THE CHARACTERIZATION OF COMPLEMENT FIXING CIRCULATING ANTIBODY BY THE JIRD (*MERIONES UNGUICULATUS*) IN RESPONSE TO INFECTION WITH *BRUGIA PAHANGI* OVER AN EXTENDED PERIOD OF TIME.

A Thesis

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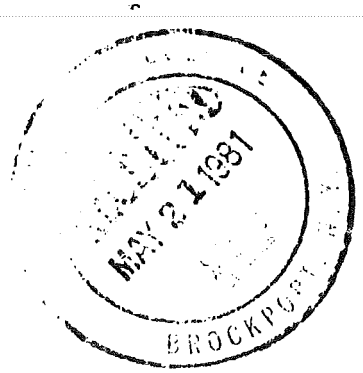

SUMMARY

Male outbred jirds (Meriones unguiculatus) were innoculated with doses of 100 or 200 infective Brugia pahangi larvae (l_3's), and tested for complement fixing circulating antibody. Following innoculation, all test animals became positive for microfilaria in peripheral blood. Seventeen of 20 test animals developed patent, sustained infections within, or shortly after 60 days. The longest prepotent period was measured at 118 days. Readings of microfilarial densities were taken after the first month, through 12 months, with the final microfilarial reading between 12 and 21 months. Microfilarial densities vacillated through the course of infection.

At the time of necropsy, adult worms were recovere recovered from the testes, heart and pulmonary regions, pelt and carcass and viscera. Recovered adults were sexed and counted for their particular region of residence. The majority of the recovered adults were found in either the testicular region or the pulmonary region with small percentages in other areas. Total worm recovery was between 25% and 30% of the l_3 dosage innoculated.

Complement fixation reagent concentrations; Rabbit Hemolysin, Guinea Pig Complement, and B. pahangi antigen were titrated and standardized (HEW Technical Report, 1965). Complement fixation tests were performed with test sera from infected animals

divided into 2 groups (A & B), Group A animals had consistently low microfilarial densities throughout the course of infection (<500) with Group B maintaining high densities throughout (>500). The lowest positive titer was determined at 1:4 with the highest titer observed at 1:256. Animals of Group A were demonstrated at having significantly greater mean antibody titers during the third, sixth and post twelve month periods with relation to Group B. The complement fixation titers showed a correlation between microfilarial densities and mean antibody titers, between the two test groups, at significant time periods.

Dedication

This thesis is gratefully dedicated to the following persons:

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For all the help, encouragement and love given throughout the project and my career.

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For all his help, advice, and encouragement without which this project would not have been completed.

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Several hundred species of nematodes belonging to the Order Filariidea are known to be parasitic in the tissues of various vertebrate hosts, with several species being disease causing agents in man (Sasa, 1976). The species which can infect humans are: Wuchereria bancrofti, Brugia malayi, B. pahangi, Loa loa, Dipetalonema perstans, D. streptocerca, Mansonella ozzardi, and Onchocerca volvulas. The World Health Organization indicated that at least 250 million people were infected with the lymphatic-dwelling filaria, W. bancrofti and B. malayi (WHO Technical Report, 1974). The taxonomic status of these two closely related species has been fully described in literature, (Lichtenstien, 1927; Burg, 1927; Rao and Maplestone, 1940; Buckley and Edeson, 1956; and Buckley, 1960).

"W. bancrofti infection in humans has been recorded in nearly all countries or territories in the tropical and subtropical zones of the world, with the exceptions of the deserts or very dry areas. The disease has also been noted from some temperate zone districts such as the mainland of Japan, middle China, and some European countries. Major endemic areas include the South Pacific, South East Asia, South and Central America, and Africa". (Sasa, 1976) Bancroftian filariasis was reported in Charleston, N.C. and through some of the Southern United States in the 1800's until approximately 1920. The disease disappeared spontaniously before World War II due to improvements in general sanitary conditions and the reduction of its disease vector, Culex pipiens.

Malayan filariasis is endemic exclusively in South and East Asia

Major endemic regions include: India, Burma, Thailand, Vietnam, the Philippines, Malayia, Indonesia, Southern Japan, China, and Korea. The spread of malayan filariasis has been apparently barricaded to the North and West by the desert zone extending from West Pakistan to Mongolia. Temperature is probably another factor that has restricted its distribution northwards and beyond the Southern regions of Japan, Korea, and China (Sasa, 1976).

B. pahangi is principally a lymphatic-dwelling parasite in carnivores, with a limited range in primate hosts and man. Differential diagnosis of B. malayi and B. pahangi in humans can be rather difficult with both species existing simultaniously in the same host (Denham and McGreevy, 1977). B. pahangi is endemic in West Malaysia exclusively.

