreting antibody (OD > 0.20 up to 1.65 at 490 nm by Titertek Multiskan Spectrophotometer II) were then assessed for crossreactivities with panel of antigens composed of extracts of adult male B. malayi, D. immitis (male and female), A. cantonensis (male and female), and third-stage larvae of G. spinigerum. Selected wells were then cloned by limiting dilution. Approximately 14 days after cloning the culture supernatants from each initial well were assessed for antibody production to B. malayi antigens. Selected colonies were then grown in tissue culture, frozen, and used for production of antibodies for immunoblotting assay later.

The second fusion was performed by a protocol slightly modified from the first one as follow.

1. Immunizing antigens were partially purified by SDS-PAGE. Protein molecules 19.5-25.0 kd and 14.3-19.0 kd were cleaved off from the gel and were given to Balb/c/J mice in order to prepare the animals for fusion nos. 105, 107 and 106 respectively. The fusion no. 105 and 106 were proformed by

using immune spleen cells, while the fusion no. 107 was performed by using immune lymph node cells. The summarization of the fusion protocol of the 3 fusions are shown in Tables 1.1, 1.2 and 1.3.

2. Time of fusion, we have to note at this point that we did not check polyclonal titer of the serum on Day 28 which was the second immunization. When we checked the polyclonal titer at Day 47 which was the 3rd immunization the O.D. might have already reached the peak. Unfortunately our myeloma cells (X 63-Ag 8, 653) seemed to have some toxicity and/or a slight contamination as it looked unhealthy. Therefore, we decided to postpone the fusion to day 57 (the fourth immunization) waiting for another batch of myeloma. After we sacrified the mice (Fusion 105 and 107 for 19.5-25.0 kd, Fusion 106 for 14.3-19.0 kd) we found that both immunized spleens had fibrosis. However, we still fused the immunized spleen cells the same way as Fusion no. 93.

4.7 Sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PADE)

Protein components of the parasite antigens were analysed by the technique previously described by Laemmli (1970). The samples to be electrophoresed were prepared in SDS-PAGE sample buffer containing 0.625 M Tris, 1% SDS, 10% glycerol and 5% 2-mercaptoethanol and boiled for 2 1/2 min. The gel was composed of a 3 cm- long 4% stacking gel buffered with 0.25 M Tris-HCl pH 6.8 and a 10 cm- long 10% separating gel buffered with 0.75 M Tris-HCl, pH 8.9 and electrophoresis was performed at 10 mA during the run in the stacking gel and at 5 mA during the run in the separating gel. At the end of the electrophoresis,

the gel was stained with Coomassie Brilliant Blue R or Silver stain (Merril et al, 1981). It was calibrated for molecular weight determination by running protein marker in parallel to the sample. The markers used were as followed: phosphoylase b (97,400), bovine serum albumin (66,000), egg albumin (45,000), trypsinogen (24,000) and lysozyme (14,300). All the markers were purchased from Sigma Chemical Company, USA. Approximate molecular weights were determined by the method of Weber and Osborn, 1979.

4.8 Countercurrent immunoelectrophoresis (CIEP)

The technique previously described by Laurell 1965 was used in the present study for detection of anti-filarial antibodies in human sera. A volume of 15 ml melted 1.5% agarose dissolved in barbitone buffer pH 8.6 was layered over a 8x10 cm^2 glass plate and allowed to solidify at room temperature. Fifty pairs of 2 mm diameter wells were made. The distance between wells of each pair was 5 mm. To run the electrophoresis, glass plates with the agarose gel were placed in a horizontal electrophoretic chamber containing barbitone buffer pH 8.6 having ionic strength 0.1. Two pieces of filter paper was used as bridges between the agarose and the buffer in the tank. Pre-electrophoretic running was performed by applying an electric current of 0.4-0.5 mA/cm width of the glass plate for 10-15 minutes in order gel to equilibrate and remove any salts from the agarose gel. Then equal volumes of 10 ul antigen and antiserum were applied in each pair of the wells. Electrophoresis was preceeded for 100 min at room temperature. After

electrophoresis, nonreacted proteins were removed from the gel by soaking in several changes of 0.85% NaCl. The salt was then removed by soaking in water and the gel was dried at 37°C. Precipitin bands were stained with 0.6% Amido Schwartz 10B.

4.9 Electrophoretic blotting and immunoenzymatic staining of protein antigens on nitrocellulose paper

4.9.1 Electrophoretic blotting

The parasite antigens already subjected to SDS-PAGE (50 ug/0.5 cm width) were electrophoretically transferred from the gel to sheets of nitrocellulose filter (pore size 0.45 um, Schleicher and Schuell, W. Germany) by the technique previously described by Towbin (1979). The process was performed by using an LKB 2051 (Bromma 1, Sweden, equipped with an iced-water cooling system operated at 60V for 3 hrs.

4.9.2 Immunoenzymatic staining of protein antigens on nitrocellulose paper

The blotted nitrocellulose filter was soaked in PBS containing 1% Tween 20 (PBST) for 30 min at room temperature. The filter was cut into 0.4 cm- width strips and immunological reaction was performed by standard technique using 5% skim milk as blocking agent and 2% skim milk as diluent for immune serum (polyclonal mouse antiserum or human serum) and the peroxidase conjugate second antibodies (HRP-conjugated rabbit antihuman immunoglobulins). The substrate was 0.5 mg/ml of 4-chloro-1-napthol containing 0.01% H_2O_2.

4.10 Enzyme-linked immunosorbent assay (ELISA)

An ELISA for antibody detection was performed by the technique slightly modified from the one previously described by

McLaren et al. (1981). Flat bottomed-96-well EIA plates (Costar, Cambridge, M.A.) were coated with optimum concentration of worm antigens at 4°C overnight. When the technique was used as the screening test for monoclonal antibody activities, the generally used concentration of the antigen at 5 ug/ml was coated onto the plate.

On the other hand, when anti-worm antibodies were detected in human sera, the optimum conditions of the assay were titrated by checker board titrations. A pool of sera collected from 17 healthy normal people in nonendemic area was used as negative reference serum and a pool of sera from 17 microfilaraemic patients who live in endemic areas of B. malayi was used as positive reference serum. Bovine serum albumin (BSA, Fraction V, Sigma, USA) at 2% concentration was used as blocking agent, while 0.05% Tween 20 in PBS (pH 7.2) was used as washing solution and 1% BSA in PBS was used as serum diluent. The second antibodies were horseradish peroxidase conjugated (DAKO, Denmark) and 0.01% o-phenylenediamine in 0.025 M phosphate-citrate buffer pH 5.5 containing 0.003% H_2O_2 was used as the enzyme substrate. The reaction was terminated with 8 N H_2SO_4 before the optical density (OD) was determined by a Titertek Multiskan spectrophotometer at 490 nm. Variations which occurred from plate to plate on different occasions were corrected by standardizing the OD of the positive reference serum which was included in every plate.

4.11 Monoclonal antibody based ELISA (MAb-ELISA) for detection of Brugian parasite antigen

The technique slightly modified from the MAb-ELISA

described by Sirisinha et al. (1991) was used to determine the diagnostic potential of McAbs obtained in the present study. The technique is principally a sandwich ELISA based on an idea that the McAbs are to be sticked onto an EIA plate (Costar) which have already been coated with rabbit-anti-mouse IgG (DAKO, Denmark) and use this to detect for the filarial antigen in patient serum. The reaction between McAbs and circulating antigen in a clinical specimen is then visualized by using an amplification system composing of biotinylated-Rabbit-IgG-anti-B. pahangi somatic antigen, Streptavidin-peroxidase labeled and o-phenylene diamine substrate. The technique was performed stepwise as followed:

4.11.1 Purification and concentration of McAbs

McAbs were precipitated from culture supernatants with half saturated ammonium sulfate solution at $4^{o}C$. The precipitin was redissolved in 0.85% NaCl to a 12.5 times concentrated solution and dialysed against several changes of 0.85% NaCl until no sulfate salt was detected in the dialysate. McAb solutions were stored at $4^{o}C$ in the presence of 0.05% NaN_3.

4.11.2 Preparation of Biotinylated rabbit-anti-B. pahangi somatic extract

4.11.2.1 Preparation of rabbit IgG-anti-B. pahangi somatic extract

Two adult rabbits weighing about 2 kg were hyperimmunized with 750,400 and 400 ug of aqueous somatic extract of adult B. pahangi by the schedule previously described in paragraph 4.5 of the present report. Hyperimmune serum obtained from these rabbits were ammonium precipitated and

purified by passing through a DEAE-cellulose column buffered with 0.02 M phosphate buffer pH 8.0. The filtrate was concentrated by ultrafiltration to a twice concentrated solution and stored frozen at -20°C.

4.11.2.2 Biotinylation of rabbit IgG-anti-*B. pahangi* somatic extract.

The purified rabbit IgG-anti-*B. pahangi* somatic antigen was biotinylated by the technique previously described by Nerurkar *et al*, 1984. The antibody solution was diluted to 2 mg/ml with 0.1 M $NaHCO_3$ pH 8.2 and dialysed at 4°C, overnight. The solution was centrifuged at 1,000 rpm, 4°C for 10 min before its concentration was adjusted to 1 mg/ml with 0.1 M $NaHCO_3$. A 2 mg/ml solution of N-Hydroxysuccinimidobiotin (SIGMA) in DMSO was freshly prepared just before it was immediatedly added to the IgG solution to make the biotin/IgG ratio equal to 0.17/1. The solution was mixed gently at room temperature for 4 hrs. The resulting solution was dialysed against 0.15 M PBS at 4°C for 48 hrs. It was mixed with an equal volume of glycerol, aliquotted and stored at -20°C.

4.11.3 MAb-ELISA

Flat bottomed-96-well EIA plates (Costar) were coated with a 2-fold serial dilutions of 40-2.5 ug/ml Rabbit-anti-mouse IgG at 4°C overnight and blocked with 2% BSA. McAb at concentration 0.01125 or 0.0225 or 0.045 or 0.450 mg/ml was added to the wells and allowed to incubate at 37°C for 1 hr. It was followed by an addition of a 2-fold serially diluted *B. pahangi* somatic extract at 2000-0.95 ng/ml. The reaction was allowed to

take place overnight at 4°C before 10 ug/ml of biotinylated rabbit IgG to B. pahangi was added to each well. It was incubated at 37°C for 2 hr and the colour reaction was proceeded by an addition of a precalibrated quantity of peroxidase-conjugated streptavidin (Sigma, St. Louis, Mo., U.S.A.) and incubated at 37°C for 30 min followed by an addition of o-phenylenediamine substrate. The colour reaction was allowed to take place in the dark at room temperature for 1 hr before it was stopped with 5 N H_2SO_4. The O.D. values was measured at 490 nm by an ELISA reader (MINIREADER II, DYNATECH, U.S.A.).

4.12 Immunofluorescence Assay

The localization of the tissue sources of antigens in adult worms was performed by indirect immunofluorescence technique. The worms were fixed in 2% paraformaldehyde in 0.1 M sodium cacodylate buffer for 30 min at 4°C. After washing with the same buffer, they were mounted in a -24°C Cryocut cabinet with the Tissue Tek O.C.T. embedding medium. The 6 um sections were transferred on to slide which had been coated with 0.5% gelatin. After drying at room temperture, the sections were incubated with the primary antibodies which included immune sera from human infected by Brugia malayi and Wuchereria bancrofti, cat immune sera infected by B. malayi and B. pahangi and jird immune sera infected by B. malayi and B. pahangi. The incubation were done in the moist chamber at 37°C for 30 min. Following extensive washing with 0.1 M PBS, the sections were incubated with the corresponding fluorescein-conjugated secondary antibodies at 37°C for 30 min in the moist chamber. After washing 3 times with the same buffer, the sections were mounted

in 9% buffered glycerol. The slides were examined under Olympus fluorescent microscope, using incident illumination.

The cuticular antigens of the L3 were localized by incubating the whole parasites in the primary antibodies described above for 30 min at 37°C. After extensive washing with 0.1 M PBS, they were incubated in the corresponding fluorescien-conjugated secondary antibodies for another 30 min. After washing, the parasites were transferred onto the gelatin coated slides and mounted with buffered glycerol. Examination of the cuticular antigens of L3 was also carried out under the fluorescent microscope.

5. Results

5.1 Immunological characterization of human sera

Since normal human sera and sera from filarial patients were to be used for characterizing filarial worm antigens, we needed to know some of their characteristics such as their specificities and levels of their antibody activities. Therefore in our work the human sera collected were tested for the presence of antibodies to somatic antigens of adult female *B pahangi* by CIEP and tested aainst adult female *B. malayi*, adult female *D. immitis* and advanced third-stage larvae of *G. spinigerum* by ELISA. Optimum concentrations of antigens and sera to be used in the assay were titrated by checker board titrations (see Figs. 5 and 6). A pool of normal human sera from non-endemic areas was used as negative reference serum and a pool of sera from 17 microfilaraemic patients living in endemic areas of *B. malayi* was used as positive reference serum. Results of the checker board

titrations indicated that the optimum concentration of the antigens to be used in the ELISA is 5 ug/ml and the optimum concentration of the filarial patient sera to be tested is 1:1600.

5.1.1 Detection of serum antibodies in filarial patients in endemic areas of Brugia malayi infection

5.1.1.1 Detection by counter current immuno-electrophoresis

Human sera were roughly categorized into 3 groups and were tested for the presence of precipitating antibodies against adult female B. pahangi somatic extract. The 3 groups of sera include:-

a) B. malayi infected patients showing elephantiasis of the leg(s). They are all amicrofilaraemic.

b) W. bancrofti infected patients suffering from hydrocoele for 1-10 years. All are amicrofilaraemic.

c) People living in the endemic areas of B. malayi who had experience of lymphadenitis and lymphangitis but show no symptoms at present time. They had never been found to be microfilaraemic.

As shown in Fig. 7 and 8 most of the sera obtained from amicrofilariaemic patients infected with B. malayi reacted strongly with the somatic extract of B. pahangi adult worms. Sera obtained from symptomatic amicrofilaraemic patients infected with W. bancrofti did not react with the B. pahangi antigen in this experiment. It was clearly demonstrated that persons having experience of lymphadenitis and lymphangitis developed strong

antibody response against the parasite antigen (see Fig. 8; S3 h-j, S4 and S5).

5.1.1.2 Detection by ELISA

As shown in Table 2 100% of normal control sera gave negative reaction while about 40-60% of people in the endemic areas gave positive reaction to the three antigens. Figs. 9-12 show that mean OD value obtained from sera of normal people in endemic areas is significantly higher than the mean OD obtained from normal control ($P<0.05$). This result seems to suggest subclinical infections to those people who live in endemic areas of the filarial parasite. When comparing between the reactions of each group of patient sera and the reactions of normal control, all patient sera gave significantly stronger reaction ($P<0.05$), while only sera in group I and II reacted significantly stronger to the three antigens when compared to the reaction of sera from normal people in the endemic areas ($P<0.05$). When comparing among groups of the patients, those who have microfilaraemia either without (group I) or with symptoms of lymphadenitis and lymphangitis (group II) gave the strongest ELISA reactions to the three antigens (Fig. 9). When tested with *B. malayi* antigen, sera in group I gave the mean OD value at 0.9 and sera in group II gave the value at 0.7 (Figs. 9 and 10). While 95% of cases in group I gave positive reaction, 100% of group II gave positive reaction. As can be noticed in our results, sera obtained from normal people and also filarial patients in endemic areas of *B. malayi* reacted significantly with antigens of *G. spinigerum* (Figs. 9 and 12) when comparing to the reaction obtained with the negative control sera ($P<0.05$).

However, the OD values obtained with G. spinigerum are always lower than those obtained with the two filarial antigens. These differences are statistically significant in almost every group ($P<0.05$) except in normal from endemic areas and group IV, V and VII. The antibody reactivities of these sera to G. spinigerum antigen is more likely to be due to cross reactivities among different nematode antigens than to be due to the experience of these people with G. spinigerum infection. This idea is based on the fact that most people in the endemic areas of B. malayi in Southern Thailand do not eat raw meat. Moreover, the antigen of G. spinigerum advanced third-stage larvae has been proved to be good at detecting the presence of infection with this parasite (Dharmkrong-at et al, 1986). The ELISA value obtained with this antigen is expected to be much higher (1.00) if those people are carrying the infection. The knowledge that our filarial sera reacted with antigen of G. Spinigerum will be applied to our further study when we make use of these sera in characterizing specific antigens of our lymphatic filarial worms i.e., B. malayi and W. bancrofit

5.1.2 Detection of serum antibodies in filarial patients in endemic areas of Wuchereria bancrofti

Since we still do not have enough W. bancrofti antigens to be used in ELISA, we therefore tested antibodies in sera of patients in W. bancrofti endemic areas with the previously described antigens. Figures 13-16 show the OD values obtained from the ELISA of sera from normal people in endemic areas and sera from different groups of W. bancrofti infected

patients tested against antigens of B. malayi, D. immitis and G. spinigerum. Results are quite similar to those obtained with B. malayi infected patient sera. Sera from normal people in endemic areas of W. bancrofti and also patient sera in group I and III gave higher ELISA values than those given by the negative control ($P<0.05$) when tested with the three antigens. Moreover, sera from microfilaraemic patients gave the highest antibody reaction against B. malayi antigen when comparing among all groups of the filarial sera ($P<0.05$). As summarized in Table 3, 84.78% of sera from microfilaraemic patients gave positive ELISA result with B. malayi antigen. However, 91.30% of group I and 84.61% of group III gave positive results with G. spinigerum antigens. Nevertheless, it is too early to make any conclusion about these filarial patient sera since too small number of cases were tested and antigens of W. bancrofti have not been used. Moreover, the possibility of these "Karen" people in acquiring G. spinigerum infection is quite high. It is therefore remained to be further investigated.

5.2 Protein profiles of filarial worms

5.2.1 Brugia pahangi and B. malayi

Five adult female and 5 adult male worms of each Brugia species were homogenized in 80 ul SDS-PAGE sample buffer and boiled for 2 1/2 min. A portion of 5 ul of each sample was electrophoresed in 10% SDS-polyacrylamide gel. After staining with Coomassie Brilliant Blue R, patterns of the protein compositions of the two Brugian parasites were compared. As shown in Fig. 17, adult Brugia worms are composed of at least 42 proteins of different sizes ranging from 180.5 kd to 13.0 kd.

They are composed of several major bands having estimated molecular weight around 150.0, 46.0, 35 and 14.3 kd. Although the protein patterns of these two species of Brugia worms looked similar, some differences are recognized in the areas equivalent to approximate molecular weight of 93.0 kd, 70.0 kd, 64.0 kd, 25.9 kd and 18.5 kd.

5.2.2 Wuchereria bancrofti

Soluble aqueous extract of W. bancrofti microfilariae was subjected to 10% SDS-polyacrylamide gel electrophoresis and stained with silver nitrate staining. As shown in Fig. 18, the somatic extract of microfilariae are composed of at least 44 proteins of different sizes ranging from 95.0 to 13.0 kd. Six major components were demonstrated to be 72.5, 47.5, 33.5, 26.5, 16.5 and 13.0 kd. When comparing to the extract of white blood cells obtained from normal human, several protein components in the microfilariae extract were found to have molecular sizes equivalent to those in white blood cell preparation (see Fig. 19). It is not known at this stage whether these components are the proteins belonging to the white blood cells contaminated into the microfilarial preparation or they are the protein of parasite that share the same characteristics with those belonging to the hosts' white blood cells.

Comparison between the soluble aqueous extract of W. bancrofti microfilariae and that of adult B. malayi was also performed. As shown in Fig. 19, the gel stained with silver nitrate staining showed the existence of proteins having the same molecular sizes in both the somatic extract of W. bancrofti

microfilariae and adult B. malayi. Sharing proteins of equivalent molecular sizes among these two worms and human blood cells were also demonstrated in the same gel.

5.3. Characterization of filarial worm antigens by immunoblotting assay

5.3.1 Immunoblotting of female adult Brugia malayi somatic extract probed with polyclonal mouse antisera

Western blots of female adult B. malayi somatic extract was probed with antisera of mice hyperimmunized with somatic extracts of adult B. malayi, adult B. pahangi, female adult T. cati, adult D. immitis and advanced third-stage larvae of G. spinigerum. As shown in Fig. 20, polyclonal mouse antisera of all specificities reacted strongly with high molecular weight antigens (40 to 200 kd) of female adult B. malayi (strips A, B, C, E, G, H and I), while pre-immune mouse serum (strip D) and reference negative human serum (strip J) did not react with any of these antigen components. It was clearly shown in this experiment that only mouse antisera to adult worms of B. malayi and B. pahangi strongly reacted with low molecular weight antigen components at 21.5 kd (strip B) and 14.3 kd (strip C) respectively. When the reference positive filarial patient serum was used, the high molecular weight antigens were also reacted, but the most distinctively created band was found to be the low molecular weight ones at 25 kd and 18 kd (strip I). However, the band 18 kd was not clearly demonstrated in the photograph.

5.3.2 Immunoblotting of female adult Brugia pahangi, Dirofilaria immitis and thid-stage larvae of Gnathostoma spinigerum probed with human sera obtained from B. malayi endemic areas

Antigenic components of female adult B. pahangi somatic extract recognized by human sera (Fig. 21.A) were compared with those of female adult D. immitis and third-stage larvae of G. spinigerum recognized by the same set of human sera (Figs. 21.B and 21.C). As shown in Fig. 21.A, only the serum from microfilaraemic (strip C) and symptomatic microfilaraemic patients (strip E) reacted strongly with the 25 kd antigen of B. pahangi. Sera from other groups of patient did not recognize this antigen of either D. immitis (Fig. 21.B) or G. spinigerum (Fig. 21.C). These results together with those demonstrated in Fig. 20 indicated that the 25 kd Brugia antigen is to be recognized by sera from patients with patent infection of B. malayi.