The adult worms of both Wuchereria and Brugia reside principally in the lymphatics of man. The female worms produce sheathed embryos called microfilaria which appear in circulating blood, occasionally showing in hydrocele fluid and chylous urine. The microfilaria are ingested by mosquito intermediate hosts, lose their sheaths, and burrow through the midgut wall into the body cavity, reaching the thoracic muscles within a few hours. They develop into mature third-stage larvae after undergoing three morphological stages. Two days after successful thoracic muscle penetration, the larvae metamorphose into a short, thick, sausage-like form. Developmental differentiation proceeds rapidly, within 5 to 6 days the second morphogenesis is achieved. At around the 10th day of development third stage differentiation begins, resulting in

elongation of the larvae to a long snake-like stage. The mature larvae leave the thoracic muscles, enter the body cavity, and migrate to the proboscus. When the mosquito takes a blood meal, the larvae enter the primary host through the puncture wound left by the mosquito.

The development of B. malayi third stage larvae to mature adults, in human hosts, will take up to a year or longer. The adult worms are thread-like and creamy white in appearance with a smooth cuticula. The adult male B. malayi taken from humans measure 13.5 to 23.3 mm in length and 0.07 to 0.08 mm in width, while mature females measure 43.5 mm to 55 mm in length and 0.13 to 0.17 mm in width (Faust et. al., 1970). The adult developmental period for B. pahangi in the jird (Gerbil) host (Meriones unguiculatus) is 55 to 60 days. The adult male B. pahangi taken from jirds measure 13.1 mm in length and 48 microns in width, while mature females measure 36.3 mm in length and 77 microns in width (Ash and Riley, 1970). The development time for W. bancrofti in the human host varies up to a year or more. The adult male worms taken from human autopsies, measure about 40 mm in length and 0.1 mm in diameter while mature female worms measure 80 to 100 mm in length and 0.24 to 0.3 mm in diameter (Faust et. al., 1970).

Microfilaria Periodicity

Microfilaria periodicity is defined as the ability of different species of microfilaria to react to the availability of its specific

intermediate vector. The result of this periodicity is that the microfilaria will have greater densities in peripheral circulating blood during the time the specific vector is present in the environment. One of the most significant aspects of infraspecific taxonomy is the occurence of clearly defined races, each characterized by the pattern of their microfilarial periodicity. Methods for surveys and the statistical analysis of such characteristics have been previously described by several researchers (Gubler and Bhattacharya, 1974; Sasa, 1976; Turner and Edeson, 1957).

"In W. bancrofti, two races differing in microfilarial periodicity have been recognized": a nocturnally periodic race distributed widely throughout tropical and subtropical zones of the world (with the exception of the Polynesian subregion); a nonperiodic, or more precisely, diurnally subperiodic race reported in a jungle area in West Thailand. These races are specific for different vector groups serving as intermediate hosts. "The microfilarial periodicity of each race coincides with the circadian rhythm of the biting activity of the principal mosquito vector". (Sasa, 1976)

In B. malayi, 2 distinct races are known to exist, a nocturnally periodic race and a nocturnally subperiodic race, first reported in Malaya. The endemic regions are geographically isolated, and each uses different mosquito vectors. Both races have been reported from a number of areas in Southern and Eastern Asia. B. pahangi is a non-periodic race known to exist concurrently with subperiodic B. malayi in the East Pahang Province of Malaya.

Pathology and Symptomatology

The pathology in humans caused by B. malayi and W. bancrofti is similar in many respects with only minor differences. The occurrence of acute symptoms such as fever attacks and lymphangitis are more frequently encountered in B. malayi infections rather than in W. bancrofti. Acute lymphodermia and elephantiasis of the legs, hands, and arms are characteristic of both, however, involvement in the urino-genital organs such as funiculitis, hydrocele, and chyluria, are almost exclusive characteristics of W. bancrofti.

Although shown experimentally transmittable in man (Buckley, 1958) B. pahangi fares poorly in the human host. Chronic elephantiasis as seen in B. malayi and W. bancrofti infections is absent in B. pahangi infections, however, central nervous system involvement can occur with worms invading the spinal area. Instances of this, however, are rare.

The course of events for Bancroftian and Malayan filariasis can be summarized as follows (from Sasa, 1976):

(A) Subclinical filariasis
  (a.1) The Prepatent Period
  (a.2) Patent phase with microfilaremia

(B) Clinical filariasis
  (b.1) Recurrent adenolymphangitis
  (b.2) Elephantiasis

A. Subclinical filariasis

(a.1) The prepatent period:

The prepatent period for both diseases refers to the period from

infection through contact with the intermediate vector to development of a microfilaremia. In W. bancrofti infections, this period has been estimated to require a year or more following continuous exposure of mature infective forms. In Brugia, the prepatent period in humans has not fully been determined. Experimentally, Edeson and Wharton (1958) found this period to be between 76 and 80 days in cats. Under natural conditions, the prepatent period is believed to be longer due to various factors inhibiting mating by adult worms. It must be noted, that only after successive exposure to the infective form larvae through the intermediate mosquito vector, can an infection be established under natural conditions.

(a.2) Patent phase with microfilaremia

The patent phase is marked by the appearance of microfilaria in the circulating blood. With both Wuchereria and Brugia, a considerable number of individuals within an endemic population may show positive microfilaremias, but are probably symptomless at first. Within this population, at least part will exhibit spontaneous remission of microfilaria and continue symptomless, while part will remain positive for microfilaria, also remaining symptomless. However, in heavy endemic areas, many will begin to show acute symptoms and later may develop chronic signs.

(B) Clinical filariasis

(b.1) The acute stage

Recurrent episodes of fever with acute inflammation of the superficial lymph nodes and their associated areas are characteristic features of filariasis due to Brugia spp. infections (Sasa, 1976).

The first symptoms are a painful tender enlargement of the inguinal nodes of one side accompanied by band like streaks of red spreading down the limbs in line with the lymphatics. Fever followed by continued swelling persists for 3 to 5 days before ending by rapid lysis and heavy sweating. The signs of adenitis and lymphagitis resolve gradually during the next 6 to 7 days. Swelling may occur at the distal portion of the limb, however, this tends to regress completely after the episode. After repeated attacks, the swelling does not subside and eventually develops into elephantiasis.

The acute stage in Bancroftian filariasis is marked by frequent fever attacks which are associated with lymphangitis or lymphadentis due to inflammation in hypersensitive reactions to the worms and or microfilaria. Reactions such as these, usually occur in the legs, hands, and urino-genital regions. The fever attacks occur at irregular intervals and become more chronic. As time goes on, permanent lesions caused by lymphatic obstructions become more evident.

(b.2) The chronic stage:

The chronic stage is marked by the permanent swelling of part of the body (elephantiasis). Elephantiasis due to Malayan filariasis usually manifests itself in the limbs and hands, rarely in the genital regions or breasts. The upper extremities are less likely to be affected than the legs with this type of filariasis.

Elephantiasis due to W. bancrofti can involve the limbs and hands, but is characterized by urino-genital complications such as hydrocele and chyluria. Blockage of the lymphatic areas leads to chronic inflammation which in turn is manifested in elephantiasis of an often

incurable nature.

Treatment with Diethylcarbamazine (DEC):

Hewitt et. al. (1947) discovered DEC to be a potent filaricide; to date it is the chemical treatment of choice. The standard method recommended for drug therapy is a dose of 6 mg per kg per person if it to be effective as a filaricide. Treatment with DEC, however, causes several side effects such as high fever reactions and inflammation due to precipitating microfilaria, after the initial dose. Due to these side effects, treatment campaigns have often failed when patients refuse to take additional doses of the drug.

Experimental Work in Filariasis

Experimental work with filarial infections offers the possibilities of better treatment procedures, control, and the study of host-parasite relationships. The need for a susceptible small animal host had been stressed by the World Health Organization (WHO Technical Report, No. 359, 1967).

Infections occuring naturally with W. bancrofti have only been demonstrated in man despite intensive surveys of a wide variety of feral and domestic animals. Attempts to experimentally infect laboratory animals have either failed or had limited success. Yokogawa (1939) found that in suckling mice, W. bancrofti larvae were surrounded by fibroblasts within 15 hours on innoculation. Laing et. al. (1961) innoculated 30 to 40 infective larvae of periodic W.

bancrofti into 4 cats and 2 Macaca mulatta monkeys, no microfilaremias developed during a 15 month period, and no adult worms were recovered at necropsy. Dissanaike and Niles (1965) gave 65 larvae to a toque monkey and 55 to each of 4 cats. A single third stage larvae was recovered from a cat which died 20 days post infection. Ramachandran et. al. (1966) innoculated larvae into 15 golden hamsters and 2 kittens, recovering only 1 larvae from a kitten at day 18. In further studies, Ramachandran and Sivanadam (1969) gave increasing larval dosages (40 l_3 to 170 l_3), to 6 cats, 10 to 37 days old, and necropsied them 7 to 27 days later, recovering only 14 larvae. Ash and Schacher (1971) innoculated 60 to 300 infective W. bancrofti larvae into each of 74 male jirds (M. unguiculatus). Fourth-stage larvae were recovered up to 126 days post innoculation, but the fourth molt did not occur and only limited progression was seen beyond 6 weeks. Dissanaike and Mak (1968) testicularly innoculated a splenectomized cacaque monkey with 286 infective W. bancrofti larvae, and treated with Depomedrol for 6 weeks post innoculation. The monkey was necropsied 231 days later recovering 11 mature adult worms. Cross et. al. (1979) innoculated infective larvae of W. bancrofti in Taiwan monkeys (Macaca cyclopis) through subcutaneous foot puncture, or by permitting infected mosquitoes to feed weekly on the monkeys. Some animals were splenectomized and others were treated by varying regiments of immunosupressants. A total of 78 monkeys were exposed to infection with parasites recovered from 63% of the males infected and 29% of the females infected. The prepatent period was determined to be between 8 and 18 months with patent infections