5.3.3 Immunoblotting of female adult Brugia pahangi somatic extract probed with human sera obtained from Wuchereria bancrofti endemic areas

As shown in Figs. 22.A and 22.B, most of the human sera from Tak and Kanchanaburi provinces reacted strongly with 92.5 and 69 kd antigenic components of both B. malayi (22A) and B. pahangi (22B) somatic extracts. When compared these results to those obtained with polyclonal mouse antisera hyperimmunized against other nematodes, it was clearly demonstrated in Fig. 20 that the 92.5 kd molecule was also reacted with antisera against T. cati (strip E), D. immitis (strips F and G) and G. spinigerum (strip H), while the 69 kd

molecule did not react with any of these mouse antisera. The results suggested that the molecule 69 kd might carry the antigenic epitope(s) of female adult Brugia worms to be cross-reacted with antisera of most of the W. bancrofti cases. In the present experiment (Fig. 22A and B), it was found that the sera that reacted with the epitope(s) on the 69.0 kd molecule are those collected from DEC-treated amicrofilaraemic cases (strip B), normal individuals from endemic areas (strip C), DEC-treated elephantiasis cases (strip D), microfilaraemic elephantiasis cases (strip E) and a microfilaraemic patient who lately turned to amicrofilaraemic status spontaneously (strip G). However, the band was shown up more solidly with the molecule from B. malayi than with the molecule from B. pahangi. Apart from these, the results obtained in the Fig. 22 also confirmed our early conclusion that the antigenic epitope(s) carried by the 25 kd molecule is of Brugia specific and is recognized by sera from individuals patently infected with B. malayi (see also Figs. 20 and 21). It was also demonstrated in the experiment that the band 14.3 kd was heavily shown up when antigen of B. pahangi was tested with sera from amicrofilaraemic elephantiasis patients (strip D, Fig. 22-B) as well as when tested with the reference positive sera (strip H, same Fig.). This molecule was also shown up, but faintly, when the reference positive serum was allowed to react with B malayi antigen (see Fig. 22-A, strip H). We still cannot make any conclusion whether the 14.3 kd molecule has any immunological significance.

5.4 Localization of the source of antigens in Brugia pahangi and B. malayi

5.4.1 Morphology of adult Brugia pahangi

Semithin plastic embedded adult worm sections stained with hematoxylin-eosin (HE) were also carried out in parallel with the immunofluorescence staining, since the former could reveal the detail of the morphology which will be used as a guideline for the immunostaining. The HE sections revealed the organization of organs in the worms' bodies at the light microscopic level. As shown in Fig. 23 A-G, the body wall of the adult worms was composed of the cuticle, lined by the hypodermis and somatic musculature. The smooth cuticle was about 1 um thick, with the outermost layer appear most delicate and basophilic. The hypodermis was thickened to form four hypodermal cords which divided the somatic musculature into four quadrants. The lateral cords were prominent except when compressed by gravid uteri (Fig. 23 A-D), and each occupied about one-fifth of body circumference and appeared granular or vacuolated and basophilic. The somatic musculature appeared as large dorsal and ventral bundles, where the dorsal and ventral cords were small. An extensive system of basal laminae segregated all structures from the pseudocoel which was occupied by the gut and the reproductive tract. The gravid uteri contained several stages of developing microfilariae within the uterine lumen. The uterine wall has two distinct layers, with the thin epithelial cells bound by a thick basal lamina and layers of visceral muscle cells. The testis (Fig. 23 E, F) surrounded by a thin wall whose lumen was occupied by closely packed immature spermatocytes. Seminal

vesicle (Fig. 23 G) wall was not uniform in thickness but appeared distinctly basophilic. Sperms appeared as dotlike basophilic granules.

5.4.2 Detection of the tissue sources of antigens in adult *Brugia pahangi*

The indirect immunofluorescence technique was carried out on 50 samples of both sexes for one type of primary antibody. The sections showed satisfactory fluorescence at 1:2 to 1:4 dilution of the primary antibodies together with 1:20 dilution of the fluoresceinated secondary antibody. Nonspecific fluorescence appears as pale green while the specific fluorescence appears as bright green. The intensity of the fluorescene in cuticles and internal organs when stained with different antibodies was compared and the result was shown in Table 4.

The cryocut sections processed by the indirect immunofluorescent technique were compared to HE sections for the structural organization and the approximate thickness of various layers, and the basal laminae. To ensure the unbias comparison of fluorescence between slides, photographs of sections were taken using equal exposure times, and the positives subsequently compared for the intensity of reaction. All antisera, when used at the dilution of 1:2 to 1:4 followed by fluoresceinated goat antibody to IgG of the corresponding animals at 1:20 dilution gave the most satisfactory result. The fluorescence was present on the cuticle, hypodermis, somatic musculature, the gut wall, the uterine wall, the egg shell, and the male reproductive tract.

The most intense fluorescence appeared as bright green zones and were confined along the basal lamina lining the body wall (Figs. 24, 25, 26), the basal lamina and luminal aspect of gut epithelium (Figs. 24 A, B; Fig. 25 A, B, D), the basal lamina of testis and seminal vesicle (Fig. 35, D, E) and the basal lamina and luminal aspect of the uterine epithelium. The continuous and intensely green lines appear on the egg shells enveloping the developing microfilariae in utero (Fig. 24, A, B; Fig. 25 A, B; Fig. 26 A, B). In some preparations (Fig. 24 D, E; Fig. 25 D, E; Fig. 26 E) the outermost layer of the cuticle also showed the characteristic bright green line.

The hypodermis and the musculature showed uniformly homogeneous fluorescence with moderate intensity. Microfilariae within the bright egg shell and the gut content showed moderate to light fluorescence, while content of the male reproductive tract showed a varying degree of fluorescence among different sections, with the high intensity on the spermatogonia within the testis (Fig. 26 E) and the sperms within the seminal vesicle (Fig. 24 D, E; Fig. 25 D)

5.4.3 Detection of the cuticular antigens of L3 *Brugia pahangi* third-stage larvae

The whole mount of L3 processed for indirect immunofluorescent technique, using the same dilution of primary and secondary antibody as indicated above, showed intense fluorescence along the entire cuticle of L3 which appeared as a continuous bright green line in all samples and all kinds of antibodies being used (Fig. 27, A-F), while in the control sections , using PBS instead of the primary antibody there was no

fluorescence (Fig. 27 G).

5.4.4 Morphology of adult Brugia malayi

In cross sections of the female (Fig. 28 A, B), the pale curticle covered the whole body wall of the worm. The cuticle thickened in the region of lateral cords at both sides. Underlying the cuticle was the darkly stained hypodermis which expanded into pseudocoel along mid dorsal, mid ventral and mid lateral of body wall to form longitudinal cords that extended through the length of the body; thus there were one dorsal, one ventral and two lateral cords, respectively. The dorsal and ventral cord were pedunculated in shape while lateral cords were rectangular. The muscular layer was composed of a single row of cells lying immediately below the intercordal hypodermis between each pair of hypodermal cords, and was divided by the cords into four quandrants. The pseudocoelom was occupied by two female reproductive tracts and the gut. The female reproductive tracts could be either ovary, oviduct, seminal receptacle or the uterus depending on the level of the sections. Sections in Fig. 28 A and B at the uterus level showed the epithelium enclosed the developing microfilariae, each of which was surrounded by the egg shell. Figure 28 A should be at the upper level and 28 B at the lower level of the uterus. At the upper level of the uterus (Fig. 28 A) the epithelium was thick and the young microfilariae, while was individually surrounded by an egg shell, were packed close to one another, and almost occupied all the lumen of the uterus. Some dead embryos were also found in the centre of the lumen. The epithelium of the uterus at lower level was very thin

and the more mature microfilariae were loosely packed together leaving some space in the uterine lumen. The intestine lying at one side of the section, showed distinct epithelium and lumen.

The cross sections of the male (Fig. 28 C, D) appeared almost the same as the female except for the much smaller body size and the presence of only one reproductive tract. The pseudocoelom was occupied by only one reproductive tract and gut. The male reproductive tract could be either testis, spermatic duct, seminal vesicle or vas deferens depending on the level of the sections. Fig. 28 C showed the testis with rather indistinct epithelium occupied by tightly packed developing spermatozoa. Fig. 28 D showed the seminal vesicle and vas deferens which was filled with spermatozoa. This section showed thicker somatic musculature and narrower lateral cords than in Fig. 28 C and in the sections of female. The intestine lied at one side of the sections showing distinct epithelium and lumen.

5.4.5 Detection of the tissue sources of antigens in adult *Brugia malayi*

All the cryostat sections of both male and female worms reacted with all antisera from various hosts, and showed similar result except for variation between the reproductive tracts of different sexes. Any tissue areas having antigen specific to each antiserum appeared as greenish fluorescence. The intensity of fluorescence of positive areas can be graded into 3 levels, which are : intense, moderate, and light fluorescence, while there was no label on the areas devoid of antigens and in control sections. The comparison of intensity in different tissues were shown in Table 5. The basal lamina of

the body wall lining the pseudocoel of the gut, of the uterus, of the testis, and the uterine epithelium, the egg shell and the sperm surface showed intense fluorescence. The cuticle, hypodermis, all of the cords, muscle, gut epithelium, uterine epithelium, testis epithelium and sperm interior showed moderate fluorescence while the egg interior showed only light fluorescence (Figs. 29-31).

5.4.6 Detection of the cuticular antigens of *Brugia malayi* third-stage larvae

The infective stage larvae showed similar fluorescence at the cuticle in all antisera (Fig. 32 A-F). No fluorecence had been found on the cuticle of control infective stage larvae (Fig. 32 G).

The microfilarial stage was observed in the uterus of adult female surrounded by the sheath of the egg shells (Figs. 24 A, B; 25 A, B; 26 A and B). These developing microfilariae showed light fluorescence while the sheath or egg shell showed intense fluorescence (Table 8; Figs. 24 A, B; 25 A, B; 26 A, B).

In summary, in both species of *Brugia* the staining pattern and the intensity of fluorescence in tissues in parasites bodies are quite similar regardless of the source of immune sera. Hence it could be concluded that the tissue sources of antigens in both species of parasites are the same and consist, in order of fluroescence intensity and presumably the quantity of antigens of epicuticle, all basal laminae, uterine epithelium, egg shell, sperm membrane, testicular epithelium and its content, the gut

epithelium and its content, the cuticle, hypodermis and muscle, respectively. The antigens in each parasite also exhibit strong cross reaction to antisera against another parasite species. Therefore, the major antigenic sources in these filariae are epicuticle, epithelia of reproductive tract and the surface of egg and sperm cells; and that antigens present in these structures show extensive interspecific cross reactions.

5.5 Characterization of monoclonal antibodies

5.5.1 Anti-nematode activities

5.5.1.1 Characterization by ELISA

The average values obtained in the fusion no. 93 (F93) experiment were (Table 1): 93% of the total initial wells plated showed growth of hybrid colonies (225/240); 55% of this hybrid-containing wells (133/240) produced antibodies to *B. malayi* antigens (OD > 0.20) and 2.25% of hybrid containing wells (3/133) were producing *Brugia* sp. - specific antibodies. These three hybrids designated F93-3B11, F93 3C7, F93-4E10 from initial well were scaled up in tissue cultures and would be used for production of ascites fluid in Balb/c mice. The titer of polyclonal antibody from immunized mouse on Day 50 (3-days before fusion) was 1:51:200 (OD = 0.78) and this serum was used as positive control, while serum from normal BALB/C/J mice was used as negative control and 1% BSA as blank. We have tested for the suitable blocking agent to be used in the ELISA system and found out that 2% BSA gave the same result as 5% skim milk and 1% ovalbumin. Therefore, we decided to use 2% BSA according to the convenience. In the second set of fusions consisting of F 105, F 107 and F 106 the results of no. wells containing hybrid cells

no. total wells were 203/360 (56.38%), 71/120 (59.1%) and 173/300 (57.67%) respectively (see Table 1.1, 1.3 and 1.2). Among all the 203 wells containing hybrid cells from F 105, only two wells (0.98%) namely F 105-4E8 and F 105-1D10 gave positive ELISA values at OD >0.15 and >0.74 respectively. Fusion no. 107 which was performed by using immune lymph node cells of the same mouse as F 105 gave four positive clones namely F 107-1D11 (OD = 0.22), F 107-1G6 (OD =0.51), F 107-1E11 (OD = 0.66) and F 107-2G2 (OD = 0.71) (see Tables 1.3 and 6).

On the other hand, Fusion no. 106 which was performed by using immune spleen cells from the mouse hyperimmunized with the 14.3-19.0 kd antigen gave four positive clones namely F 106-1E4 (OD=0.52 and increased to 1.02 after 2-3 wks), F 106-3G2, (OD = 0.17), F 106-4F9 (OD = 0.11) and F 106-5G6 (OD = 0.21) respectively (see Tables 1.2 and 6).

5.5.1.2 Characterization by immunoblotting assay

Immunological specificities of the monoclonal antibodies 3B11, 3C7 and 4E10 were further analysed by immunoblotting assay against somatic extracts of both the filarial and non-filarial nematode. The McAbs were taken to the London School of Hygiene and Tropical Medicine by the PI when he visited Dr. D.A. Denham in May 1990. The McAbs were tested in his laboratory against western blots of somatic extract of *B. pahangi* third-stage larvae. It was found that the McAbs 3C7 and 4E10 reacted strongly in Fig. 33, the 3C7 supernatant reacted with epitopes on molecules 74, 71, 55, 51, 47, 27 and 25 kd, while 4E10 reacted with epitopes on 28.5, 27.5 and 24 kd

molecules (the 24 kd band was not clearly reproduced when photographed). The McAb 3B11, on the other hand, did not react with any molecule of the antigens.

We have further characterized these McAbs in our laboratory at Mahidol University by allowing them to probe onto western blots of somatic extracts of female adult B. malayi, female adult D. immitis and third-stage larvae of G. spinigerum. We found that all the three McAbs reacted with somatic antigens of B. malayi. The McAbs 3B11 and 3C7 reacted with the high molecular weight antigens, while the McAb 4E10 reacted with the low molecular weight ones. As shown in Fig. 34, the McAb 3B11 reacted with 95 and 93 kd antigens, while the 3C7 reacted with several antigenic components having molecular weight ranging from 123 to 64. The McAb 4E10, on the other hand, was found to react with epitope on 32, 30 and 27 kd molecules. No reaction was detected when these McAbs were tested against antigens of D. immitis (Fig. 35). When somatic antigens of G. spinigerum third-stage larvae was used, we found only the reaction with McAb 3B11 which recognized the epitopes on molecules 35 and 32 kd molecules as shown in Fig. 36.

It can be concluded from the results of ELISA and immunoblots that the McAbs 3C7 and 4E10 do not react with antigens of D. immitis or G. spinigerum, but reacted with Brugian antigens. If we could find out that they do not react with W. bancrofti antigen, we will be able to make a more solid conclusion that the McAbs 3C7 and 4E10 are the antibodies of Brugian specificities. Unfortunately we do not have any more

supply of W. bancrofti antigens to perform any further analysis.

5.5.2 Isotype

Immunoglobulin isotype of the McAbs 3B11, 3C7 and 4E10 were identified by using a reagent kit supplied by ZYMED, California, U.S.A. It was found that all of these McAbs are IgG1 having kappa type light chains.

5.6 Demonstration of an evidence indicating the presence of circulating Brugia malayi antigens in our filarial patient sera.

We have tried to demonstrate an evidence showing that the filarial parasite antigen does exist in our filarial patient sera by using both a direct and an indirect techniques as follow.

5.6.1 Demonstration by a direct technique

In this experiment Western blots of B. malayi microfilaraemic patient sera diluted to 1:5, 1:10 and 1:20 were prepared and they were analysed by immunoenzymatic reactions against hyperimmune mice sera. The sera were obtained from animals that had been immunized with aqueous somatic extract of adults B. malayi, B. pahangi, D. immitis, A. cantonensis and advanced third-stage larvae of G. spinigerum. The tests were also performed with sera of normal individuals living in B. malayi endemic areas and sera of normal individuals in non-endemic areas.

It was found that we could not demonstrate any distinct specific antigenic band on any of the reactions as shown in Figs. 37, 38 and 39. We therefore, moved to an indirect technique.

5.6.2 Demonstration by an indirect technique

By an indirect way we firstly immunized rabbits

with filarial patient sera, then the rabbit sera were collected and assayed for anti-filarial antibodies against somatic extract of adult B. pahangi by ELISA. In the actual experiment, three groups of filarial sera from B. malayi endemic areas including normal, microfilaraemic and symptomatic microfilaraemic individuals were given to six rabbits. Two other rabbits were also immunized with normal human sera from non-endemic areas. The immunization was done by subcutaneous injection of 3 doses of 1.0, 1.0 and 0.5 ml of serum mixed with equal volume of Freund's complete adjuvant, Freund's incomplete adjuvant and 0.85% NaCl respectively. Each injection was done at two weeks interval. Five ml of blood was collected from each rabbit 4 days after the third injection and ELISA activity of the serum was checked against the immunizing serum. We found a very high antibody activities in these sera. Therefore, 40 ml of blood was collected from each rabbit and tested for the anti-B. malayi antibody activity. Results are shown as histograms in Fig. 40. The microfilaraemic patient sera at dilution 1:400 gave the OD value at 490nm as high as 1.05 while sera from normal and symptomatic microfilaraemic individuals at the same dilution gave the OD values as low as 0.10 and 0.12. We concluded at this stage that a detectable level of filarial antigen does exist in our microfilaraemic patient sera. However, further analysis by using immunoblot reactions against filarial and non-filarial antigens will be carried out. If we find the antibodies to be Brugia specific or even-filarial specific we will try to make use of these antibodies in the development of serodiagnostic assay.

5.7 Study for the diagnostic potential of Monoclonal antibodies 3B11, 3C7 and 4E10.

We have tried to test for the diagnostic potential of the three McAbs which we have partially characterized by using a monoclonal antibody based-ELISA. The checker board titration between serial dilutions of 2000-0.95 ng/ml of adult B. pahangi somatic extract and serial dilution of 40-2.5 ug/ml of Rabbit-anti-mouse IgG at concentrations of 0.01125, 0.0225, 0.045 and 0.450 mg/ml of McAbs 3B11, 3C7 and 4E10. The titration between the Rabbit-anti-mouse IgG and the antigen indicated that the varying concentrations of the rabbit antibodies resulted in only a slight variations of ELISA values. However, we decided to use the rabbit antibodies at the most reasonable and economical concentration of 5 ug/ml. On the other hand, we have found the optimum concentration of the McAb to be 0.045 ug/ml. As shown in Fig. 41, comparison among the three McAbs at 0.045 ug/ml when the plate was coated with 5 ug/ml of Rabbit-anti-mouse IgG indicated that the McAb 3C7 is the best one having the strongest binding affinity to the filarial antigen. We also tried to analyse the reactivity of a 'cocktail' composed of the three McAbs mixed at the ratio 1:1:1 and found the reactivity to be as high as that of the McAb 3C7 (Fig. 41). However, the sensitivity of the assay we have obtained is still very low. It is therefore doubtful whether the McAbs 3B11, 3C7 and 4E10 will have any diagnostic potential. We therefore, tried to regrow 15 frozon clones obtained from the previous fusion (fusion no. 93). These clones have ELISA characteristics against antigens of B. malayi, D. immitis, A. cantonensis, G. spinigerum L3 and T. cati as shown in

Table 7. Unfortunately the cells grew up weakly and finally died. We therefore stopped at this point.

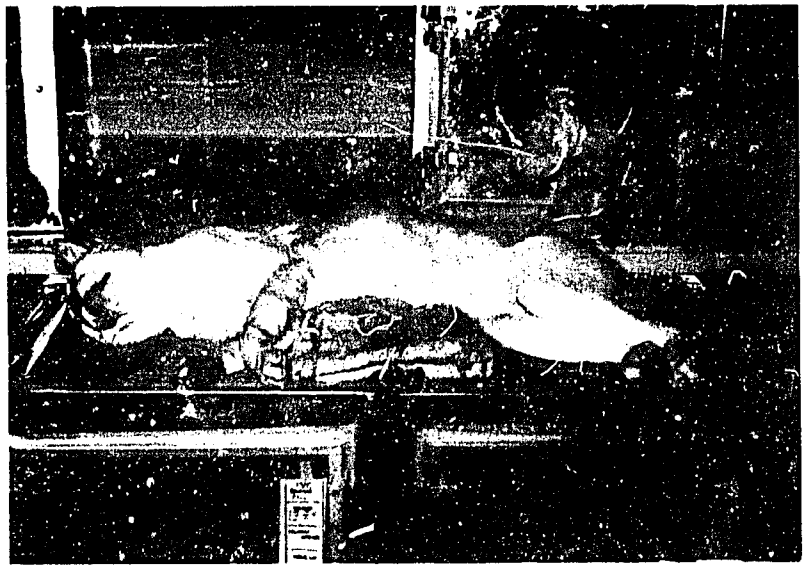


Figure 1. Feeding of mosquitoes on an anaesthetized microfilaraemic cat

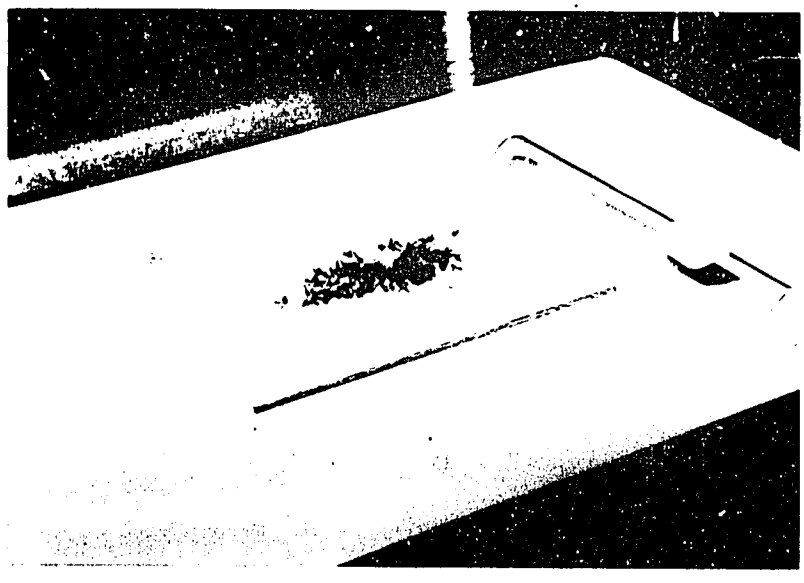


Figure 2. Crushing of mosquitoes with a glass test tube on a glass plate

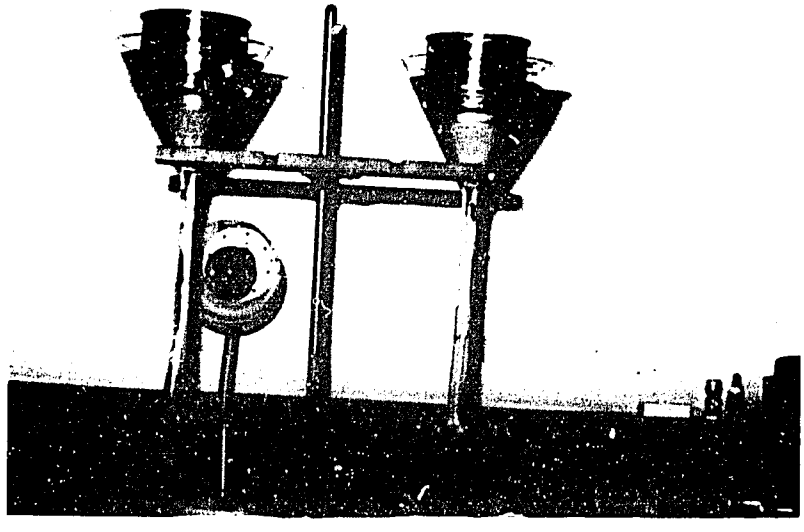


Figure 3. Baermann's apparatus for the collection of third-stage larvae of lymphatic filarial worm.