lasting between 5 and 21 months. Successful experimental transmission of W. bancrofti from patent monkeys to uninfected monkeys was also demonstrated.

Experimental transmission of both B. malayi and B. pahangi has been shown to be successful in cats, dogs, and monkeys (Edeson and Wharton, 1958; Schacher, 1962; Edeson, 1960; and Nelson, 1959). The use of cats, dogs, or monkeys as laboratory hosts has severe limitations due to size, handling problems, and expense. Attempts to introduce Brugia spp. into commonly available laboratory rodents has met with varying degrees of success.

Brugia spp. infections in the multimammate rat (Praomys natalensis) have been shown to be successful. Petranyi and Mieth (1974) found that 76% of 92 males became patent after innoculation with 100 infective B. malayi larvae. Ahmed (1966) successfully infected 6 animals with B. pahangi with Benjamin and Soulsby (1974) employing this system in immunologic studies.

The golden hamster, Mesocritcetus auratus, has also been successfully infected with Brugia spp., 35% will develop patent infections with B. pahangi and 20% with B. malayi (Ash and Riley, 1970; Harbut, 1973; and Malone et. al., 1974). Adults worms have been recovered from the heart, pulmonary vessels, lymphatics, and testes. Infections in this system have been shown to be less desirable in comparison with other systems (as opposed to a jird system), due to its rather weak susceptability to infection. Recently, Malone et. al. (1979) indicated that the inbred Syrian hamster (M. auratus), showed increased susceptability to infection with B. pahangi such that it could constitute

a suitable alternative to the jird. Attempts to infect the white rat with Brugia spp. have met with little success. Experimental transmission of B. malayi in rats by Laing et. al. (1961) and Cheong et. al. (1967) found only 19% of 26 animals infected developed patent infections. In other studies by Ash and Riley (1970); Sucharit and MacDonald (1973); and Harbut (1973) rats were innoculated with infective B. pahangi with only 15% showing patent infections.

Experimental infections using Brugia spp. in mice has shown little encouragement. Laing et. al. (1961) and Ahmed (1967) could not demonstrate any signs of infection in mice innoculated with B. pahangi. Cheong and Wong (1967) innoculated 3 strains of mice with B. pahangi and were able to develop patent infections, with the recovery of adult worms from 2 individuals. However, Vincent et. al. (1980) demonstrated that all of 16 nude mice infected with 100 l_3 B. pahangi would develop patent infections with worm recoveries in all animals at necropsy. Among 14 normal mice given 100 infective B. pahangi larvae, 13 worms were seen in short term infections (8 days), but nine recovered from mice necropsied after 50 days.

The most successful attempts to infect small rodents with Brugia spp. have been demonstrated with the black clawed mongolian jird, M. unguiculatus. The jird has shown to be a susceptable host for B. malayi, B. pahangi, and B. patei (Ash and Riley, 1970 and Ash, 1971, 1973). Infections were established by subcutanious innoculations with varying numbers of infective Brugia spp. larvae. Animals infected subcutaneously were shown to have adult worms establishing in the heart, lungs, pulmonary arteries, viscera, subcutaneous es-

tablishment in the pelt and muscle tissue, in the lymphatics associated with the testes, and in the seminiferous tubules (Ash and Riley 1970; Ash, 1971, 1973; Ah and Thompson, 1973; El Bihari and Ewert, 1971). Infective larvae innoculated intraperitoneally were found to develop freely to adults in the peritoneal cavity with little or no migation to the lymphatics and other organs (Ah and Thompson, 1973; MaCall et. al., 1973). Ash (1971) reported a mean prepatent period of 72 days for male jirds and 80 days for females infected with B. pahangi. El Bihari and Ewert (1973), found the mean prepatent period in jirds infected with B. malayi to be 129 days in males and 117 days in females.