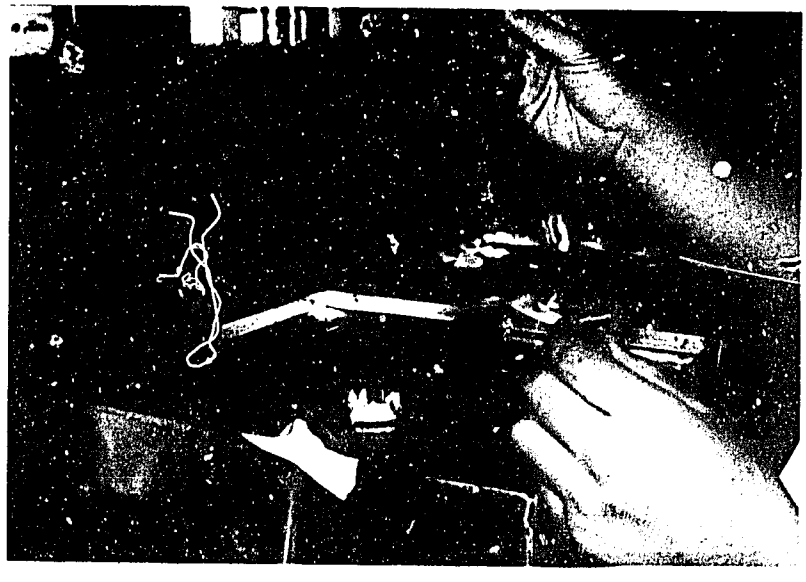


Figure 4. Feeding of mosquitoes on a membrane feeding apparatus containing microfilaraemic blood.

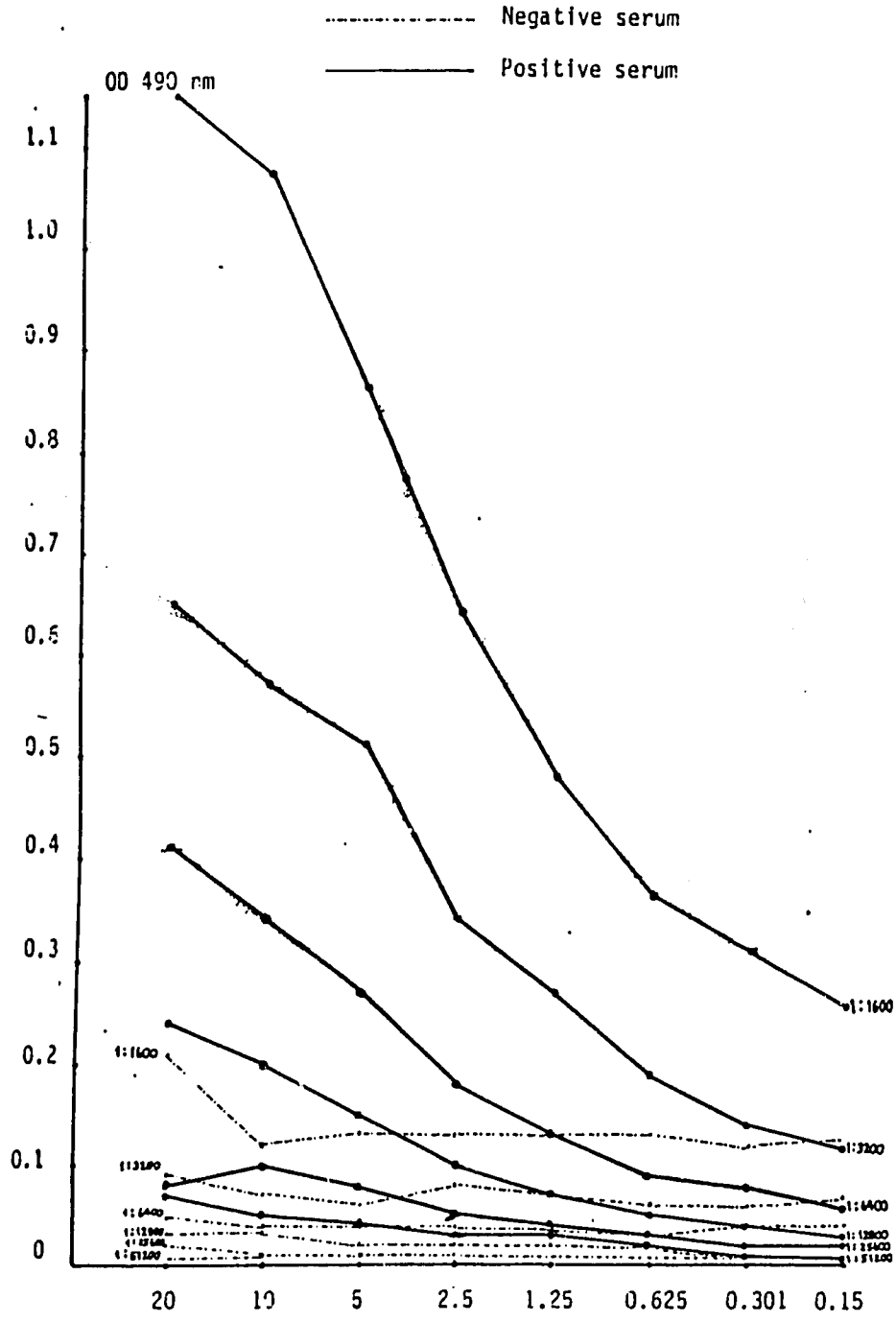


Figure 5. Checkerboard titration of somatic antigens of B. malayi against negative and positive reference sera. The negative serum was a pool of sera obtained from healthy normal people with no history of infection. The positive serum was a pool of sera from microfilaraemic patients in endemic areas of B. malayi.

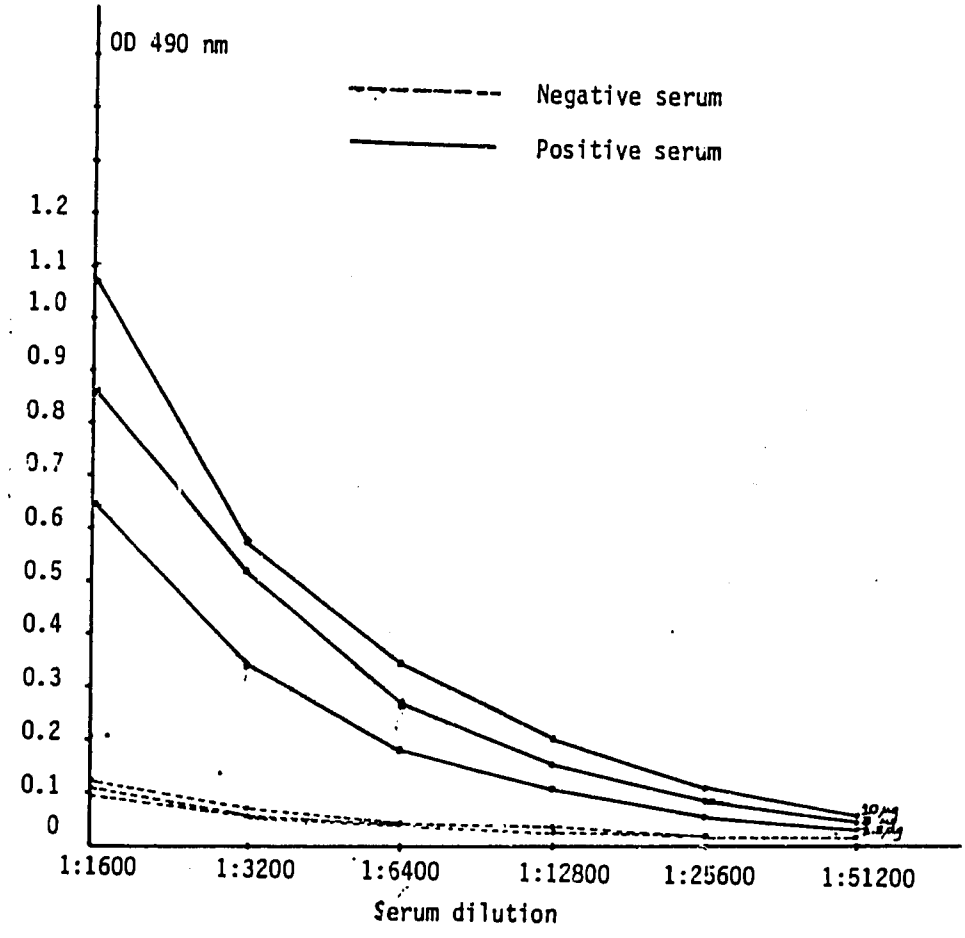


Figure 6. Titration curves of positive and negative sera diluted from 1:1600 to 1:51200 against somatic antigens of female adult B. malayi at concentrations 10, 5 and 2.5 ug/ml.

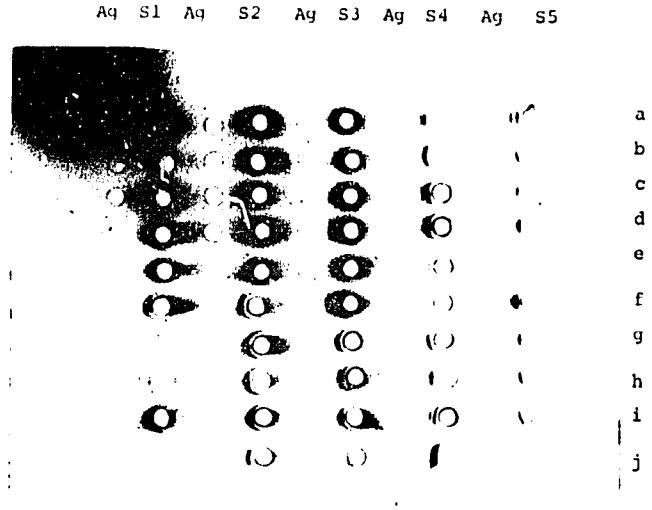


Figure 7. Amido black staining of a countercurrent electrophoresis of aqueous soluble extract of adult B. pahangi (Ag) against positive control sera (S_1 a-f), negative control sera (S_1 g-i), sera from symptomatic amicrofilaraemic patients infected with B. malayi from Southern Thailand (S_2-S_5).

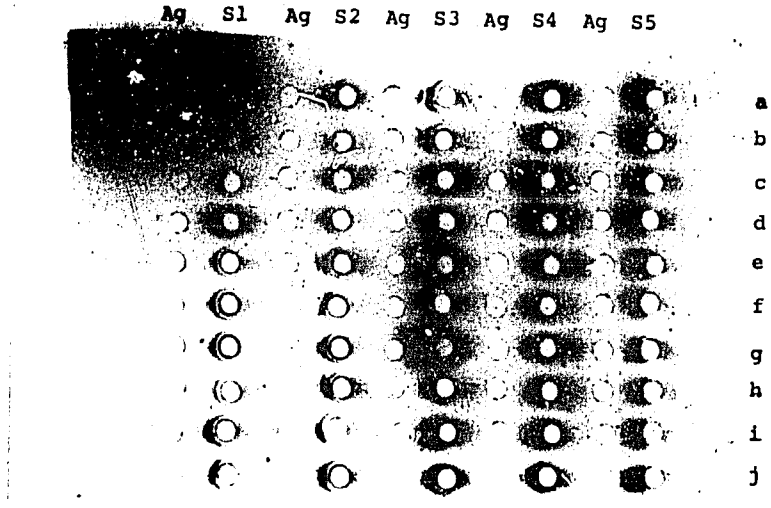
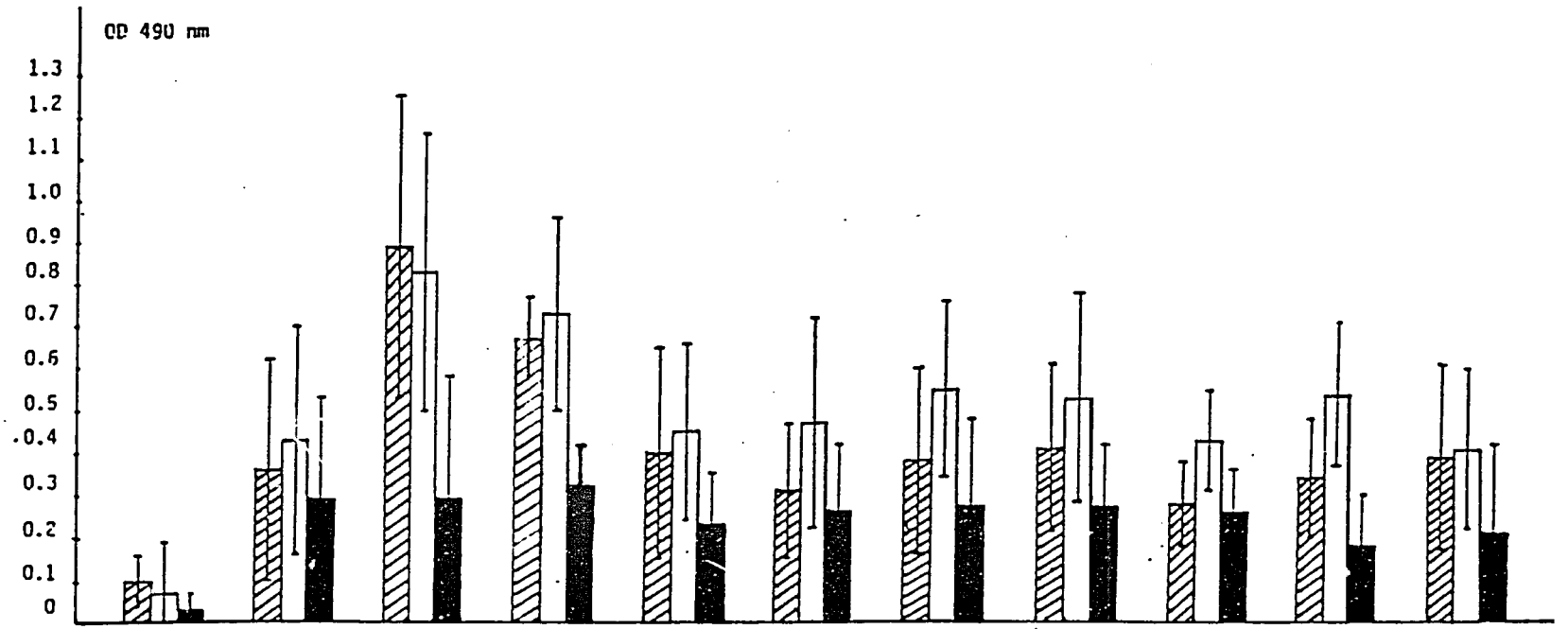
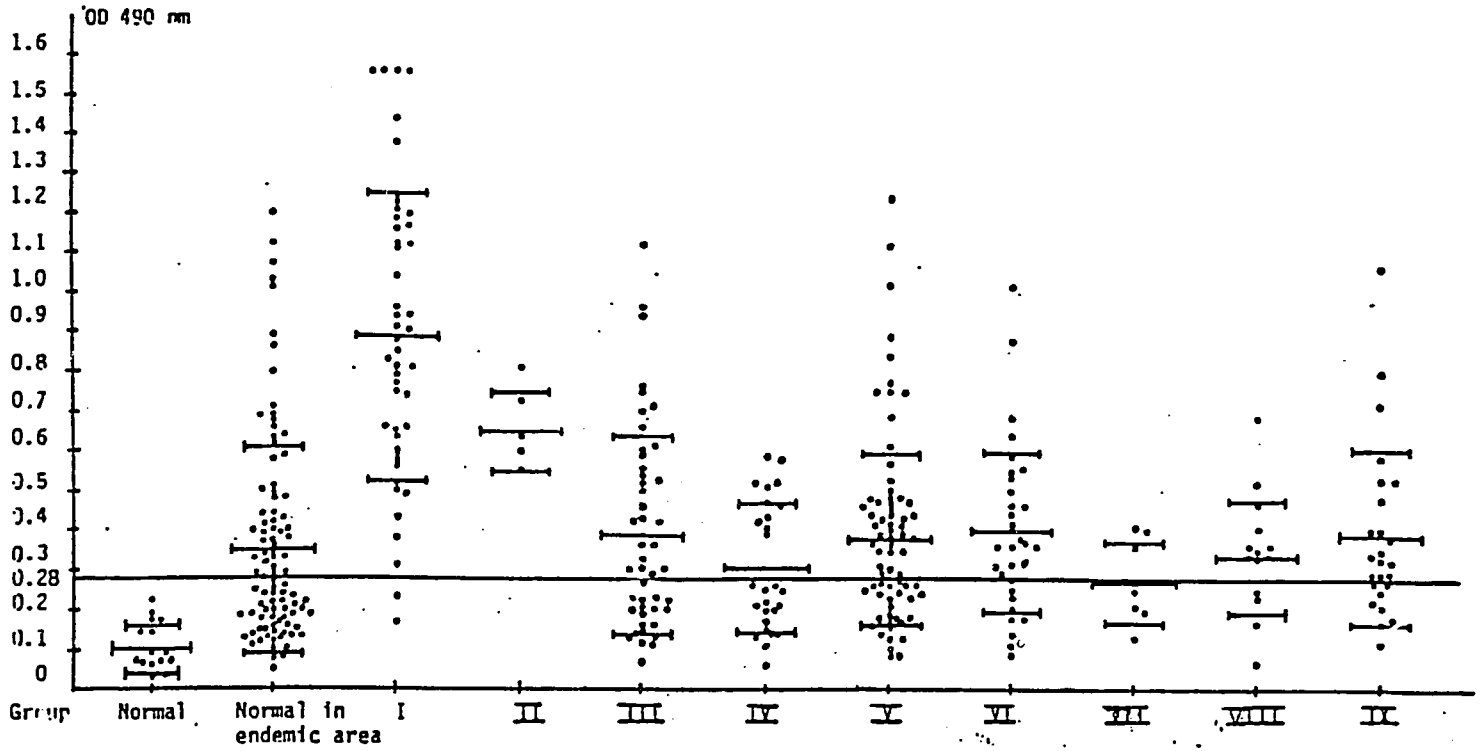


Figure 8. Amido black staining of a countercurrent electrophoresis gel of aqueous soluble extract of adult B. pahangi (Ag) against sera from symptomatic amicrofilaraemic patients infected with B. malayi from Southern Thailand (S_1, S_2, S_3 a-d), sera from symptomatic amicrofilaraemic patients infected with W. bancrofti from Western Thailand (S_3 e-g) and sera from normal persons who had experience of lymphadenitis and lymphangitis from the endemic area of B. malayi, Southern Thailand (S_3 h-j, S_4 and S_5).



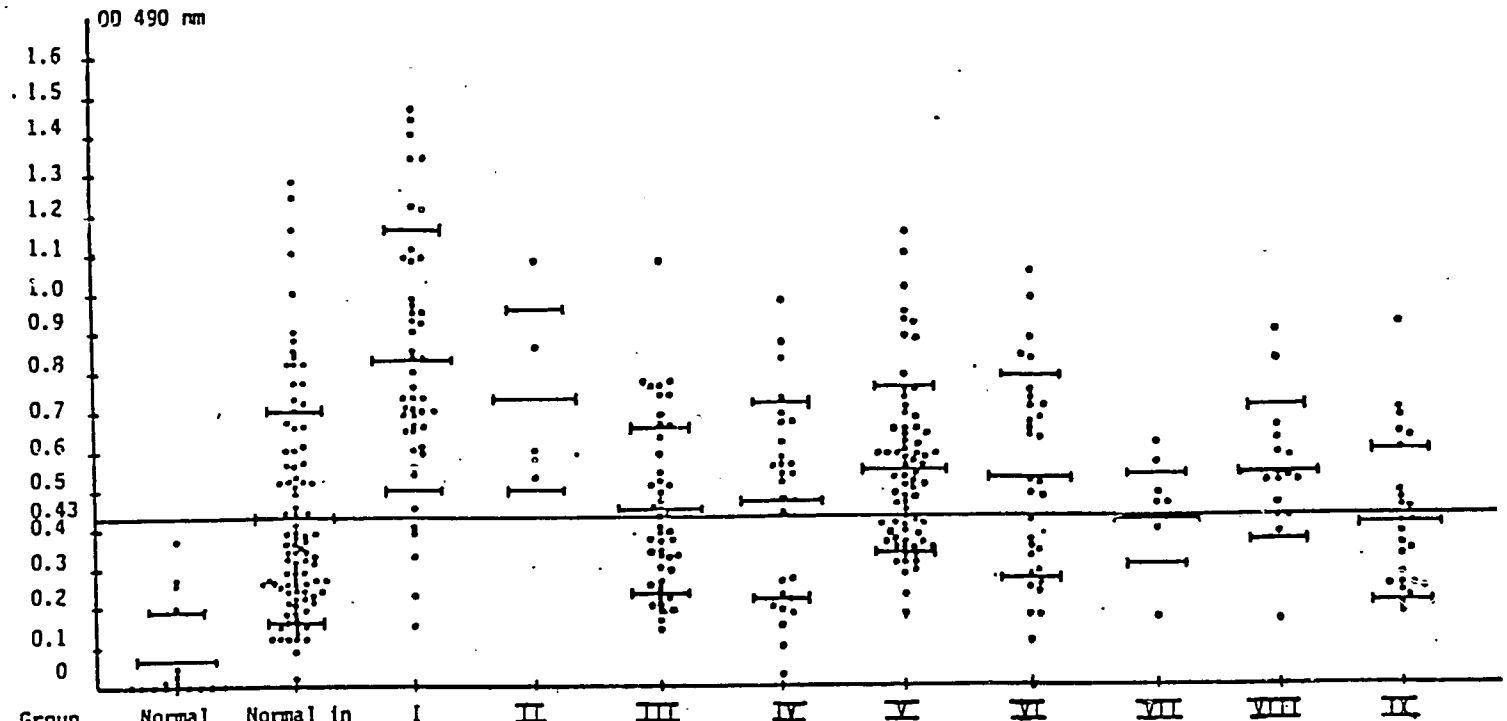
Group	Normal			Normal in endemic area			I			II			III			IV			V			VI			VII			VIII			IX		
N	17			95			47			5			47			27			73			31			8			16			25		
$\bar{X}$	.10	.07	.03	.36	.43	.29	.89	.83	.29	.67	.73	.32	.40	.45	.23	.31	.47	.26	.28	.55	.27	.41	.53	.27	.28	.43	.26	.34	.54	.18	.39	.41	.21
SD	.06	.12	.04	.26	.27	.24	.36	.33	.29	.10	.23	.11	.25	.21	.13	.16	.25	.16	.22	.21	.21	.20	.25	.15	.10	.12	.10	[illegible]	.17	.12	[illegible]	.19	.22

Figure 9. Means and Standard deviations of the ELISA values of total antibodies in the sera from different groups of filarial patients in endemic areas of B. malayi in Thailand and normal controls tested against antigens of B. malayi (▨), D. immitis (□) and G. spinigerum (■). See materials and methods for details about each group of the filarial patients. Sera were diluted 1:1600.