Ash (1971) found preferential susceptibility to infection among male jirds over female jirds to B. pahangi; 82% of 17 males and 24% of 17 females became patent after subcutaneous innoculations of 25 to 75 infective larvae. El Bihari and Ewert (1973) made similar observations between the 2 sexes when they were infected with B. malayi; 92% of 26 male and 43% of 21 female jirds became patent after subcutaneous innoculations with 20 to 105 infective larvae. Westley (1973) through endocrine manipulation of both male and female jirds infected with B. pahangi, was able to show that resistance was androgen dependent. Resistance in female jirds was apparently due to estrogen. Devereux and Ash (1978) showed increased susceptability of female jirds with age.

Pathology in M. Unguiculatus due to Brugia spp.

Pathology due to lymphatic-dwelling Brugia spp. has been demonstrated in a variety of different anatomical locations. Ah and Thompson (1973) reported migration of infective larvae of B. pahangi to the regional lymphatics of M. unguiculatus. After being innoculated subcutaneously, infective larvae would migrate as early as 1 to 4 days post infection. Some injected larvae remained in the skin for as long as 30 days. Localization occured in the heart, lungs, pulmonary arteries, pleural cavity, with three-fourths recovered filaria found in the regional lymphatics. Major pathological changes were noted in involved regions. Severe inflammation appeared throughout the migratory pathways and infected tissues. Granuloma formation appeared around dead and developing larvae creating lymphatic and pulmonary obstructions. Lesions were noted in infected organ and tissue systems, both as a result of damage incurred by larval migration, as well as hyperimmune responses to invading larvae.

Vincent et. al. (1976) described the pulmonary pathology in M. unguiculatus due to Brugia spp. infections. The major pathological changes include: (1) granulomas induced by larvae and adults; (2) obstructive endarteritis and; (3) chronic interstitial inflammation with degrading microfilaria. The majority of pulmonary granuloma formation occurred near the time of the final molt at 30 days post infection followed by involution and formation of residual vascular lesions over the next several months. A minority of granulomas a-

rose about sexually mature adult worms and showed a characteristic sequence of development along the length of the worms. Obstructive endarteritis appeared to peak at about the same time as the final molt and became largely fibrotic by 125 days post infection.

Vincent and Ash (1978) investigated splenic indices and histological changes in jirds infected with B. pahangi and B. malayi. Low splenic indices and a lack of histologic changes were observed for the first 2 months post innoculation. At the end of the prepatent period, spleen weights were shown to be increasing with full mean value attained by the fourth month (post innoculation). Spleens from animals infected with B. malayi were roughly double the normal splenic index while in B. pahangi infections, indices were larger.

"Histologic sections showed expansion of both red and white pulp; maximum follicular activity was observed in the third and fourth months post-innoculation and gradually waned in long term infections." Granulomas associated with microfilaria in tissue was a conspicuous feature with B. pahangi. Although no consistant relationship has been established between splenic reactions and microfilaria, Vincent and Ash feel that the increased splenic indices are due to increasing levels of microfilaria as well as excretory, secretory, products released by the adult worms.

Immunology

W. bancrofti

Our present knowledge of the immune response to lymphatic-dwelling filarial species is limited. Immunological studies with W. bancrofti are severely limited due to the lack of a suitable laboratory host, and the scarcity of homologous antigen. In the past, immunological studies involving Wuchereria spp. have involved cross-reaction tests with heterologous antigen in immunodiagnostics (Sasa, 1976).

Natural and Acquired Resistance

Brugia spp.

The susceptability of vertebrates to infection with Brugia spp. varies between and within species. Studies on domestic cats have shown Malayan cats to have a higher rate of patency than do English cats. Ash (1971) demonstrated preferential susceptability of male jirds to infection with Brugia spp. as opposed to female jirds. Wesley (1973) showed that this resistance was androgen dependent, not due to the presence of estrogen, Wesley and Ash (1972) showed gonadectomy and injections of testosterone considerably altered patency rates in jirds but do not vary according to sex.

Denham et. al. (1972) repeatedly infected cats every 10 days by injecting 50 infective B. pahangi larvae into their left hind foot pads and found of the animals had suddenly lost their circulating microfilaria. These studies were extended to include the specific

response of repeatedly infected cats. Denham found that after repeated infections, the cats became resistant to new challenges by infective larvae. At necropsy, live adult worms were found in the lymphatics and microfilaria in the popliteal nodes. Resistance was theorized as probably the result of acquired immunity and of pathological changes in the lymphatics. The degree of resistance was found to be directly proportional to the degree of lymphatic damage.

Kowalski and Ash (1975) studied repeated infection with B. pahangi in M. unguiculatus. After repeated subcutaneous infections (innoculations), a moderate degree of resistance was seen. Adult worm recoveries from singly infected animals was 15% to 16% as opposed to repeatedly infected animals at 8% to 10%.