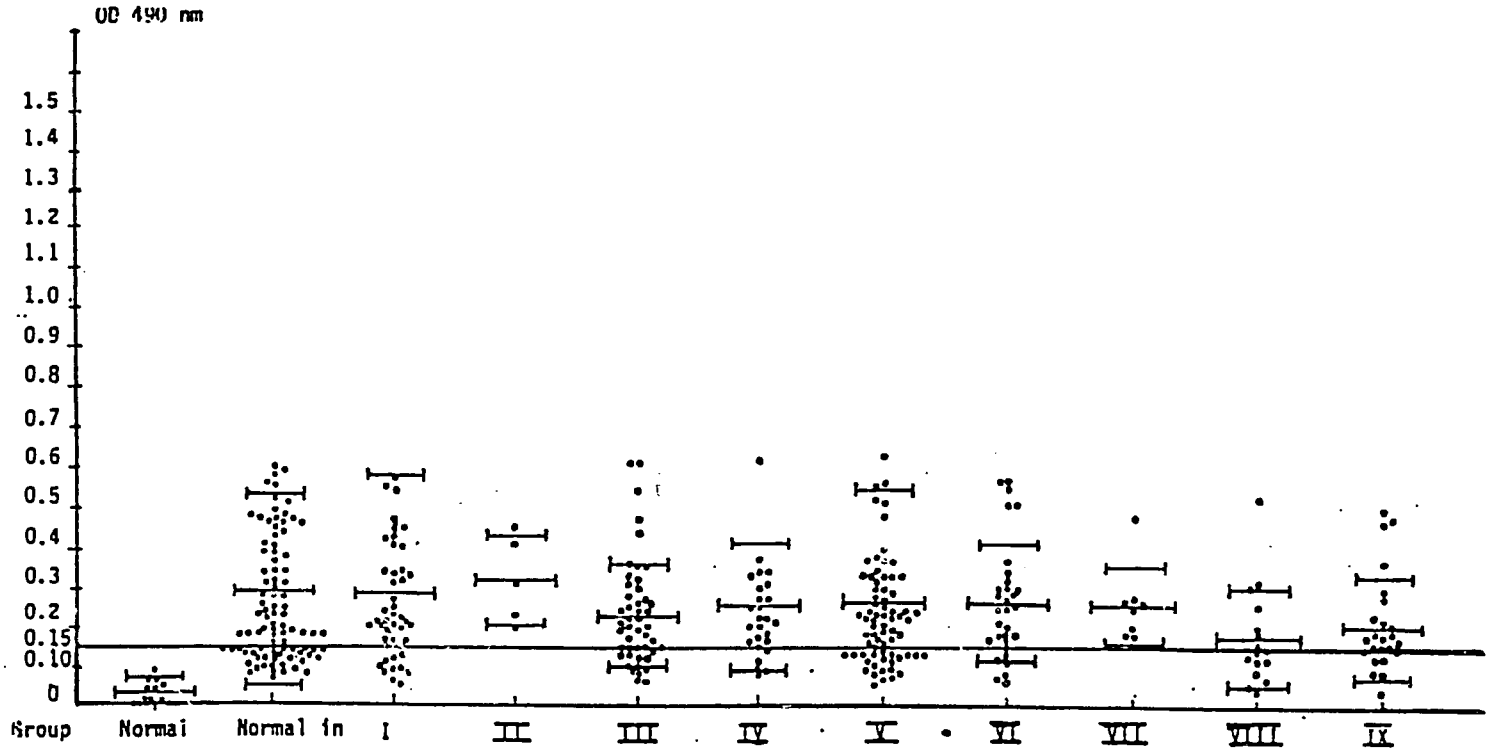
	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	17	95	47	5	47	27	73	31	8	16	25
$\bar{X}$	0.105	0.357	0.887	0.666	0.390	0.310	0.383	0.407	0.278	0.340	0.392
SD	0.060	0.256	0.364	0.104	0.251	0.163	0.216	0.205	0.101	0.143	0.218

Figure 10. ODs of the ELISA reaction for antibodies to somatic antigen of B. malayi in the sera from different groups of filarial patients in endemic areas of B. malayi in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.



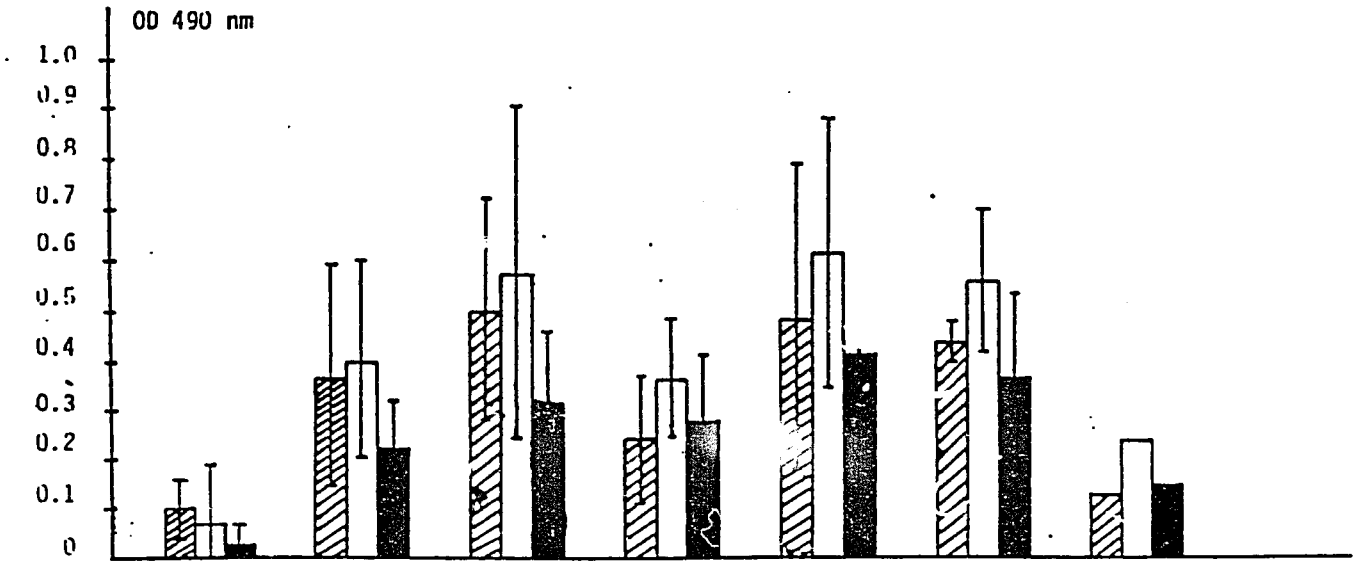
Group	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	17	95	47	5	47	27	73	31	8	16	25
$\bar{X}$	0.07	0.43	0.833	0.730	0.446	0.469	0.548	0.527	0.426	0.543	0.413
SD	0.12	0.274	0.331	0.234	0.210	0.254	0.211	0.255	0.115	0.171	0.190

Figure 11. ODs of the ELISA reaction for antibodies to somatic antigen of D. immitis in the sera from different groups of filarial patients in endemic areas of B. malayi in Thailand and normal controls. Sera were dil ted 1:1600. See materials and methods for details about each group of the filarial patients.



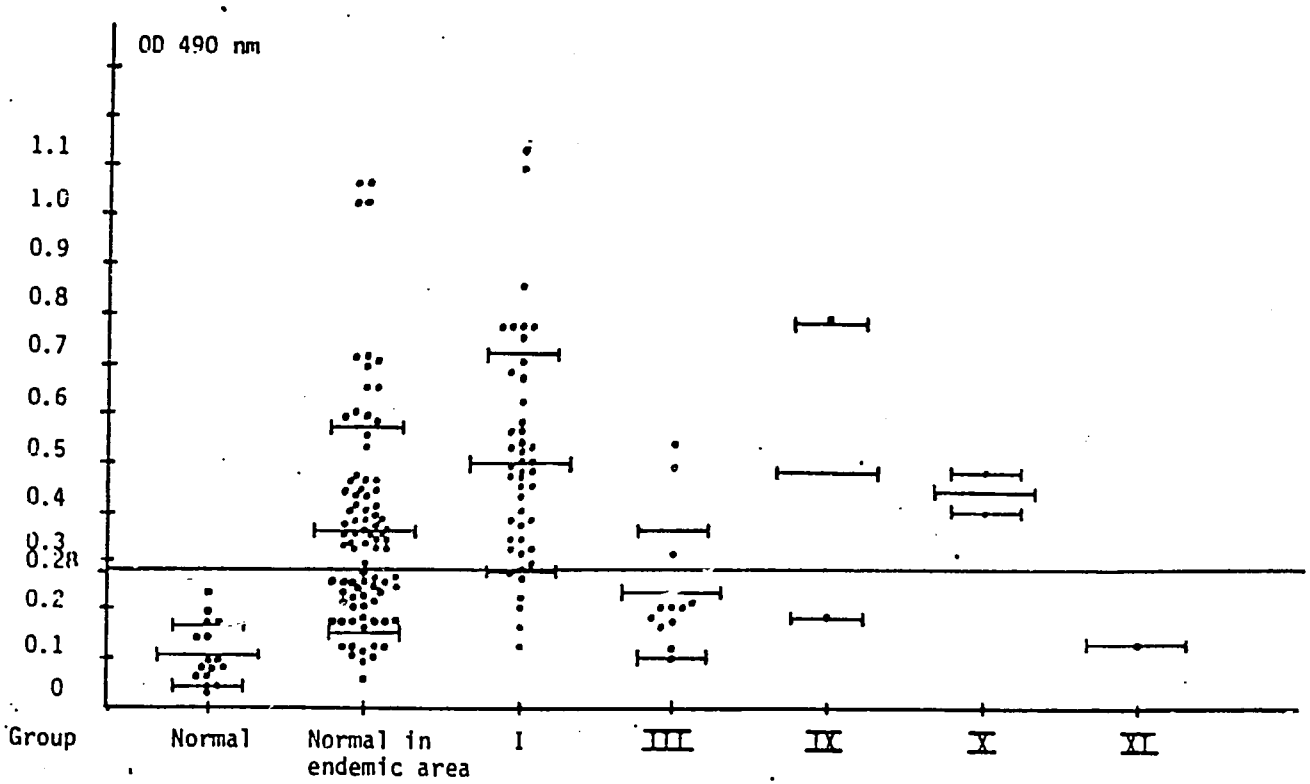
	Normal	Normal in endemic area	I	II	III	IV	V	VI	VII	VIII	IX
N	15	95	45	5	47	27	73	31	8	16	25
$\bar{X}$	0.03	0.290	0.281	0.320	0.231	0.255	0.266	0.263	0.263	0.180	0.209
SD	0.04	0.244	0.291	0.109	0.134	0.165	0.208	0.147	0.097	0.125	0.129

Figure 12. ODs of the ELISA reaction for antibodies to somatic antigen of G.spinigerum in the sera from different groups of filarial patients in endemic areas of B.malayi in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.



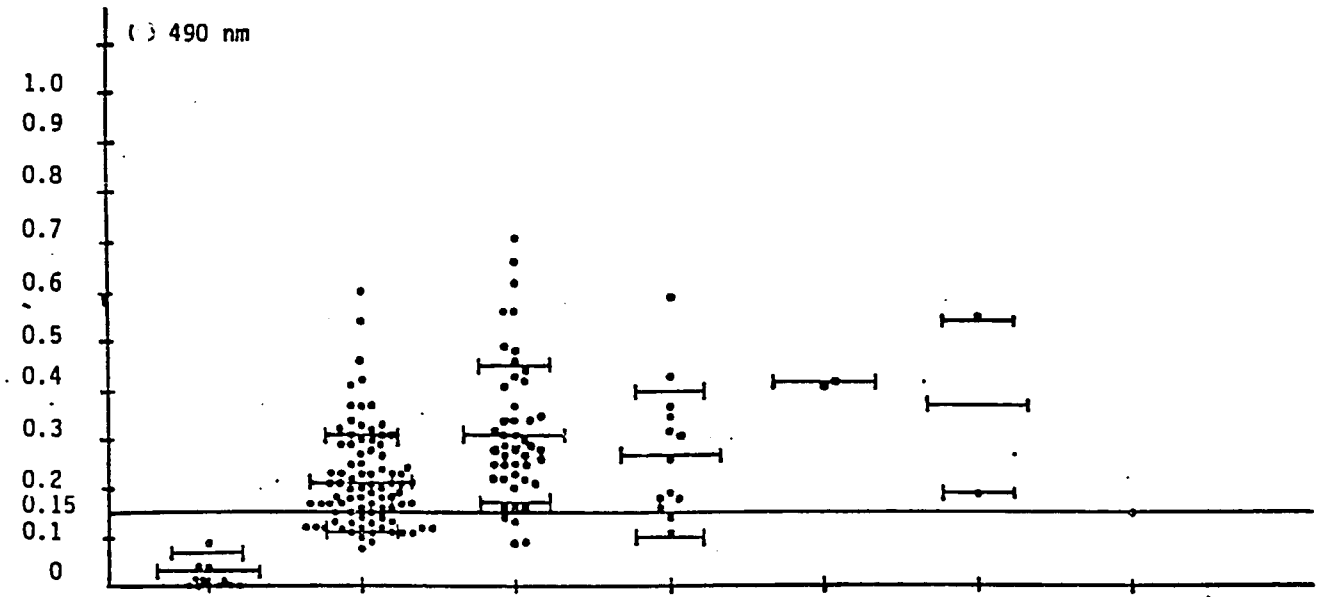
Group	Normal	Normal in endemic area	I	III	IX	X	XI
N	17	86	46	13	2	2	1
$\bar{X}$	.10.07.03	.37.40.22	.50.57.32	.24.36.28	.48.61.41	.44.56.37	.13.24.15
SD	.06.12.04	.22.19.10	.22.33.14	.13.12.14			

Figure 13 Means and Standard deviations of the ELISA values of total antibodies in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls tested against antigens of B.malayi (▨), D.immitis (□) and G.spinigerum (■). See materials and methods for details about each group of the filarial patients. Sera were diluted 1:1600.



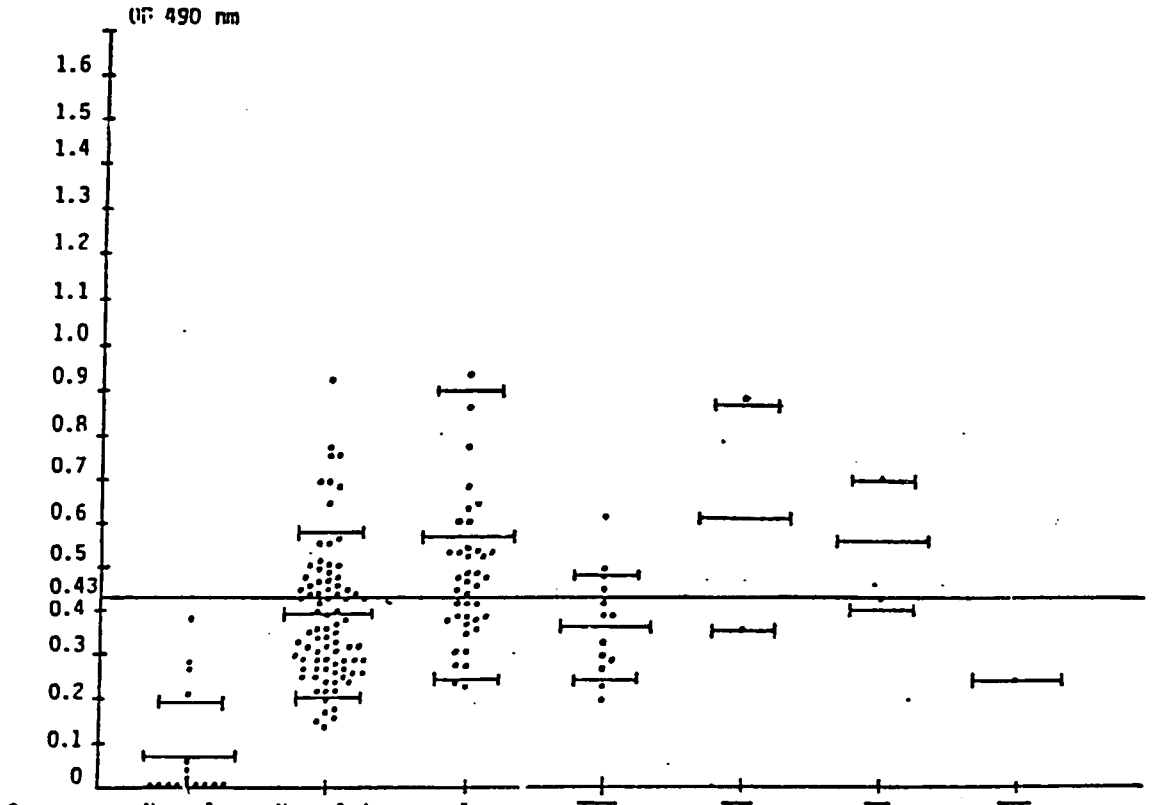
	Normal	Normal in endemic area	I	III	IV	V	VI
N	16	86	46	13	2	2	1
$\bar{X}$	0.105	0.366	0.50	0.239	0.485	0.44	0.13
SD	0.05	0.218	0.22	0.133			

Figure 14 ODs of the ELISA reaction for antibodies to somatic antigen of B.malayi in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls. Sere were diluted 1:1600. See materials and methods for details about each group of the filarial patients.



Group	Normal	Normal in endemic area	I	III	IX	X	XI
N	15	86	46	13	2	2	1
$\bar{X}$	0.03	0.218	0.318	0.276	0.415	0.37	0.15
SD	0.04	0.100	0.145	0.137			

Figure 15 ODs of the ELISA reaction for antibodies to somatic antigen of G.spinigerum in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls. Sera were diluted 1:1600. See materials and methods for details about each group of the filarial patients.



Group	Normal	Normal in endemic area	I	III	IV	V	VI
N	17	86	46	13	2	2	1
$\bar{X}$	0.07	0.396	0.572	0.365	0.615	0.56	0.24
SD	0.12	0.194	0.331	0.120			

Figure 16 ODs of the ELISA reaction for antibodies to somatic antigen of D.immitis in the sera from different groups of filarial patients in endemic areas of W.bancrofti in Thailand and normal controls. Sere were diluted 1:1600. See materials and methods for details about each group of the filarial patients.

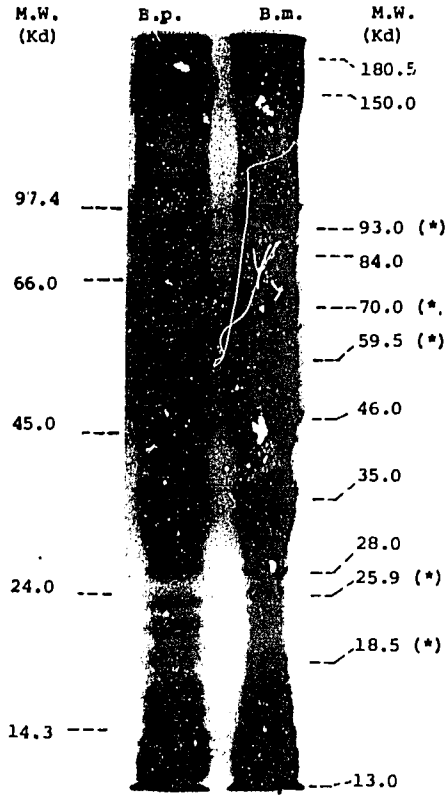


Figure 17. Brillient blue R staining of whole B. pahangi (B.p.) and B. malayi (B.m.) extracts in 10% SDS-polyacrylamide gel. Differences in protein profile of these two worms are demonstrated (*).

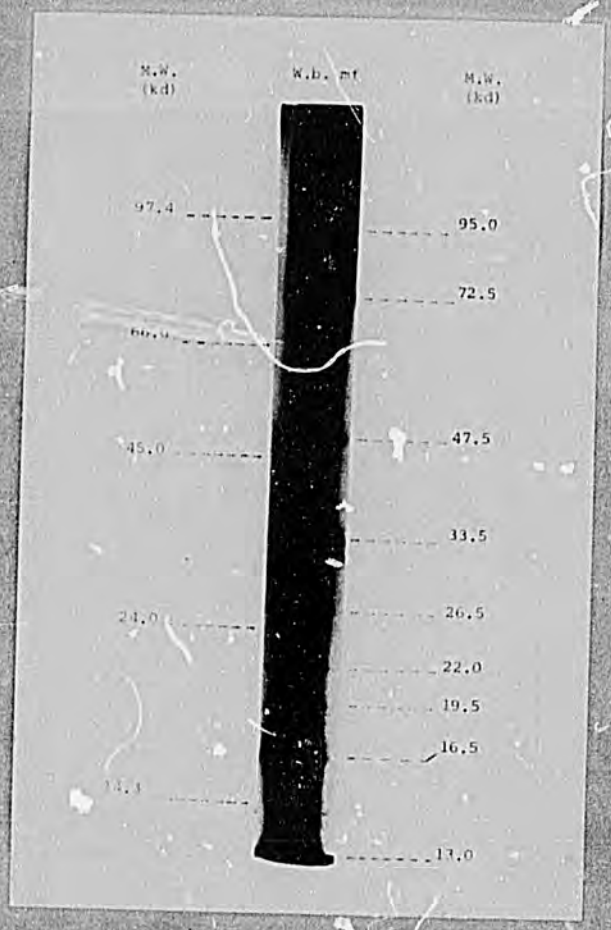


Figure 18. Silver staining of aqueous soluble extract of *Wuchereria bancrofti* microfilariae (W.b. mf) in 10% SDS-polyacrylamide gel.