Attempts at vaccinations against filarial infections using gamma-irradiated larvae of Brugia spp. has met with varied success. Wong et. al. (1969) conducted the first filarial vaccination experiments using subperiodic B. malayi in Macaca mulatta. Two innoculations were able to stimulate protective immunity which lasted 10 to 12 months. The vaccinated animals never became patent, however, developed cryptic infections.

The induction of immunity against developing larvae and adults was demonstrated by Ah et. al. (1974) using B. pahangi in dogs. Six dogs were vaccinated subcutaneously with 500 infective larvae attenuated with 22.5 krad of gamma radiation. All 6 dogs became patent, however, 4 of the dogs showed suppression of microfilaria. In addition, the recovery of adult worms was reduced by 57%, with

2 dogs showing no adult worms at necropsy. Ramachandran (1970) in an attempt to immunize cats against B. malayi was unable to induce immunity. The failure to induce protection with Brugia spp. in cats appears to be dose related; a large stimulus is needed to invoke protective immunity.

Schistosoma mansoni has been shown to evade the hosts immune response by the acquiring of host, or host-like antigens on their surfaces (Smithers et. al., 1969; McLaren et. al., 1975). Mcgreeve et. al. (1975) attempted to show that B. pahangi protects itself in a similar fashion. The first attempt was to demonstrate the incorporation of mosquito antigen on third stage larval cutica. Cats immunized with thoracic flight muscles of Aedes aegypti followed by immunization with third stage larvae from this vector showed no differences in infectivity and subsequent worm recoveries. A second approach of immunizing jirds with cat antigens then transfering adult worms and microfilaria from the cats to the immunized hosts, also met with failure, showing that B. pahangi does not evade the immune response as do schistosomes (Denham and McGreevy, 1977).

Basic Studies

Weller (1978) studied cell mediated immunity during chronic infections with B. pahangi in inbred Lewis rats. Microfilaria were demonstrated in over 90% of the rats by 16 weeks. Using in vitro lymphocyte blastogenesis, cell mediated immune responses of blood, splenic, and mesenteric node lymphocytes were followed during a year

and 6 months of infection. Lymphocyte responses to infective stage larvae were shown demonstratable in the first weeks of infection, whereas responses to microfilarial antigen only developed later on in the course of infection as microfilaremias waned. Lymphocyte responses to the adult filaria vacillated during the infection. Portaro et. al. (1977) employed lymphocyte transformation techniques for the detection of B. pahangi antigens in infected jirds. Portaro demonstrated that lymphocyte populations could determine and discriminate different types of filarial antigens.

Vincent et. al. (1980) infected normal and nude mice with 100 infective larvae of B. pahangi subcutaneously. Normal mice showed only short term infections (8 days) with no adult worms recovered after 50 days. In contrast, all nude mice developed patent infections persisting as long as 240 days post innoculation, with microfilaremias ranging from 2 to 600 per 20 mm^3 of peripheral blood. In the absence of a functional thymus, the nude mouse is severely deficient in its ability to generate cytotoxic effector cells to respond to T-dependent antigens. The results suggested that resistance to B. pahangi infections is directed against immature stages and depends upon the presence of T-lymphocytes.

Piessens et. al. (1980) investigated the mechanisms of specific immune unresponsiveness to microfilarial antigens in the blood of (human) patients infected with B. malayi. Blood from these individuals was shown to contain an adherent cell type that specifically suppresses reactions to microfilarial antigens, but not other antigens. In the absence of continued stimulation by parasite antigens,

this suppressor cell looses its functional activity after overnight culture *in vitro*. These suppressive reactions were also found in serums from patients without microfilaremias, suggesting that immune unresponsiveness in humans with patent infections is due to active suppression of immune responses directed against the parasite by the patients own immune system, but not an intrinsic inability of infected patients to react to parasite antigens.

Immunodiagnostics

Lymphocyte Transformation:

The use of lymphocyte transformation for the detection of filarial infections was employed by Portaro et. al. (1977). Using *B. pahangi* infected jirds, Portaro was able to demonstrate that lymphocyte populations could discriminate between 2 types of filarial antigens. This technique was also shown to be capable of distinguishing filarial antigens from those of *Trichinella*, suggesting its usefulness in the specific detection of filarial infections for clinical and epidemiological work.

Immunofluorescent Antibody Test

The application of the indirect fluorescent antibody test for the diagnosis of filarial infections has been widely employed (Ridley and Hedge, 1977; Sasa, 1976; Barbosa et. al., 1972; Grove and Davis, 1978).