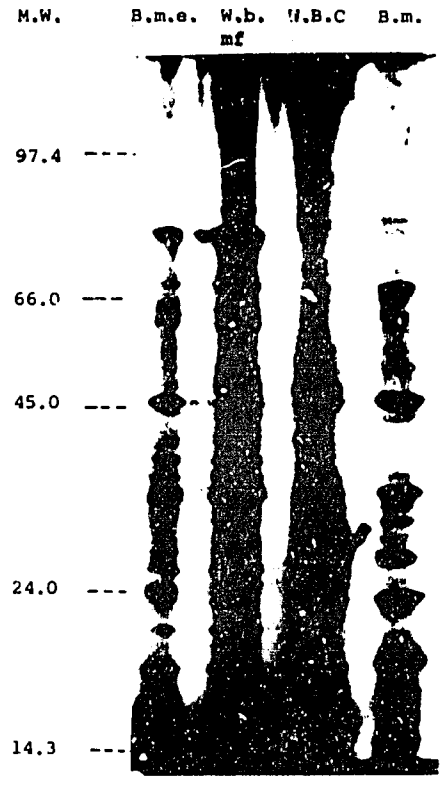


Figure 19. Silver staining of soluble aqueous extracts of adult B. malayi (B.m.e.), microfilariae of Wuchereria bancrofti (W.b.mf.) and normal human white blood cells (W.B.C.) in 10% SDS-polyacrylmide gel.

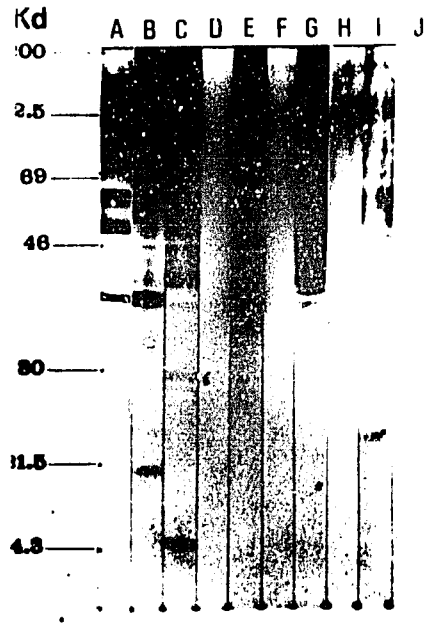


Figure 20. Immunoenzymatic analysis of Western blots of female adult B. malayi somatic extract probed with polyclonal antisera obtained from mice hyperimmunized with somatic extract of male adult B. malayi (A), male and female adult B. malayi (B), male and female adult B. pahangi (C), male and female adult T. cati (E), male adult D. immitis (F), female adult D. immitis (G) and advanced third-stage larvae of G. spinigerum (H). Normal mouse serum (D), reference positive (I) and reference negative (J) human sera for lymphatic filariasis were also used. Rainbow markers (Amersham, U.K.) were used as molecular weight standard.

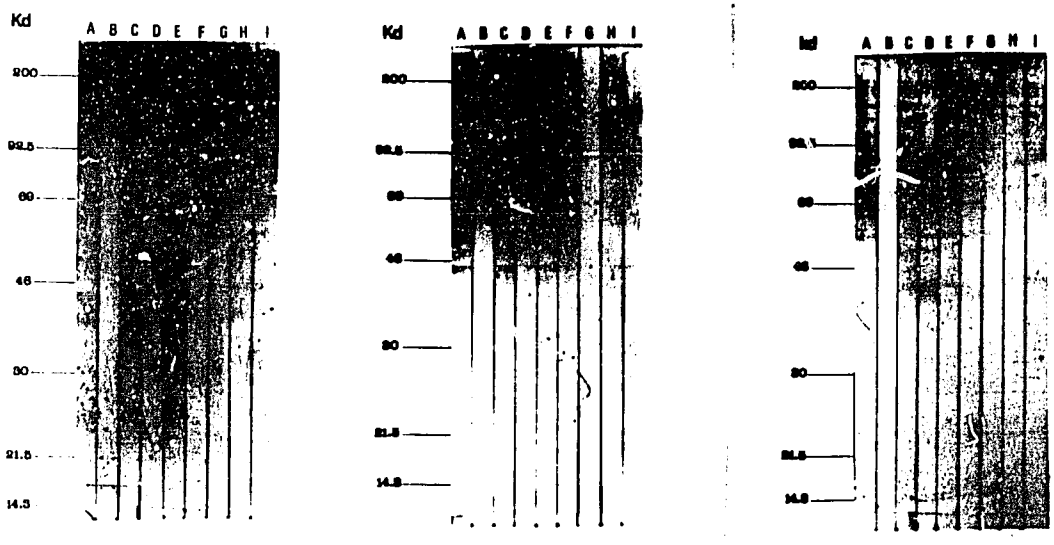


Figure 21. Immunoenzymatic analysis of western blots of somatic extracts of female adult B. pahangi (21.1), female adult D. immitis (21.2) and G. spinigerum advanced third-stage larvae (21.3) probed with sera obtained from individuals who have been living in endemic areas of B. malayi. The individuals include microfilaraemic patients (C), normal people (D), symptomatic microfilaraemic patients (E), symptomatic amicrofilaraemic elephantiasis patients (F), amicrofilaraemic elephantiasis patients (G), anthelminitics treated symptomatic amicrofilaraemic patients (H) and symptomatic amicrofilaraemic filarial patients (I). Reference positive (A) and reference negative (B) sera for lymphatic filariasis were also used. Rainbow markers (Amersham, U.K.) were used as molecular weight standard.

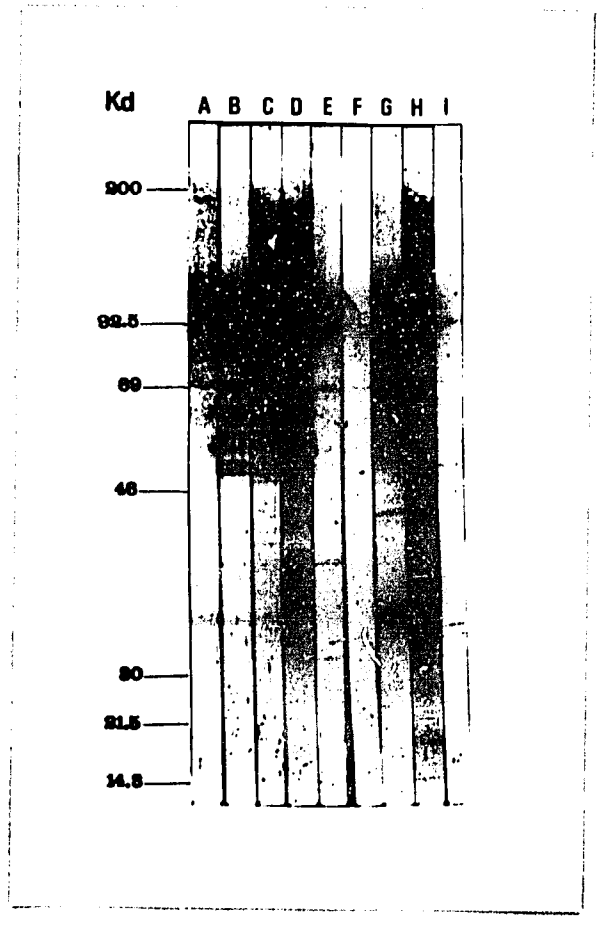


Figure 22.1 Immunoenzymatic analysis of western blots of female adult B. malayi somatic extract probed with sera obtained from individuals who have been living in endemic areas of W. bancrofti in Thailand. The individuals include microfilaraemic patients (A), anthelmintics treated amicrofilaraemic patients (B), normal people (C), anthelmintics treated elephantiasis patients (D), microfilaraemic elephantiasis patients (E), a patient who turned to amicrofilaraemic status spontaneously (F) and a microfilaraemic patient who lately turned to amicrofilaraemic status spontaneously (G). Reference positive (H) and reference negative (I) sera for lymphatic filarial patients were also used. Rainbow markers (Amersham, U.K.) were used as molecular weight standard.

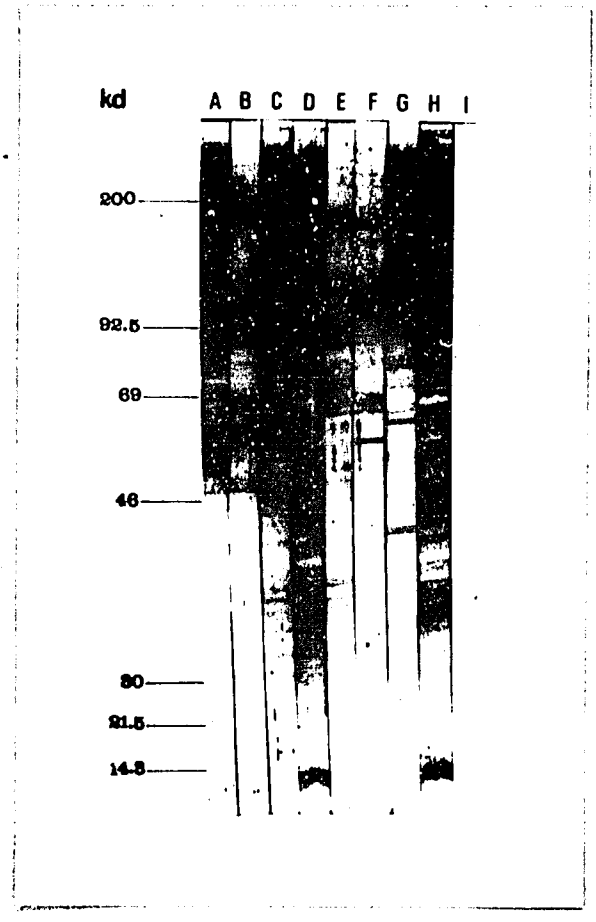


Figure 22.2 Immunoenzymatic analysis of Western blots of female adult B. pahangi somatic extract probed with sera obtained from individuals who have been living in endemic areas of W. bancrofti in Thailand. The individuals include microfilaraemic patients (A), anthelmintics treated amicrofilaraemic patients (B), normal people (C), amicrofilaraemic elehantiasis patients (D), microfilaraemic elephantiasis patients (E) patient who turned to amicrofilaraemic status spontaneously (F) and a microfilaraemic patient who lately turned to amicrofilaraemic status spontaneouly (G). Reference positive (H) and reference negative (I) sera for lymphatic filarial patients were also used. Rainbow markers (Amersham, U.K.) were used as molecular weight standard.

Figure 23. Hematoxylin-eosin staining of semithin sections of adult B. pahangi: A-D are females; E-G are males. A-G revealed the body wall of the adult worms consisting of the cuticle (C), lined by the hypodermis and the somatic musculature (M). The cuticle was about 1-2 um thick, smooth throughout with the outermost layer appearing more basophilic. The hypodermis thickened to form four hypodermal chords and divided muscle into four quadrants. The lateral chord (LC) was prominent, except when compressed by gravid uteri (Ut). The somatic muscle (M) occupied about one-fifth of body circumference and appeared basophilic. The dorsal and ventral chords were not prominent in HE sections. An extensive system of basal laminae (BL) segregated all structures from the pseudocoel (P). x600.

A-D) In the pseudocoel (P) of female worm was occupied by the uteri (U) and gut (g). The gravid uteri contained several stages of developing microfilariae (MF) in the lumen, each surrounded by loosely stretched egg shell (ESh).

E-F) Germinal testis (rt = reproductive tract) consisting of thin wall occupied by closely packed immature spermatocytes.

G) Seminal vesicle wall was not uniform in thickness and appeared distinctly basophilic.

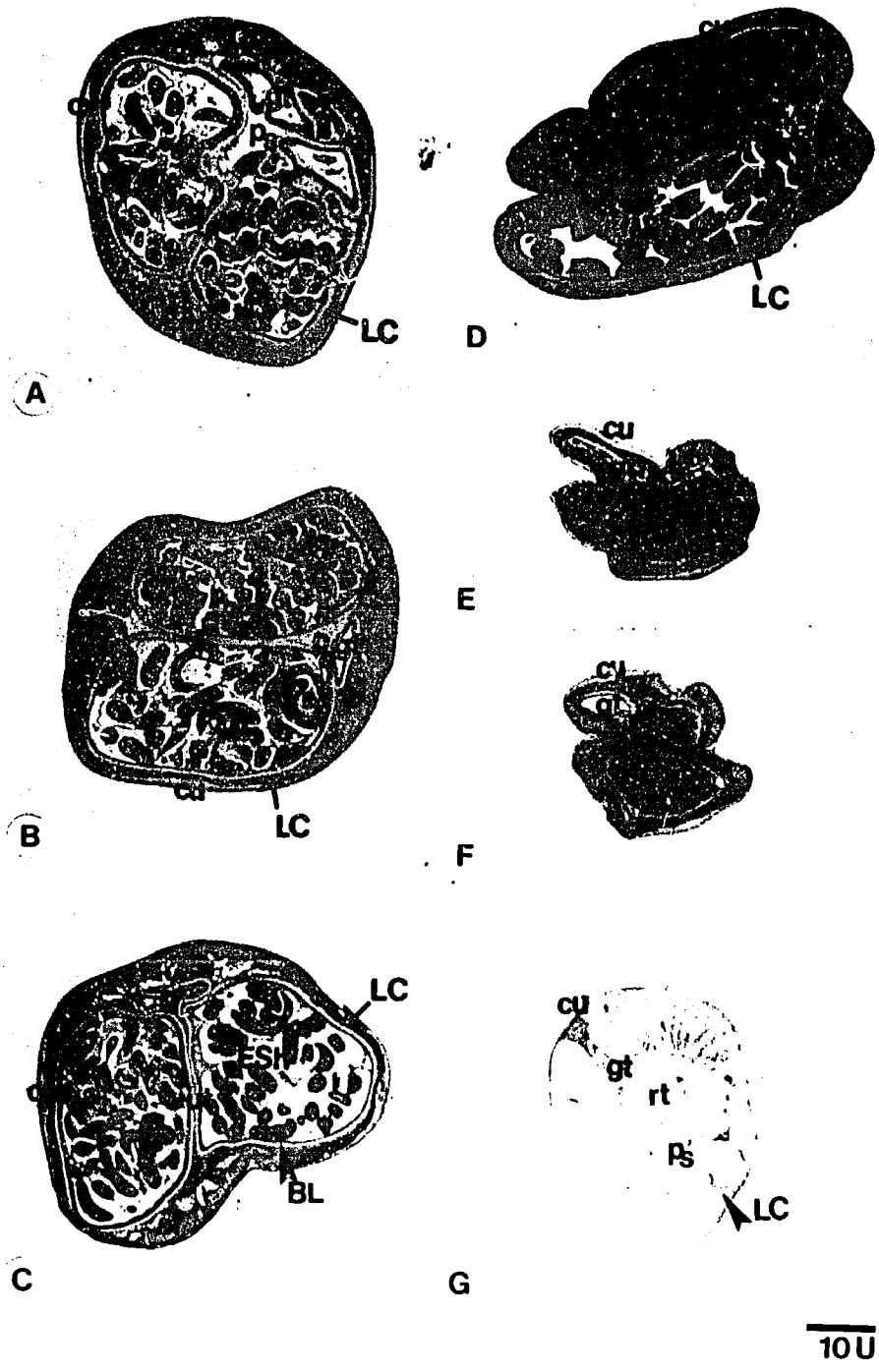
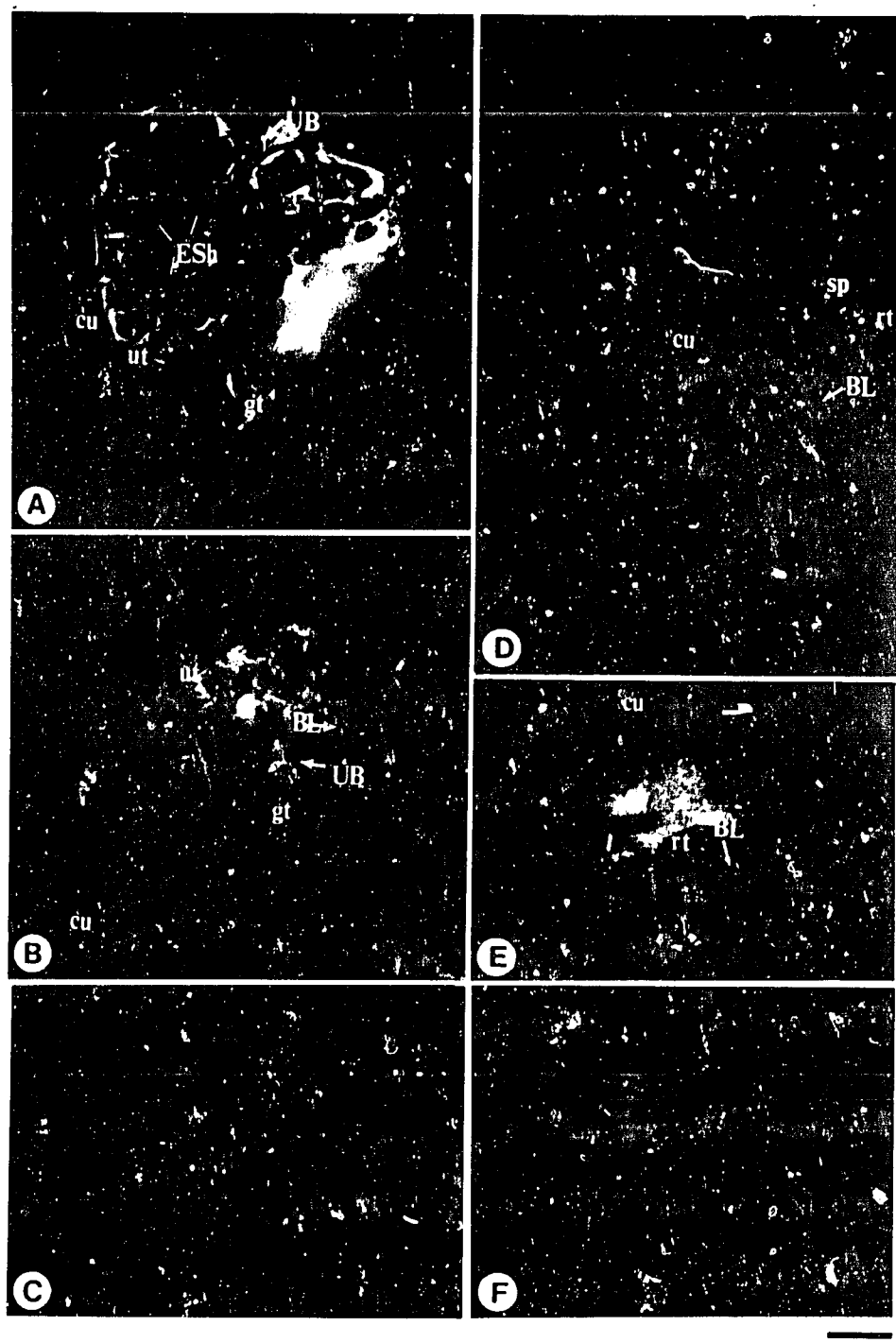

LC
A
D
LC
cu
E
B
LC
F
LC
ESH
BL
C
cu
gt
rt
ps
LC
G
10 U

Figure 24. Indirect immunofluorescence assay to localize antigen sources in the cryocut sections of female (A-C) and male (D-F) B. pahangi, by using human antisera against W. bancrofti (A-D) and against B. malayi (B-E) as primary antibodies; C = control of female, F = control of male. x 600.

A-B) Cross sections of a female worm showing equal fluorescence despite different types of antibodies that were used. The fluorescence was intense along the outermost layer of the cuticle, the basal lamina of the body wall, the uterine and gut basal laminae, egg shell covering the in utero microfilariae. The labelling was moderately at the deeper part of cuticle, hypodermis, musculature, epithelial cell of uterus and gut. Weak fluorescence was confined to the egg interior.

D-E) Cross sections of a male worm showing equal fluorescence on the body wall as in female. The male reproductive tract luminal content and spermatocytes were also intensely fluorescent.

C-F) Control experiment using PBS instead of the primary antibodies, there was no fluorescence except a weak autofluorescence in the gut.



10 U

Figure 25. Indirect immunofluorescence assay to localize antigenic sources in cryocut sections of female (A-C) and male (D-F) B. pahangi by using cat antisera against B. malayi (A, D) and against B. pahangi (B, E) as primary antibodies; C = control of female, F = control of male tissue. x 600.

A-B, D-E) Cross section of adult worm of both sexes showed equally strong fluorescence and at the same sites as indicated in Fig. 2, although different antisera were used as primary antibodies.

C-F) Control sections which are stained with PBS instead of primary antibodies showed no fluorescence.