Barbosa et. al. (1972) used heterologous antigen fragments of

O. volvulus in the detection of L. loa and W. bancrofti infections in human subjects. Successful cross reactivity was demonstrated; reactions in which antibody titers were shown to be 1:40 or higher were considered positive. Nineteen out of 20 patients infected with either L. loa or W. bancrofti, or both, were shown to be positive. To further demonstrate the sensitivity and specificity of the test, filarial antigens were used in sera from 50 ancylostimiasis or strongyloidiasis cases, and 50 control seras, in all cases, the tests were negative. Grove and Davis (1978) employed indirect immunofluorescence to measure antibody titers to B. malayi and W. bancrofti in human subjects living in endemic regions. B. malayi was used as the source of antigen with a positive reaction demonstrable at 1:8 or higher. All the subjects tested showed positive titers ranging from 1:8 to 1:1,024 with control subjects negative.

Indirect Hemagglutination

Work done by Benjamin and Soulsby (1976) on the homocryptic and hemagglutinating antibody responses to B. pahangi infections in Mastomys natalensis indicates the level of free antibody in the sera remains relatively low (peak titer; 1:512) and does not increase 60 days post infection (in patent infections). Kowalski (unpublished, work in progress) indicates that hemagglutinating antibody levels in jirds infected with B. pahangi appear about 3 to 4 weeks post infection.

In other work done with L. Carinii in hemagglutination studies, Tanaka et. al. (1969) and Sasa (1976) show titers as high as 1:16,384,

demonstrating a high degree of sensitivity and specificity with this test.

The Complement Fixation System

Complement is a thermolabile complex of 11 serum proteins present in most vertebrates. The concentration of complement appears to be stable except in times of antigenic challenge. In conjunction with specific antibody, this series of proteins becomes activated and will combine with an antigen-antibody complex. Following fixation, complement will proceed in an orderly and sequential fashion referred to as the complement cascade. The end result being (potentially) the elimination of the antigen. Since the fixation of complement proceeds without visible changes *in vitro*, a hemolytic system consisting of sheep erythrocytes sensitized to a rabbit anti-sheep erythrocyte antibody (hemolysin) must be used to indicate complement activity.

In the complement fixation test (Figure 1), known quantities of antigen are added to serially diluted quantities of antisera. Antigen-antibody complexes are allowed to form, followed by the addition of complement. Complement is next allowed to fix with the antigen-antibody complexes overnight before the addition of sensitized sheep erythrocytes. After these sensitized cells are added, unbound complement will combine with the erythrocyte-hemolysin complex, causing the erythrocytes to lyse. If the sensitized cells have not lysed, all the available complement was bound by the antigen and test serum complex. The degree or level of antibody in the test sera to the

specific antigen is measured by the percentage of lysis seen in the hemolytic system.

The preparation for the complement fixation test employs several biological reactions requiring careful standardization. The specific titration of hemolysin, complement, and test antigen are necessary to quantitate optimal amounts before the test can be used.

Complement fixation tests have been widely employed in both nematode infections and viral infections. Tanaka et. al. (1969) employed the complement fixation test with adult L. carinii antigen and sera from cotton rats infected with L. carinii. Antigen titers from infected serum tested showed positive titers ranging from 1:16 to 1:1024. In a related study, Tanaka et. al. (1970) demonstrated cross-reactivity in complement fixation testing among filarial species. Antigens derived from Dirofilaria immitis, L. carinii, and Setaria cervi were found to cross-react to serum from subjects infected with B. malayi, W. bancrofti, Apodems specious, D. witei, and each other, showing a small degree of species specificity.

Thesis

As has been previously described (Ash and Riley, 1970; Ash, 1971; Denham and McGreevy, 1977; Porataro et. al., 1977; Kowalski and Ash, 1975) the male jird, M. unguiculatus is an effective laboratory model for study with lymphatic-dwelling Brugia spp. The jirds size, ease of handling, cost, and relatively short lifespan (2 years or more), make it a preferred system over the normal cat host. The selection of B. pahangi over that of B. malayi is due to the latter's high infectivity

towards the human host as opposed to that of B. pahangi. Although B. pahangi has been demonstrated infective in man (Buckley, 1958) risk of infection is generally poor and less likely than to that of B. malayi.

Little or no work using homologous antigen has been done in complement fixation testing with either W. bancrofti or Brugia spp.. To date, no work has been performed to characterize antibody levels in jirds infected with B. pahangi over an extended period of time using the complement fixation system. Studies performed by Kowalski and others using indirect hemagglutination have demonstrated titers as high as 1:512 in jirds infected with B. pahangi. Further studies by Kowalski (unpublished) show antibody appearing in the sera as soon as 3 to 4 weeks post-innoculation, with titers leveling off after 3 to 6 months. Jird serum samples were collected prior to infection, with B. pahangi and post infection, varying up to 20 months. The application of these serum samples using homologous antigen in the complement fixation assay is hoped to further characterize antibody titers during infections with lymphatic-dwelling filaria. Using this system, further insight will be gained to the host-parasite relationship and the immunological response during that time.