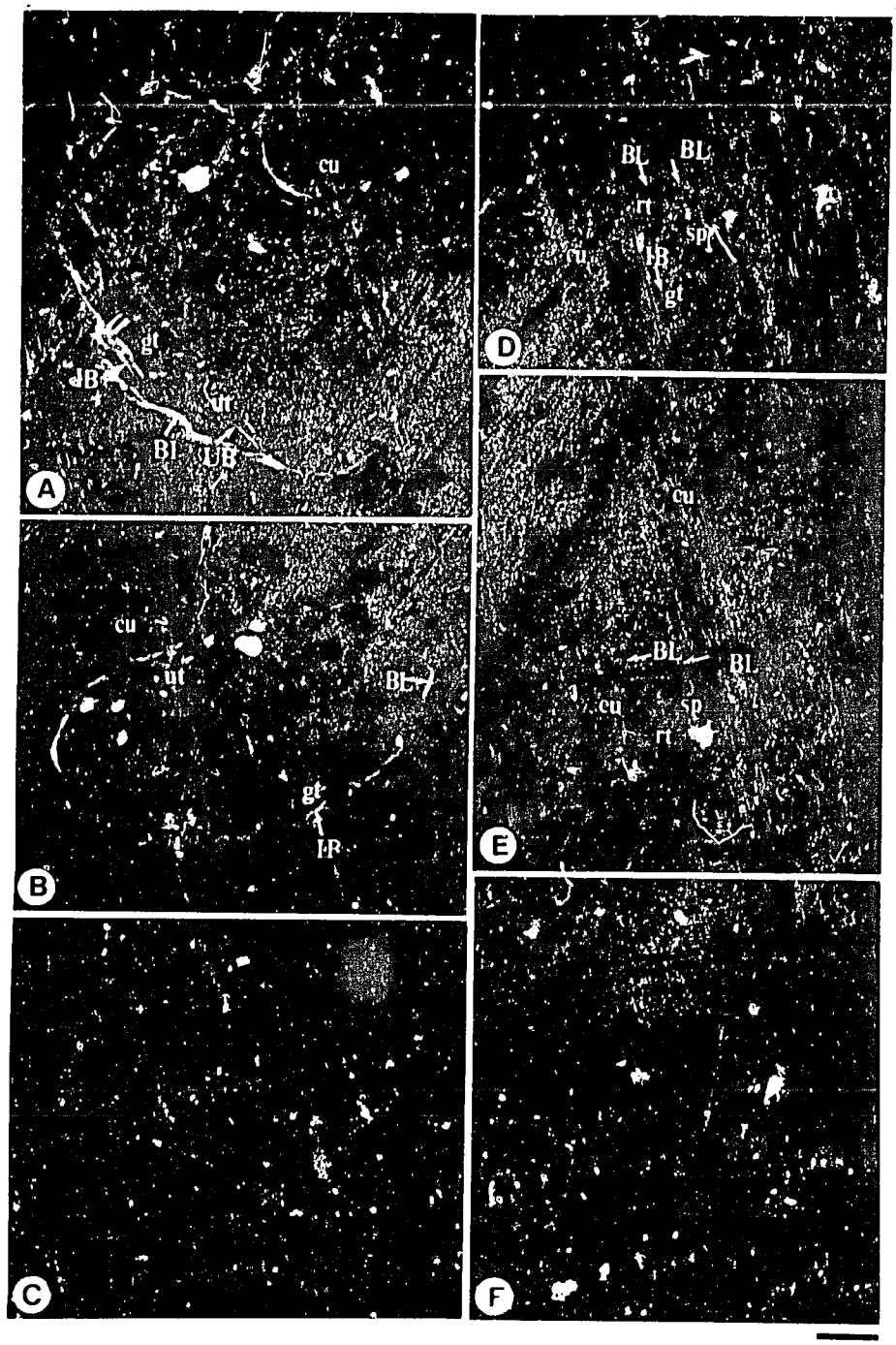

cu
gt
JB
ut
BL
LB
A
cu
ut
BL
gt
LR
B
C
BL
BL
rt
sp
cu
LB
gt
D
cu
BL
BL
cu
sp
rt
E
F
10 U

Figure 26. Indirect immunofluorescence assay to localize antigenic sources in cryocut sections of female (A-C) and male (D-F) B. pahangi by using jird antisera against B. malayi (A, D) and against B. pahangi (B, E) as primary antibodies. x600.

A-B, D-E) Different antisera showed equally strong fluorescence at the same sites in the adult worms tissue as shown in Figures 2 and 3.

C-F) Control sections stained with PBS instead of primary antibodies showed no fluorescence.

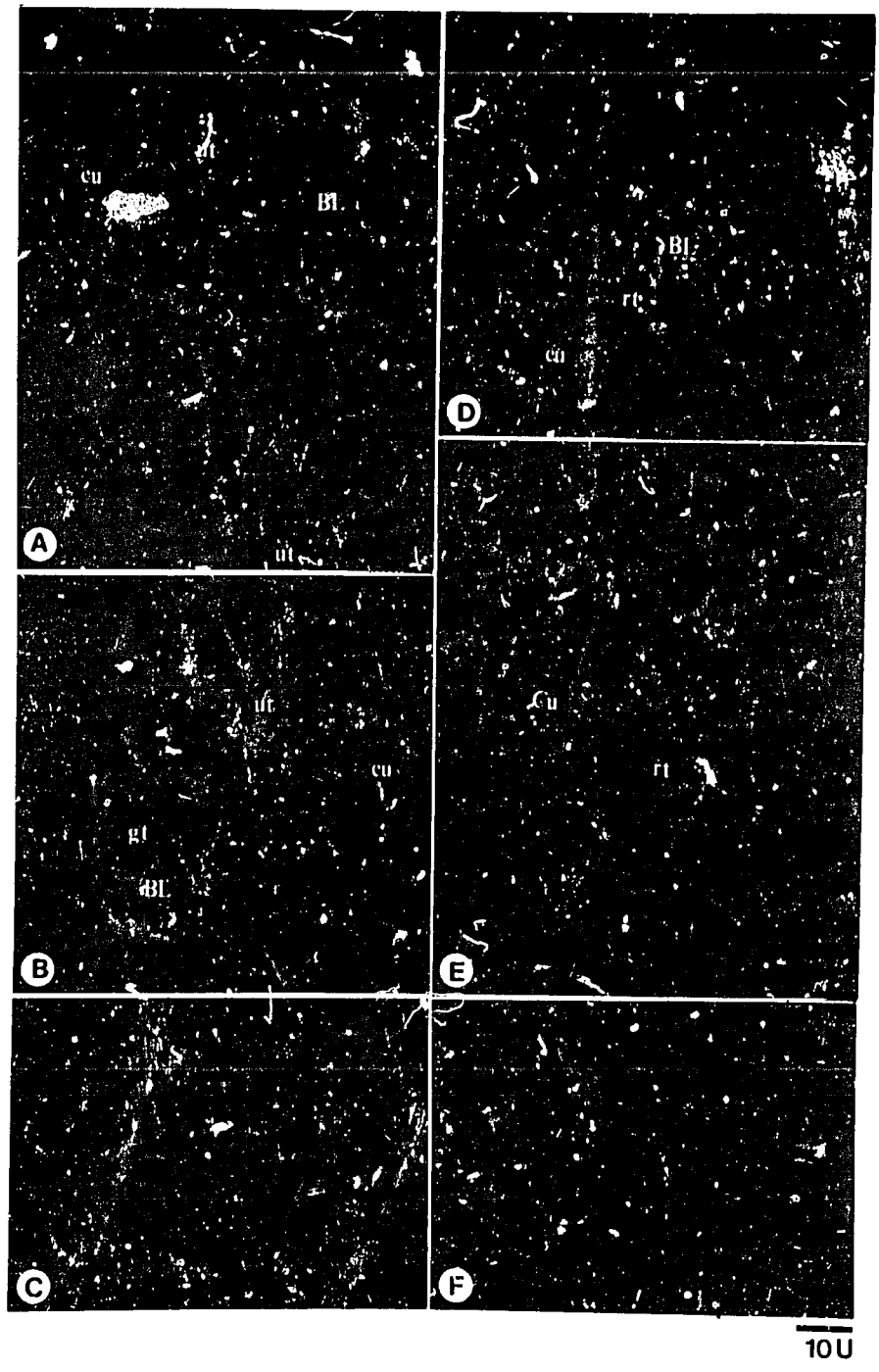

A
cu
ut
Bl
ut
B
ut
cu
gt
BL
C
D
Bl
rt
cu
E
Cu
rt
F
10 U

Figure 27. Indirect immunofluorescence assay to localize the cuticular antigens on the whole mount of L3 B. pahangi, using six categories of antisera as primary antibodies. x600.

A) Human antiserum against W. bancrofti

B) Human antiserum against B. malayi

C) Cat antiserum against B. malayi

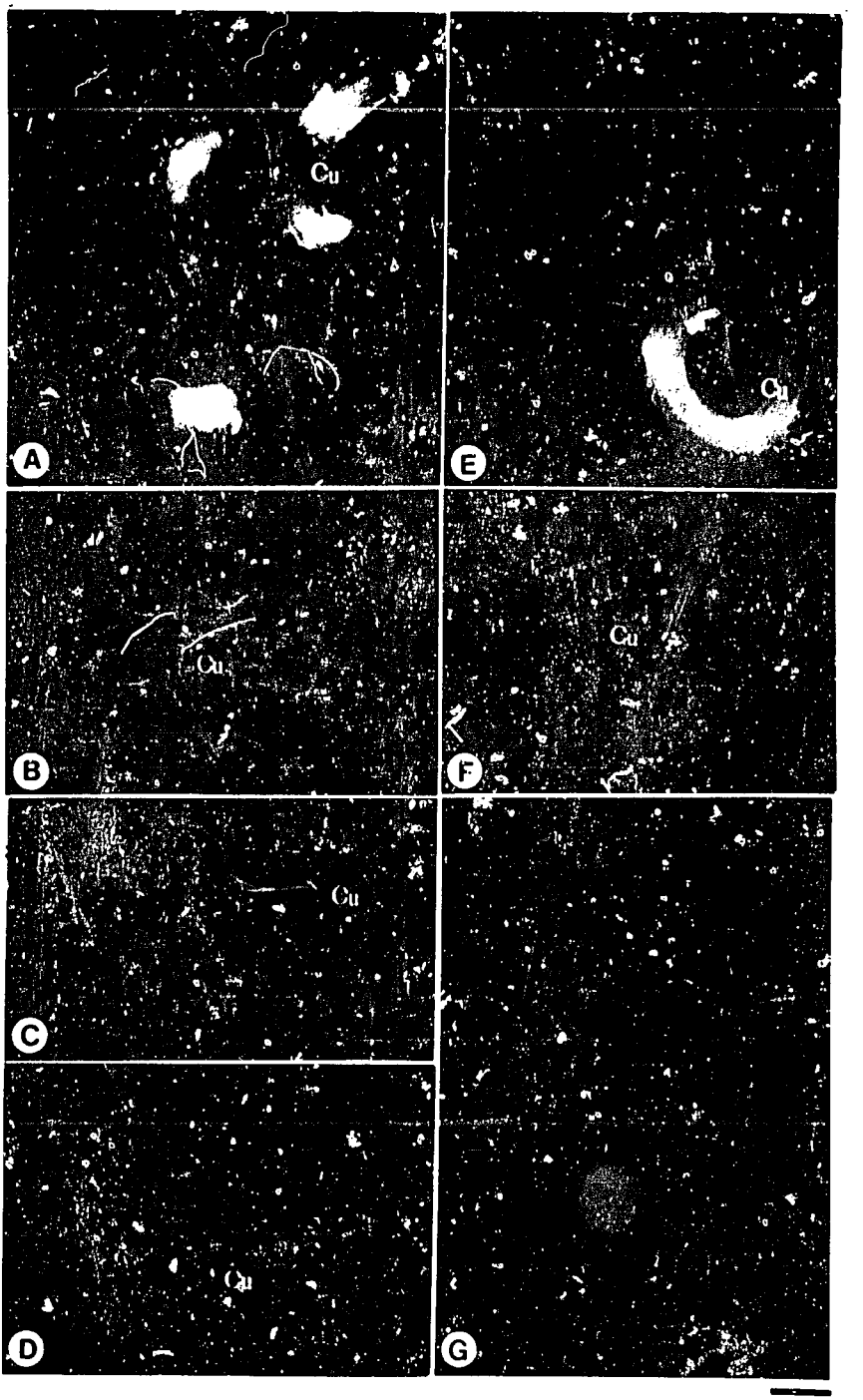
D) Cat antiserum agaisnt B. pahangi

E) Jird antiserum against B. malayi

F) Jird antiserum against B. pahangi

The fluorescence was equally along the entire cuticle of L3 B. pahangi, despite different types of antisera were being employed.

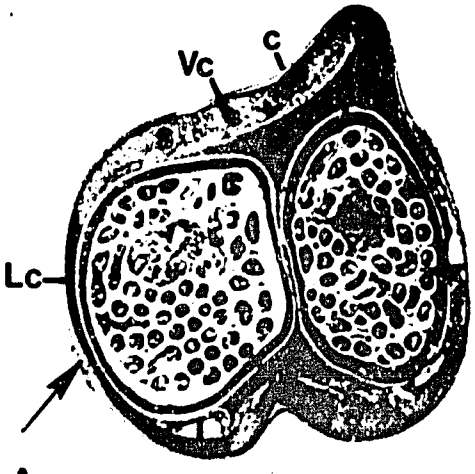
G) Control parasites stained with PBS instead of primary antibodies showed no fluorescence.



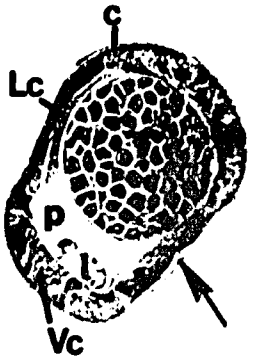
10 U

Figure 28. Hematoxylin and eosin staining of transverse plastic sections of adult B. malayi. x600. A & B) Adult female sections at different level of the uterus showing cuticle (C),somatic musculature (S), lateral chord (Lc), pseudocoelom (P), ventral chord (Vc), dorsal chord (Dc), intestine (I), developing microfilaria (Mi), dead microfilarie (Dm), uterine epithelium (E), thickened lateral cuticle over the lateral chord (arrow), egg shell (arrow head).

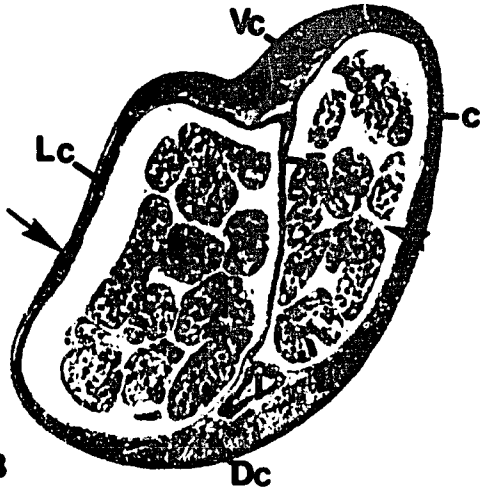
C & D) Adult male sections at different level of reproductive tract showing developing spermatozoa (Sp), testis (T), reproductive tract (rt).



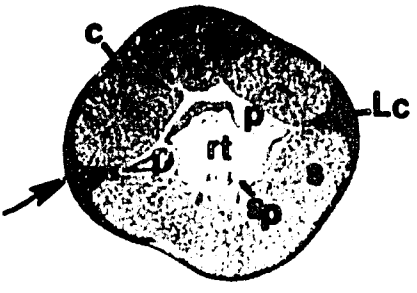
A



C



B



D

Figure 29. Indirect immunofluorescence assay to detect antigenic sources in cryocut sections of adult B. malayi by using different jird antisera as primary antibodies. x600.

A) Female section stained with jird antiserum against B. malayi

B) Female section stained with jird antiserum against B. pahangi

C) Control section of adult female showed no fluorescence

D) Male section stained with jird antiserum against B. malayi

E) Male section stained with antiserum against B. pahangi

F) Control section of adult male showed no fluorescence on the section

Abbreviation:

cuticle (C), egg shell (es), uterine epithelium (E), gut (g), body wall (Bw), developing microfilaria (mi), pseudocoelom (p), basal lamina of body wall lining pseudocoel (B), testis (T).

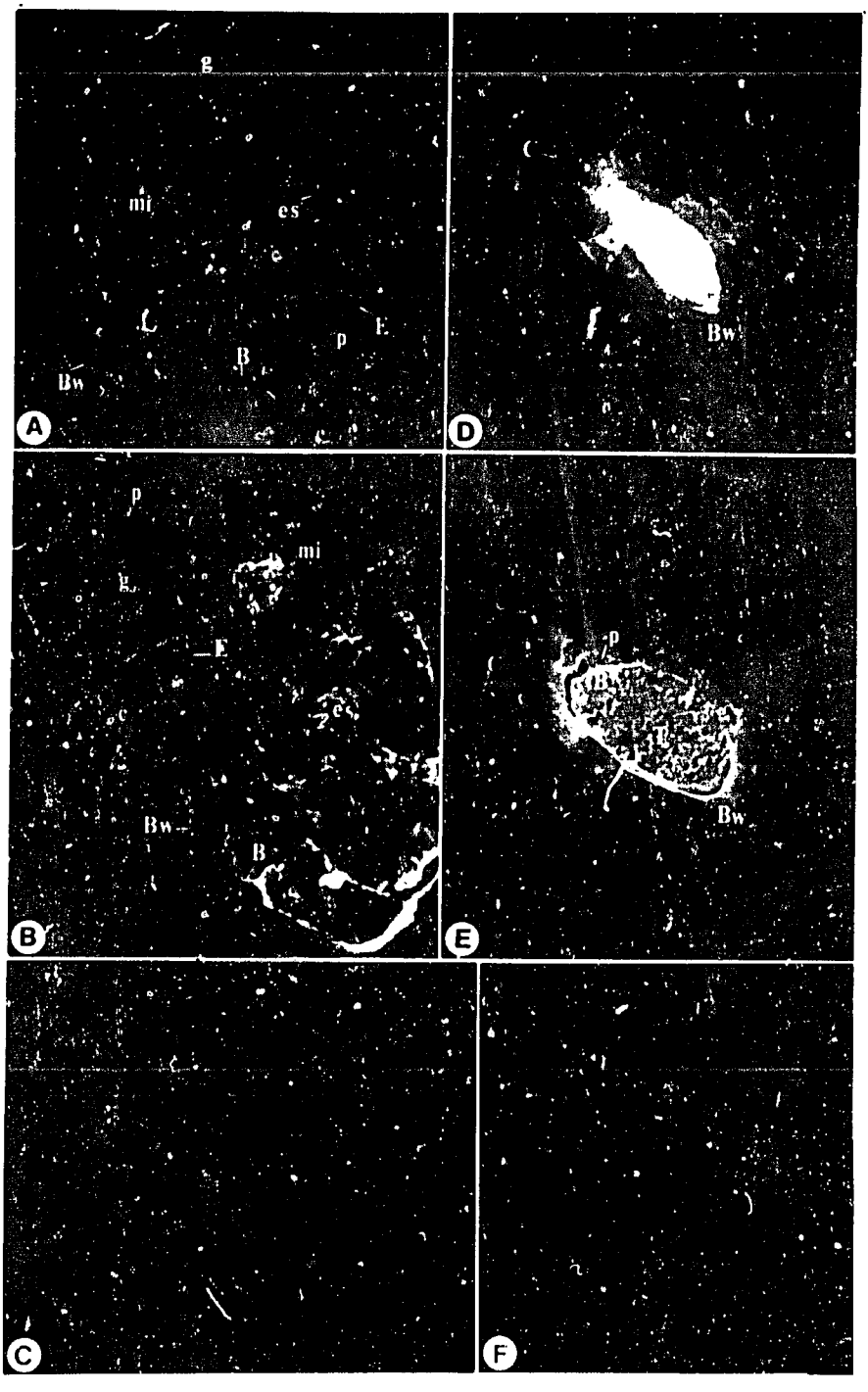

g
mi
es
C
E
p
B
Bw
A
C
Bw
D
p
mi
g
E
es
Bw
B
B
p
Bw
Bw
E
C
F

Figure 30. Indirect immunofluorescence assay to detect antigenic sources in cryostat sections of adult B. malayi using different human antisera as primary antibodies. x 600.

A) Female section stained with human antiserum against B. malayi

B) Female section stained with human antiserum against W. bancrofti

C) Control section of adult female showing no fluorescence

D) Male section stained with human antiserum against B. malayi

E) Male section stained with human antiserum against W. bancrofti

F) Control section of adult male showing no fluorescence

Abbreviations:

cuticle (C), egg shell (es), uterine epithelium (E), gut (g), body wall (Bw), developing microfilaria (mi), pseudocoelom (P), basal lamina of body wall lining pseudocoel (B), testis (T).

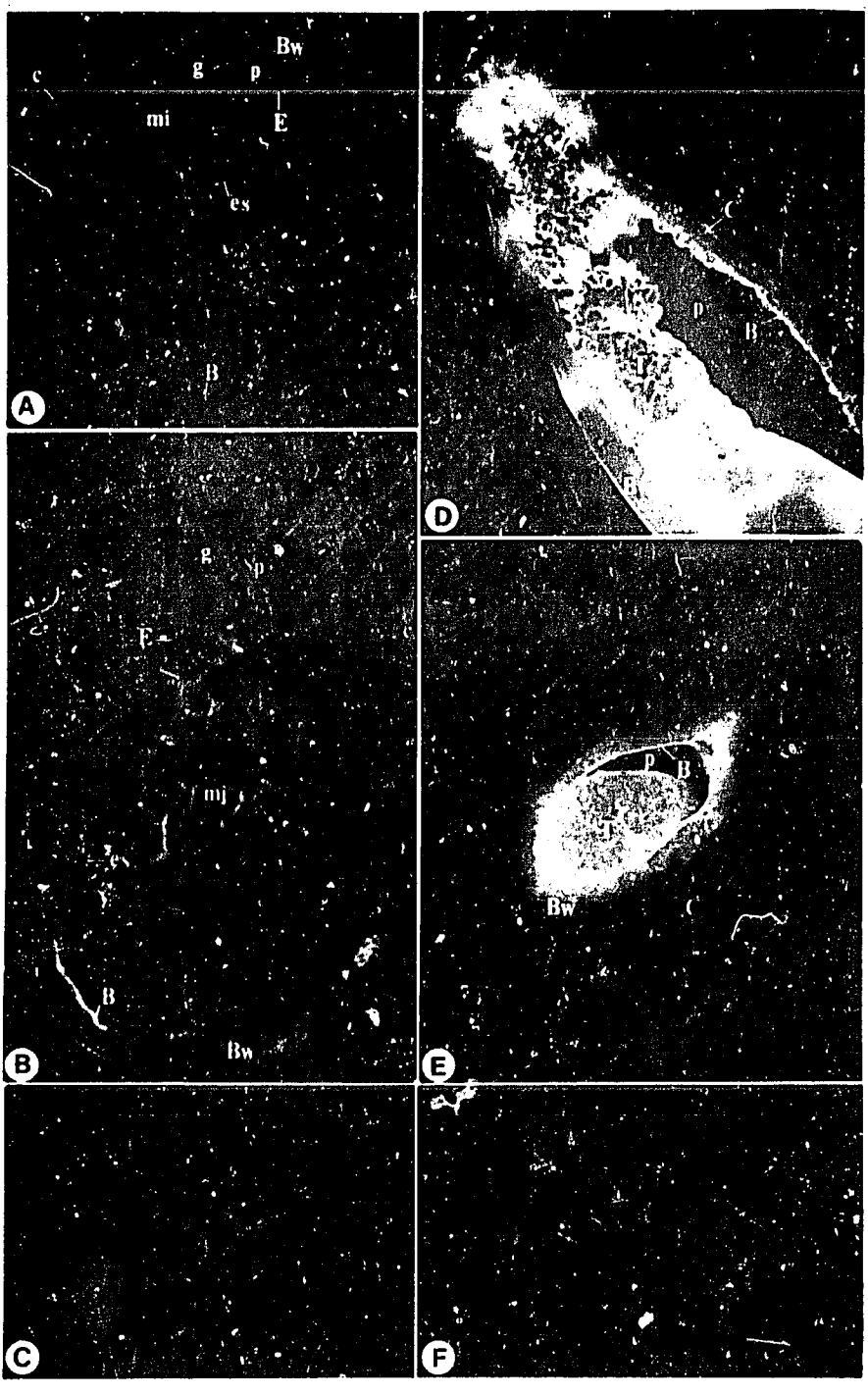

Bw
c
g
p
mi
E
es
B
A
g
p
E
mj
es
B
Bw
B
C
D
B
D
p
B
Bw
C
E
F

Figure 31. Indirect immunofluorescence assay to detect antigenic sources in cryostat sections of adult B. malayi using different cat antisera as primary antibodies. x 600.

A) Female section stained with cat antiserum against B. malayi

B) Female section stained with cat antiserum against B. pahangi

C) Control section of adult female showing no fluorescence

D) Male section stained with cat antiserum against B. malayi

E) Male section stained with cat antiserum against B. pahangi

F) Control section of adult showing no fluorescence.

Abbreviations:

cuticle (C), egg shell (es), developing microfilaria (mi), gut (g), body wall (Bw), pseudocoelom (P), basal lamina of body wall lining pseudocoel (B), testis (T).

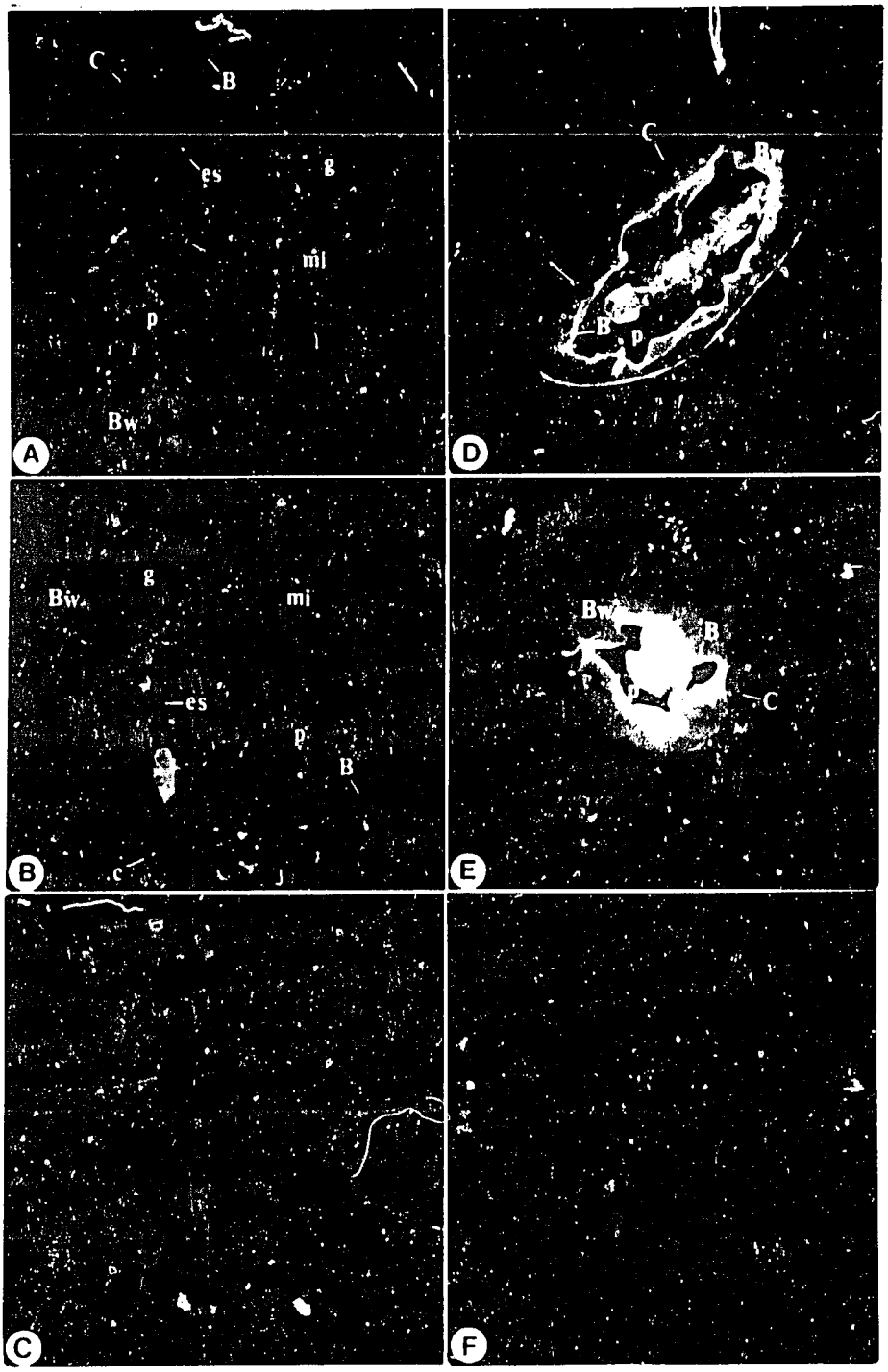

C
B
es
g
mi
p
Bw
A
C
Bw
B
p
D
Bw
g
mi
es
p
B
c
B
Bw
B
C
E
C
F

Figure 32. Indirect immunofluorescence assay to detect antigens on the whole mount of B. malayi infective stage larvae L3 using different antisera as primary antibodies. x600.

A) L3 stained with jird antiserum against B. malayi

B) L3 stained with antiserum against B. pahangi

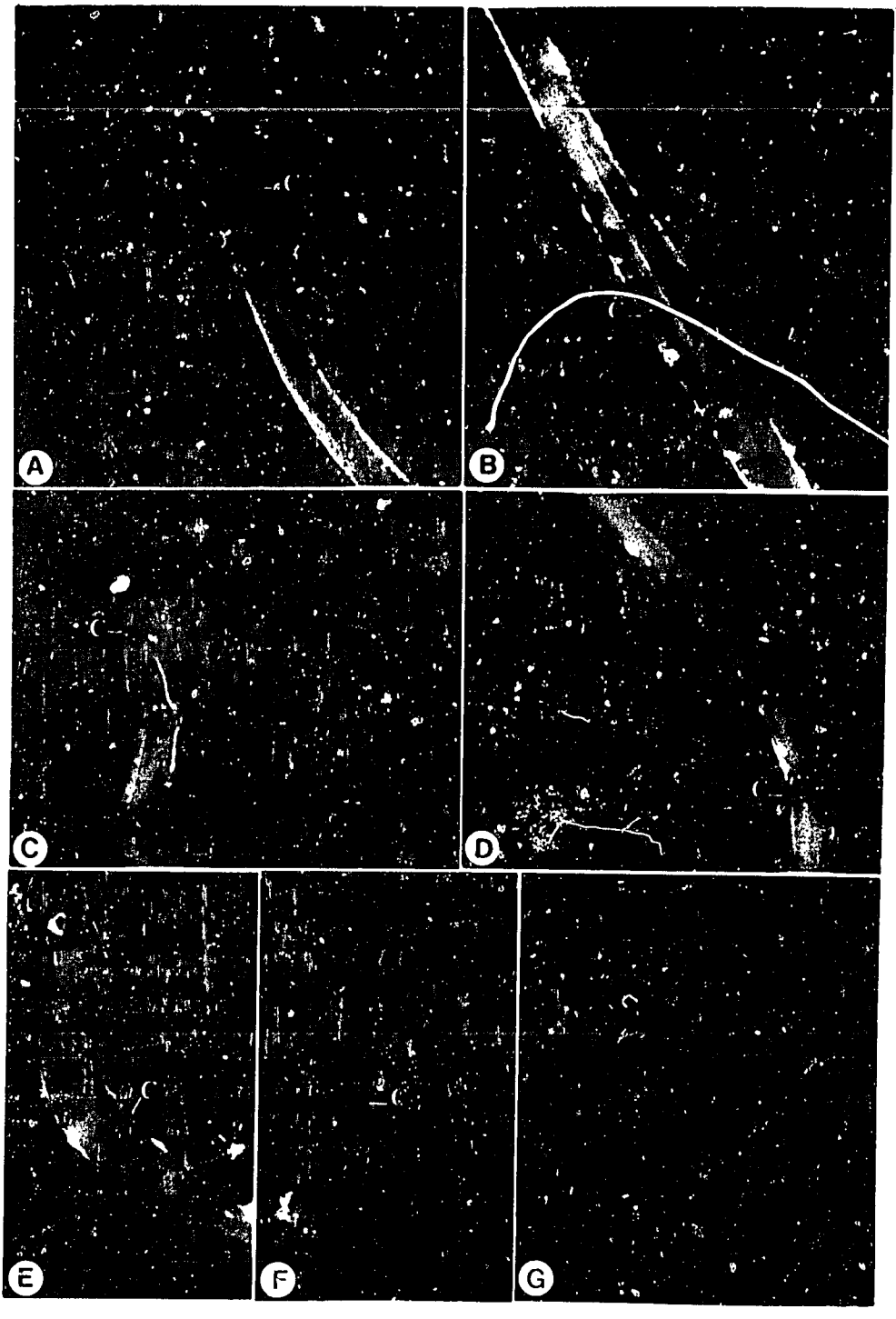
C) L3 stained with antiserum against B. malayi

D) L3 stained with antiserum against W. bancrofti

E) L3 stained with antiserum against B. malayi

F) L3 stained with antiserum against b. pahangi

G) Control L3 showed no fluorescence on the cuticle


A
B
C
D
E
F
G

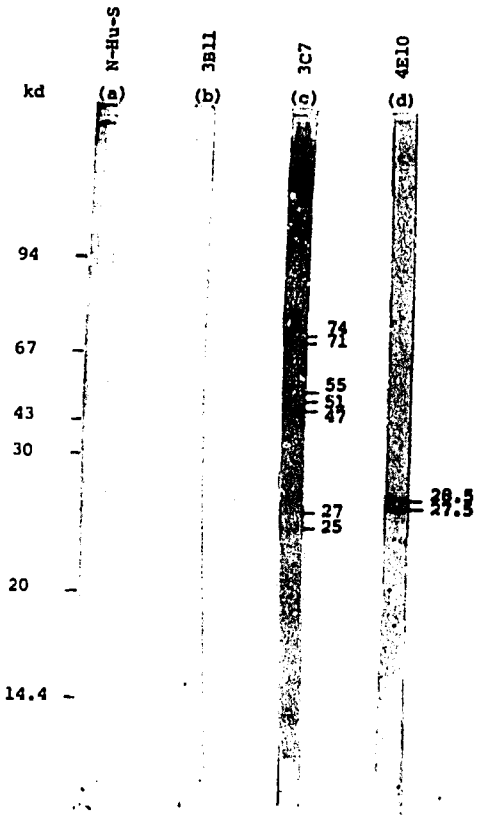


Figure 33. Western blot and immunological reactions of somatic extract of B. pahangi third-stage larvae tested against monoclonal antibodies 3B11 (b), 3C7 (c) and 4E10 (d). Normal human serum from non-endemic area of lymphatic filariasis was used as negative control (a). [Tested at London School of Hygiene and Tropical Medicine].

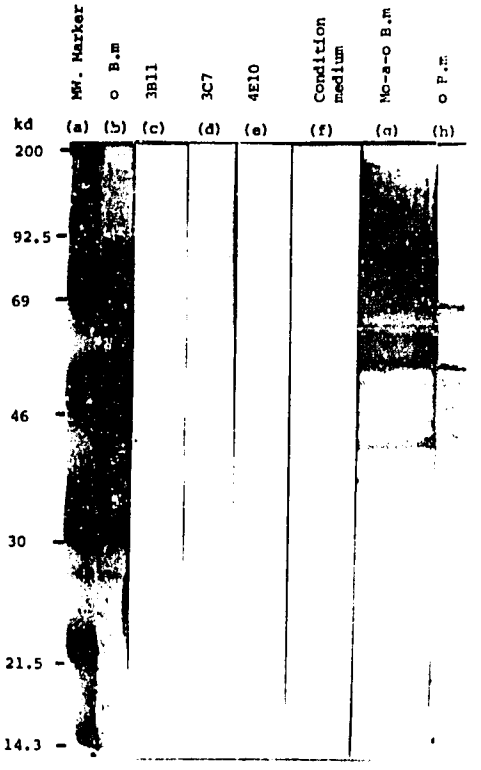


Figure 34. Western blot and immunological reactions of somatic extract of female adult B. malayi tested against monoclonal antibodies 3B11 (c), 3C7 (d) and 4E10 (e). Condition medium (f) was used as negative control and polyclonal mouse antibodies to female adult B. malayi somatic extract (g) was used as positive control. The antigen blotted onto the nitrocellulose sheet and stained with Amido Schwartz 10 B was also shown (b and h).

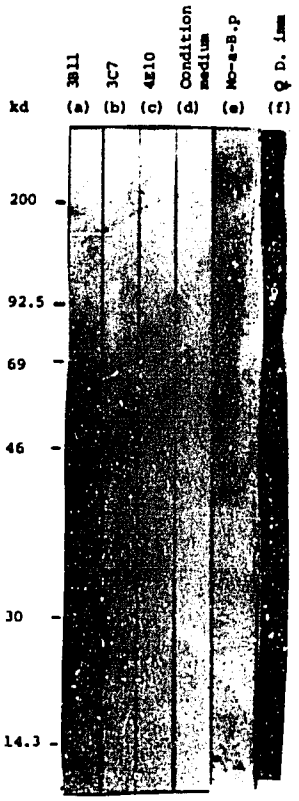


Figure 35. Western blot and immunological reactions of somatic extract of female adult D. immitis tested against monoclonal antibodies 3B11 (a), 3C7 (b) and 4E10 (c). Condition medium (d) was used as negative control while polyclonal mouse antibodies to female adult B. pahangi somatic extract (e) was used as positive control. The antigen blotted onto the nitrocellulose sheet and stained with Amido Schwartz 10 B was also shown (f).

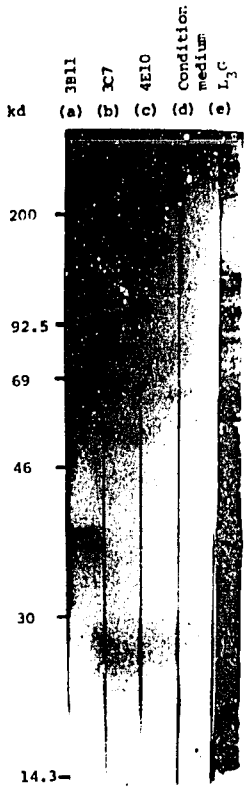


Figure 36. Western blot and immunological reactions of somatic extract of G. spinigerum advanced third-stage larvae tested against monoclonal antibodies 3B11 (a), 3C7 (b) and 4E10 (c). Condition medium was used as negative control (d). The antigen blotted onto nitrocellulose sheet and stained with Amido Schwartz 10 B was also shown (e).

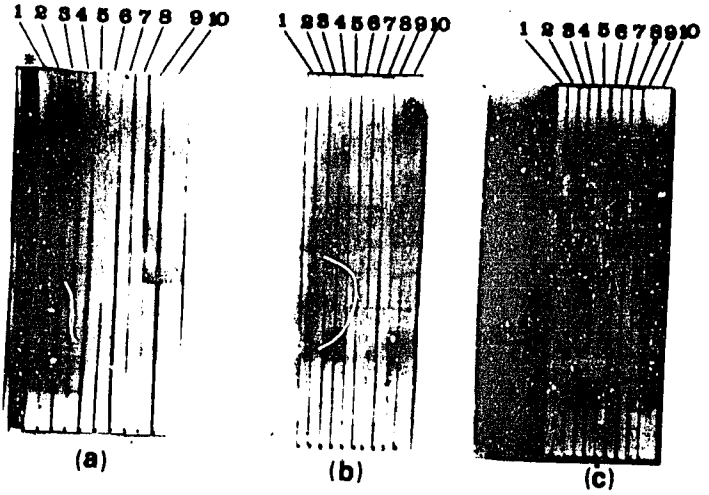


Figure 37. Immunoblot patterns of B. malayi microfilaraemic patient sera diluted to 1:5 (a), 1:10 (b) and 1:20 (c) probed with mouse antisera hyperimmunized against somatic extract of adult male B. malayi (1), adult male and female B. malayi (2), adult female D. immitis, (3), adult male D. immitis (4), third-stage larvae of, G. spinigerum (5), adult female A. cantonensis (6) and adult male A. cantonensis (7). Reactions with pre-immunized mouse serum (8), reference positive (9) and reference negative sera (10) for B. malayi infected patient sera are also included.
(*) Amido black staining of the patient sera.

Figure 38. Immunoblot patterns of human sera obtained from normal individuals in B. malayi -endemic areas diluted to 1:5 probed with mouse antisera hyperimmunized against somatic extract of adult male B. malayi (1), adult male and female B. malayi (2), adult female D. immitis (3), adult male D. immitis (4), third-stage larvae of G. spinigerum (5), adult female A. cantonensis (6) and adult male A. cantonensis (7). Reaction with pre-immunized mouse serum (8), reference positive (9) and reference negative sera (10) for B. malayi infected patient sera are also included.
(*) Amido black staining of the normal human sera in endemic areas.

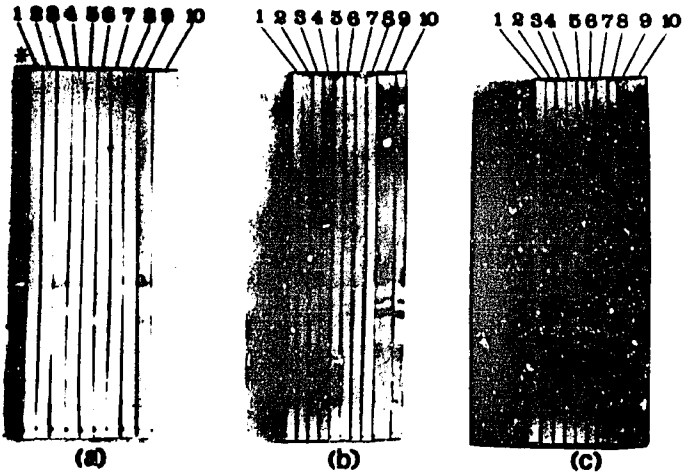


Figure 39. Immunoblot patterns of human sera obtained from individuals in B. malayi -nonendemic areas diluted to 1:5 (a), 1:10 (b) and 1:20 (c) probed with mouse antisera hyperimmunized against somatic extract of adult male B. malayi (1), adult male and female B. malayi (2), adult female D. immitis (3) adult male D. immitis (4), third-stage larvae of G. spinigerum (5), adult female A. cantonensis (6) and adult male A. cantonensis (7). Reaction with pre-immunized mouse serum (8), reference positive (9) and reference negative sera (10) for B. malayi infected patient sera are also included.
(*) Amido black staining of the normal human sera.

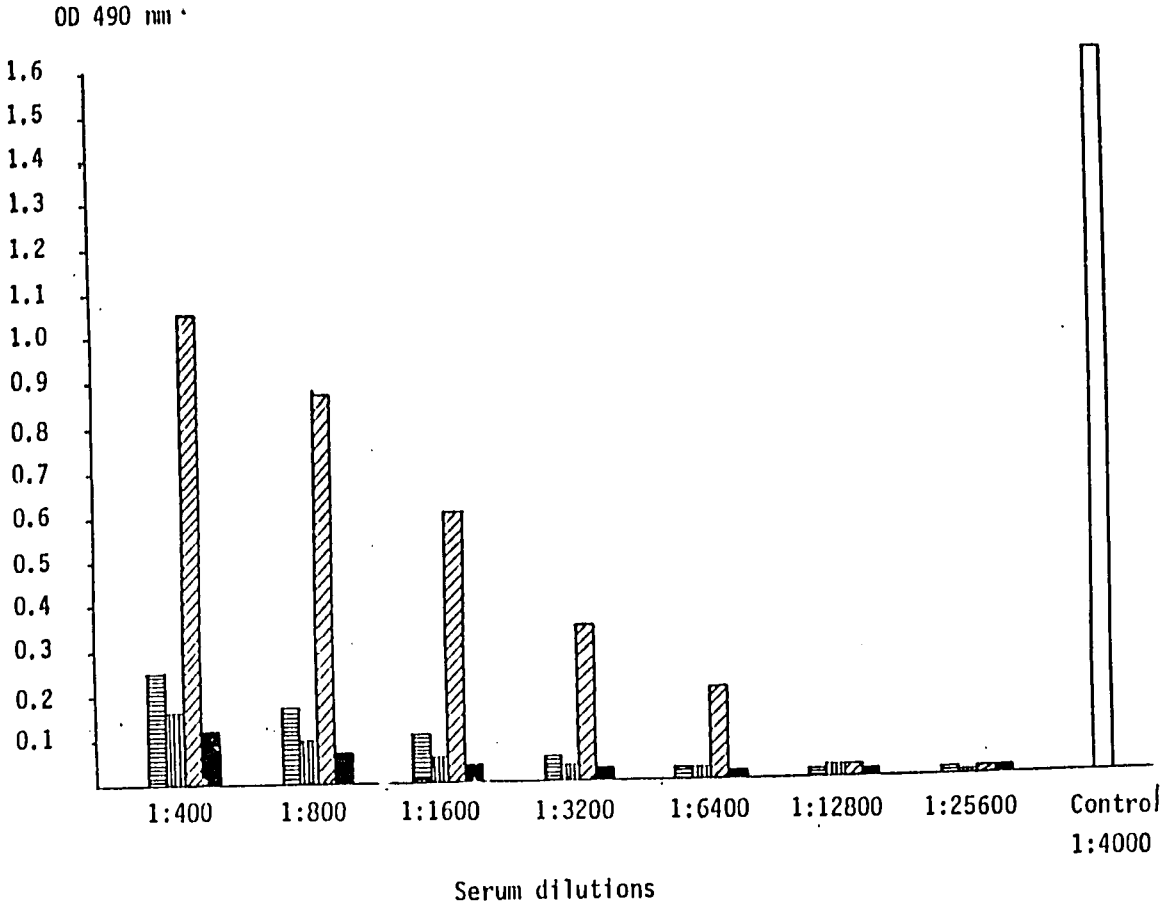


Figure 40. ELISA (OD 490 nm) values obtained from the reactions between aqueous somatic extract of adult B. pahangi and hyperimmune rabbit sera immunized with human sera obtained from normal individuals in non-endemic areas (▤), normal individuals in B. malayi endemic areas (▥), B. malayi microfilaraemic individuals (▨) and B. malayi symptomatic microfilaraemic individuals (■). Hyperimmune rabbit sera immunized with somatic extract of adult B. pahangi was used in the positive control reaction (□).

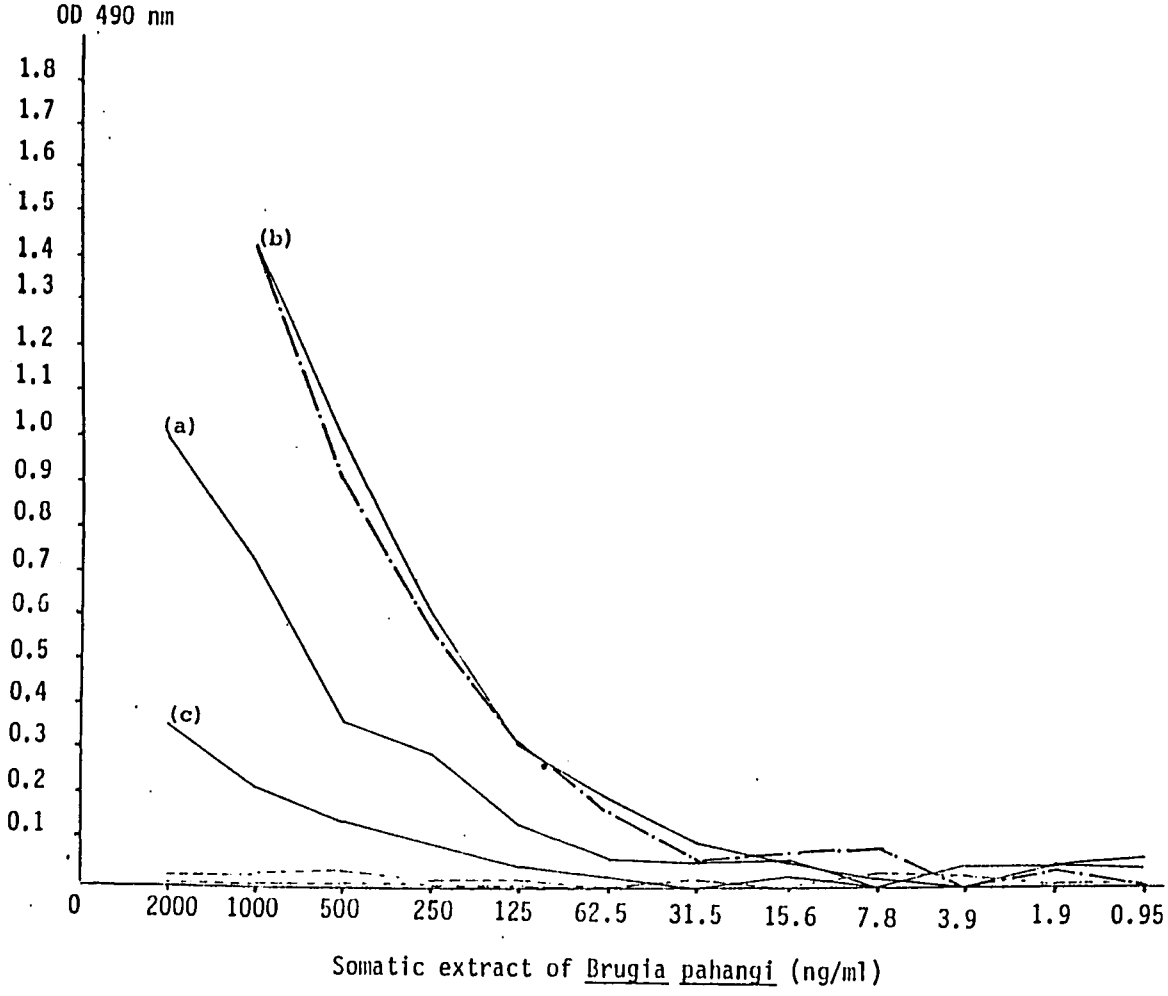


Figure 41. OD_{490nm} values obtained from Biotin-Streptavidin ELISA. An EIA plate was coated with 5 ug/ml of rabbit-anti-mouse IgG followed by 0.045 ug/ml of McAbs 3B11 (a), 3C7 (b), 4E10 (c), for a cocktail of the three McAbs mixed at the ratio of 1:1:1 (---.---.---). The plate was allowed to react with somatic extract of adult B. pahangi at concentrations of 2000-0.95 ng/ml. The antigen-antibody reaction was detected by addition of biotinylated-rabbit-IgG-anti-B. pahangi at 1:100 dilution. Streptavidin-peroxidase labeled at 1:1000 dilution and 0.5 mg/ml of O-phenylene diamine substrate. The reaction was stopped with 4.5 M H_2SO_4. The reactions in the absence of the antigen are shown as broken lines (-----).

Table 1. Immunization protocols and their effects on serum titer, on number of spleen cells stimulated and on number of specific hybridomas.

Fusion No.	93
mouse, Balb/c/J	1
Immunization (female *B. malayi* antigen form stock solution in ug)	
1 (Day 0, IP)	60
2 (Day 30, IP)	30
3 (Day 50, IV)	30
Polyclonal serum titer	
Titer OD (490nm; Titertek Multiskan spectrophotometer)	
Day 0	0
Day 50	1:51,200 (OD = 0.78)
Myeloma	X63-Ag 8.653 (.653)
Wells with growth	225/240 (93%)
Positive wells (OD > 0.20)	133/240 (55%)
Brugia specific McAb	3/133 (2.25%)

OD titers of three positive clones at the different period of time

	3 Jan 90	13 Jan 90	21 Feb 90
F93	3B11 = 0.32	3B11 = 0.39	3B11 = 0.39
	3C7 = 0.69	3C7 = 1.08	3C7 = 1.08
	4E10 = 0.42	4E10 = 0.44	4E10 = 0.49

Table 1.1.

Summarization of Fusion No. 105

1. Fusion no. 105
2. Mouse Balb/c/J amount 1
3. Immunization data
   3.1 Antigen; *Brugia* - specific band from SDS-polyacrylamide gel, molecular weight 19.5-25.0 kd
   3.2 Doses (in ug) and Route of Immunization

1st Imm. (Day 0, IP with CFA)	60 ug
2nd Imm. (Day 28, IP with IFA)	30 ug
3rd Imm. (Day 47, IP)	30 ug
4th Imm. (Day 57, IP)	30 ug

4. Polyclonal serum titer (O.D. at 490 nm; Titertek Multiskan Spectrophotometer)

Day after immunization	O.D.
Day 0	0
Day 47 (dilution 1:800)	1.37
Day 57 (" 1:800)	1.26

5. Myeloma cell X63-Ag-8.653 (.653)
6. Immunized cell: spleen cells with fibrosis
7. Result of the fusion

Wells with hybrids growth	203/360 = 56.38%
Wells of positive (before treated with chloramphenicol)	2/203 = 0.98%

Table 1.2

Summarization of Fusion No. 106

1. Fusion No. 106
2. Mouse B/C/J amount 1
3. Immunization data
   3.1 Antigen; *Brugia* - specific band from gel electrophoresis MW 14.3-19.0 kd
   3.2 Doses (in ug) and Route of administration

1st Imm. (Day 0, IP with CFA)	60 ug
2nd Imm. (Day 28, IP with IFA)	30 ug
3rd Imm. (Day 47, IP)	30 ug
4th Imm. (Day 57, IP)	30 ug

4. Polyclonal serum titer (O.D. at 490 nm; Titertek Multiskan Spectrophotometer)

Day after immunization	O.D.
Day 0	0
Day 47 (dilution 1:50)	0.87
Day 57 (" 1:50)	0.99

5. Myeloma cell X63-Ag-8.653 (.653)
6. Immunized cell: spleen cells with fibrosis
7. Result of the fusion

Wells with hybrids growth	173/300 = 57.67%
Wells of positive (before treated with chloramphenicol)	4/173 = 2.3%

Table 1.3.

Summarization of Fusion No. 107

The protocol of this fusion is the same as Fusion no. 105 (From item 1 to item 5) because it was the same B/C/J but for this fusion we used lymph node cells as immunized cells instead of spleen cells.

6. Immunized cell; cells from lymph nodes
7. Result of fusion

Wells with hybrids growth	71/120	= 59.1%
Wells of positive (Before treated with chloramphonicol)	4/71	= 5.6%

Table 2. Number of cases from endemic areas of B. malayi giving positive ELISA value with antigens of B. malayi, D. immitis and G. spinigerum.

Group	Total number of cases	% of cases giving positive ELISA values with		
		B. malayi	D. immitis	G. spinigerum
Normal control	17	0.00	0.00	0.00
Normal in endemic area	95	49.47	38.95	64.21
I	47	95.74	91.49	70.21
II	5	100.00	100.00	100.00
III	47	59.57	48.94	63.83
IV	27	40.74	62.96	77.78
V	73	60.27	60.01	68.49
VI	31	74.19	58.06	77.42
VII	8	37.50	62.50	100.00
VIII	16	62.50	75.00	43.75
IX	25	68.00	40.00	68.00

Table 3. Number of cases from endemic areas of *W. bancrofti* giving positive ELISA value with antigens of *B. malayi*, *D. immitis* and *G. spinigerum*.

Group	Total number of cases	% of cases giving positive ELISA values with		
		B. malayi	*D. immitis*	*G. spinigerum*
Normal control	17	0.00	0.00	0.00
Normal in endemic area	86	58.13	32.56	69.76
I	46	84.78	60.87	91.30
III	13	23.07	30.77	84.61
IX	2	50.00	50.00	100.00
X	2	100.00	50.00	100.00
XI	1	0.00	0.00	0.00

Table 4. Sources of antigens in various tissues of adult *B. pahangi* as detected by indirect immunofluorescence assay.

Tissues	Human antisera		Cat antisera		Jird antisera	
	+*B. malayi*	+*W. bancrofti*	+*B. malayi*	+*B. pahangi*	+*B. malayi*	+*B. pahangi*
BODY WALL						
Cuticle	++	++	++	++	++	++
Epicuticle	+++	+++	+++	+++	+++	+++
Hypodermis	++	++	++	++	++	++
Muscle	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
Lining body wall						
GUT						
Epithelial cell	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
UTERUS						
Epithelial cell	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
Egg shell	+++	+++	+++	+++	+++	+++
Egg interior	+	+	+	+	+	+
TESTIS AND SEMINAL VESICLE						
Epihelial cell	++	++	++	++	++	++
Basal lamina	+++	+++	+++	+++	+++	+++
Sperm	+++	+++	+++	+++	+++	+++
Interstitial content	++	++	++	++	++	++

+++ = Intense fluorescence, ++ = Moderate fluorescence, + = Light fluorescence

Table 5. Source of antigens in various tissues of adult *B. malayi*

Tissues	Human antisera		Cat antisera		Jird antisera	
	+*B. malayi*	+*W. bancrofti*	+*B. malayi*	+*B. pahangi*	+*B. malayi*	+*B. pahangi*
BODY WALL						
Cuticle	++	++	++	++	++	++
Hypodermis and chords	++	++	++	++	++	++
Muscle	++	++	++	++	++	++
Basal lamina (of body wall separating the pseudocoel)	+++	+++	+++	+++	+++	+++
GUT						
Eithelium	++	++	++	++	++	++
Basal lamina	+++	++	+++	+++	+++	+++
REPRODUCTIVE TRACTS						
Female						
Uterine epithelium	++	++	++	++	++	++
Uterine epithelium lining	+++	+++	+++	+++	+++	+++
Uterine basal lamina	+++	+++	+++	+++	+++	+++
Egg interior	+	+	+	+	+	+
Egg shell	+++	+++	+++	+++	+++	+++
MALE						
Testis epithelium	++	++	++	++	++	++
Testis basal lamina	+++	+++	+++	+++	+++	+++
Sperm interior	++	++	++	++	++	++
sperm surface	+++	+++	+++	+++	+++	+++

+++ = Intense fluorescence, ++ = Moderate fluorescence, + = Light fluorescence

Table 6. Results of ELISA testing for 1) monoclonal antibodies before and after treatment with 200 mg/ml chloramphenicol 2) cross-reactions with antigens from different parasites.

McAb*	Before chloram treated O.D. with B.P.* antigen	After 200 mg/ml chloramphenicol treatment								
		B.m (♀)	B.p. (♀)	B.p. (♂)	D.imm (♀)	D.imm (♂)	A. canto (♀)	A. canto (♂)	Gn.L3	T.cati(♀)
F 105 -4E3	0.15	0.37	0.36	0.37	0.20	0.33	0.19	0.27	0.47	0.34
F 105-1010	0.77	LOSS ACTIVITY NO TITER ----------								
F 106-1E4	0.52: 1.02									
F 106-3G2	0.17									
F 106-4E9	0.11									
F 106-5G6	0.21									
F 107-1D11	0.22	0.19	0.42	0.24	0.07	0.09	0.04	0.03	0.24	0.08
F 107-1E11	0.66									
F 107-1G6	0.51	----------NO TITER ----------								
F 107-2G2	0.71									

*McAb = monoclonal antibody
B.p = Brugia pahangi
B.m = Brugia malayi
D.imm = Dirofilaria immitis

T. cati = Toxocara cati
A. canto = Angiostrongylus cantonensis
Gn. L3 = Gnathostoma spinigerum third stage larvae

Table 7. ELISA (OD 490 nm) reactivities of 15 MaAbs against aqueous somatic extract of filarial and nonfilarial nematodes.

McAb	Filarial nematode antigens				Nonfilarial nematode antigens		
	B. malayi ♀	*B. malayi* ♂	*D. immitis* ♀	*D. immitis* ♂	*A. cantonensis* ♀	*A. cantonensis* ♂	*G. spinignum* L3
4E9	1.36	-	0	0.05	0.17	0	0.80
4G6	over	-	over	over	0	0	0.23
2B3	0.48	-	0.10	0.10	0.16	0.26	0.01
4D10	0.22	-	0	0.05	0.04	0	0.01
3F7	0.44	0.45	0.07	0.02	0.14	0.02	0.02
3C3	1.04	over	0.01	0	0.78	0.08	0.31
3D7	1.45	0.05	1.57	1.49	0.13	0.09	0.13
4B9	0.39	0.30	0.09	0.28	0.06	0.02	0.02
4F3	0.40	0.84	0	0	0.02	0	0.02
3D3	0	0.19	0	0	0	0	0
3D7	1.0	0.45	1.51	0.87	0	0	0.08
4B9	0.07	0.17	0	0	0	0	0
4G6	1.19	0.33	over	1.01	0	0	0.08
3F3	0	0.43	0	0	0	0	0.07
3C4	0.24	0.12	0	0.01	0.02	0	0.08

Discussion

In the present study, somatic extracts of adult Brugia malayi and B. pahangi were antigenically characterized. The almost identical protein profiles of these worms were probed with polyclonal mouse antisera obtained from animal hyperimmunized with somatic extracts of adult B. malayi, B. pahangi, Toxocara cati, Dirofilaria immitis and advanced third-stage larvae of Gnathostoma spinigerum. It was clearly demonstrated that only the anti-Brugia antisera that reacted with molecules 14.3 and 21.5 kd of the Brugian worms (see Fig. 20). We, therefore concluded at this stage that the antig ens 14.3 and 21.5 kd are of Brugian specificity. Further characterization of the Brugian antigens was performed by using clinically and immunologically characterized human antisera from endemic areas of B. malayi and W. bancrofti. These filarial human sera reacted strongly with several antigenic components of the Brugian worms. However, it was strikingly noticed that the 25 kd antigen component of these worms were shown up quite distinctively and prominently when the antigen was probed with B. malayi microfilaraemic patient sera (see Figs. 20.I and 21.1 A, C, and E). This band was not shown up when the antigen was probed with sera from W. bancrofti-infected individuals (see Fig. 22). The specific reaction of this 25 kd antigenic component of adult female Brugian worms with only the B. malayi - microfilaraemic patient serum strongly indicated that the antienic component is most likely to belong to the microfilariae of Brugian parasites. This conclusion was supported by our finding that the 25 kd antigenic component did

not appear on any of the immunoblotting reactions of the somatic antigens of female adult D. immitis or advanced third-stage larvae of G. spinigerum (see Figs. 21.2 and 21.3). Since the finding of the 25 kd antigenic component of B. malayi microfilariae has been reported previoushy by Maizels (830375, WHO Report of the steering committee of the scientific working group on filariasis, July 1983 to June 1988, p.14) it strongly suggested that this component is not likely to be of strain specificity if we consider our filarial worms as genetically distinct from those reported from other part of the world. In addition to the 14.3, 21.5 and 25.0 kd antigenic components we also noticed the appearance of 18 kd antigen of the Brugian worms shortly after the immuno-enzymatic staining of the protein profile was performed by using the reference positive patient serum. However, it disappeared completely after the reacted sheet was kept at room temperature for a few hours. We cannot make any conclusion about this molecule. Nevertheless, we considered the significance of the 14.3, 21.5 and 25 kd Brugian specific antigens as the starting materials for our goal. These components were cleaved off from the SDS-polyacrylamide gel and were used as the immunizing agent for mouse monoclonal antibody production. It was unfortunate that we did not get any monoclone out of that fusion (Fusion no. 106). The reason was because of the toxicity and/or contamination on the hybrid themselves.

We were not sure whether the causative agents came from the individual bottle of fetal calf serum or from the feeder cells (normal B/C/J spleen cells). When we treated those hybrids with 3 cycles of 100 ug/ml and 200 mg/ml of garamycin and

chloramphenicol respectively for every other days, the ELISA reactivity of the clones were rapidly dropped. This immunization protocol should have been repeated, but it was not. The reason was the lack of antigen supply.

Apart from the finding of the antigenic components 14.3, 21.5 and 25.0 kd, we also found that molecule 69 kd of the Brugian worms reacted strongly with W. bancrofti-infected human sera (see Figs. 22A and 22B). Since it did not react with any of the heterologous mouse antisera (see Fig. 20), we concluded that the 69 kd antigenic component of the Brugian worms are more likely to carry the antigenic epitope(s) of female adult Brugian worms that cross react with antisera of most of the W. bancrofti infected cases. It is still to be further investigated whether or not this 69.0 kd antigen component will be valuable for the development of serodiagnostic test for W. bancrofit. Apart from these results, we also found out that the component 92.5 kd of the Brugian parasites was recognized by almost all of the heterologous antisera (see Figs. 20 and 22-A, 22B).

As we have discussed, we did not get any monoclones out of the fusion no. 106 using the spleen cells hyperimmunized with antigenic components 14.3, 21.5 and 25 kd. However, three monoclonal antibodies namely 3B11, 3C7 and 3E10 were selected from the previous fusion (fusion no. 93) by using the criteria that they do not have ELISA reactivity with antigens of A. cantonensis, D. immitis or G. spinigerum. When the reactivity of these McAbs were further defined by immunoblotting assay, we found out that the McAb 3B11 reacted with antigenic components of

G. spinigerum. We have tested for the possibility of these McAbs as the diagnostic agent by using a monoclonal antibody based-ELISA. We found that the test was still not sensitive enough. It could only detect the soluble antigen in the test specimen at concentration of at least 7.8 ng/ml (OD 490 was ~0.1). However, further study will be carried out by using the same technique we already have in our hand at the present time.

Notice has to be made here that we have passed the very bad time during the first two years period of the project. We have started our first year with the production of parasites, collection of all parasitic materials and serum specimens and also collection of all needed equipments and supplies. At the end of the first year, the specimens we have collected in the -20°C freezer were gone because the electric supply to the building was out of order during the long weekend. We started another year trying to concentrate more seriously on the antigen production. It was unfortunately that the mosquitoes decreased their susceptibility to infection. Moreover, they produced less off-springs. This made us more difficult to work. It is noticeable that we only mentioned about the analysis of the antigenic compositions of the female adult Brugia sp. somatic extract eventhough it was mentioned in our proposal that both somatic and ES antigens of several developmental stages would have been studied. In fact, we have done some in vitro cultivation of the third-and the fourth-stages larvae and adult worms of Brugia sp. parasites. We had already been successfully culturing the third-stage larvae of the Brugian parasites in RPMI 1640 (without phenol red) in the presence of MK-2 cells. The

larvae survived for a few days in 5% CO_2 at 37°C. The fourth-stage larvae, on the other hand, could be maintained in the same recipe of RPMI 1640 but supplemented with 10% fetal calf serum in the same CO_2 incubator. We found that adult worms remained quite active when culturing in RPMI 1640 without phenol red (GIBCO, USA) for 3 days in 5% CO_2 at 37°C. It was unfortunate that we did not have chance to try to keep them longer than 3 days, eventhough we believe that they would have survived quite longer, as most of the worms were to be supplied to Dr. Billing who always needed quite a large number of freshly collected (not to be cultivated) worms for preparing his immunizing antigen. It was found out later that the technique he prepared the antigen was to collect the leaked antigen from the worms that had been alternatively frozen and thawed. It was really discouraging that he did not get any monoclone even after 2 years of his collaboration to our project. We have tried to work on the very small amount of the ES product we have collected, but found out that the amount was insufficient. We could not even demonstrate any of the protein component on SDS-PAGE after ultrafiltration. That was the reason we did not have any data on the ES product. It should also be mentioned here that the work on W. bancrofti antigens had to be stopped since our collaborator who worked for the Filariasis Division, Department of Communicable Diseases Control, Ministry of Health could not obtained the human clinical material from endemic areas of W. bancrofti. It might suggest that the eradication methods have worked in most of those areas. We should also mention here

that apart from all of the problems we have come across the important reason that made the project not successful is our lack in experience. The present study is the first large research project for most of us. Nevertheless, we have gained a lot of experience and learned valuable lessons from this project. The technologies we have gained and all the equipments originated from this grant will be our tools for the future works.

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