Materials and Methods

Experimental Animals:

The black clawed mongolian jird, M. unguiculatus served as the definitive host for B. pahangi. The animals were obtained from Tumblebrooke Farms, Westgate, Massachusetts. Seven to 16 week old male jirds were infected with either 100 or 200 infective stage larvae (l_3), of B. pahangi.

Intermediate Mosquito Host:

The intermediate mosquito vector, A. aegypti (selected Liverpool strain), were cultured using standard methods adapted from Barr (1964) and McDonald (1962). Mosquitoes were allowed to feed on anaesthetized jirds (10% Na Nembutal solution in sterile water: injected intraperitoneally in accordance with body weight: 0.01ml/gr. wt.) which had microfilarial densities between 75 and 100 microfilaria per 20 mm^3 of peripheral blood at the time of infection. Infected mosquitoes were maintained at a temperature of 80°F. to 83°F. with a relative humidity of 80% for 12 to 14 days, after which time, the mosquitoes were killed and the mature larvae were isolated.

Infection Technique:

Infected mosquitoes were vacuumed out of their cages and transfered to large test tubes immersed in ice. After cold stunning, the mosquitoes were placed on large size petri dishes, crushed and then transferred to Baerman funnels containing Hanks Balanced Saline

with 4 layers of gauze at the top. The funnels containing the crushed mosquitoes were left standing at room temperature for 1 hour to allow the larvae to migrate to the bottom of the funnels. Larvae were collected in a watch glass. Doses of 100 or 200 infective larvae were separated under a dissecting microscope using silicone treated Pasteur pipettes. Doses of infective larvae were pipetted and/or injected with tuberculin syringes. The left and/or right groins of the experimental animals were shaved and a needle inserted to make an artificial subcutaneous pocket. The larvae were injected or pipetted into these pockets with no more than 0.2 ml. of fluid.

Collection of Serum

Animals were bled via the orbital sinus using sterile 5 3/4" Pasteur pipettes. One ml. of serum at each bleeding was stored at -70°C. until used. Serum samples were taken the day of innoculation and weekly for 4 weeks, thereafter at monthly intervals. Samples were collected over a 12 month period, with a final sample being taken at necropsy. Microfilaria densities were monitored bi-weekly for up to 1 year, thereafter at monthly intervals.

Determination of Microfilaria:

Samples (20 mm^3) of peripheral blood (collected via the orbital sinus) were placed in tubes containing 0.2 ml. formalin. Microfilaria were counted under a light microscope.

Preparation of Homologous Antigen:

B. pahangi adults from jirds, previously innoculated with 400 l_3 larvae, (from Dr. John McCall, University of Georgia), served as homologous antigen. Adult male and female worms (isolated from the jird hosts), were placed in a Pyrex tissue grinder and homogenized in phosphate buffered saline (PBS), pH 7.2. The homogenate was sonicated, and ultracentrafuged at 50,000xg for 1 hour. The soluble portion served as the homologous antigen (extracted whole adult male and female B. pahangi worms).

The Complement Fixation System:

The complement fixation assay used was adapted from: Standardized Diagnostic Complement Fixation Method and Adaption to Micro-Test (HEW Report, 1965).

Preparation of Sheep Red Blood Cells (SRBC's)

SRBC's in Alserver's solution were washed 3 times in veronal buffered diluent (VBD). After the third washing, 1 ml. of packed cells was added to 34.7 ml. of VBD, effected a 2.8% suspension.

Preparation of Color Standards:

Eleven serological tubes were labeled 0% through 100% with each designate corresponding to the percentage of hemolysis as would be observed using the 2.8% suspension in the test system. A 0.28% hemoglobin solution was prepared by adding 1.0 ml. of the 2.8% suspension to 7.0 ml. of water (effecting total lysis) then adding 2.0 ml. of concentrated VBD to the solution. A 0.28% SRBC suspension with

9.0 ml. of VBD buffer. One ml. of the 0.28% suspension was placed in the 0% tube, with the 10% tube receiving 0.9 ml. of the 0.28% SRBC suspension and 0.1 ml. of the hemoglobin solution, so on in this fashion. The tubes were centrifuged and the resultant coloration of the increasing hemoglobin solutions through the tubes corresponded to the percent lysis that would be observed through the tests.

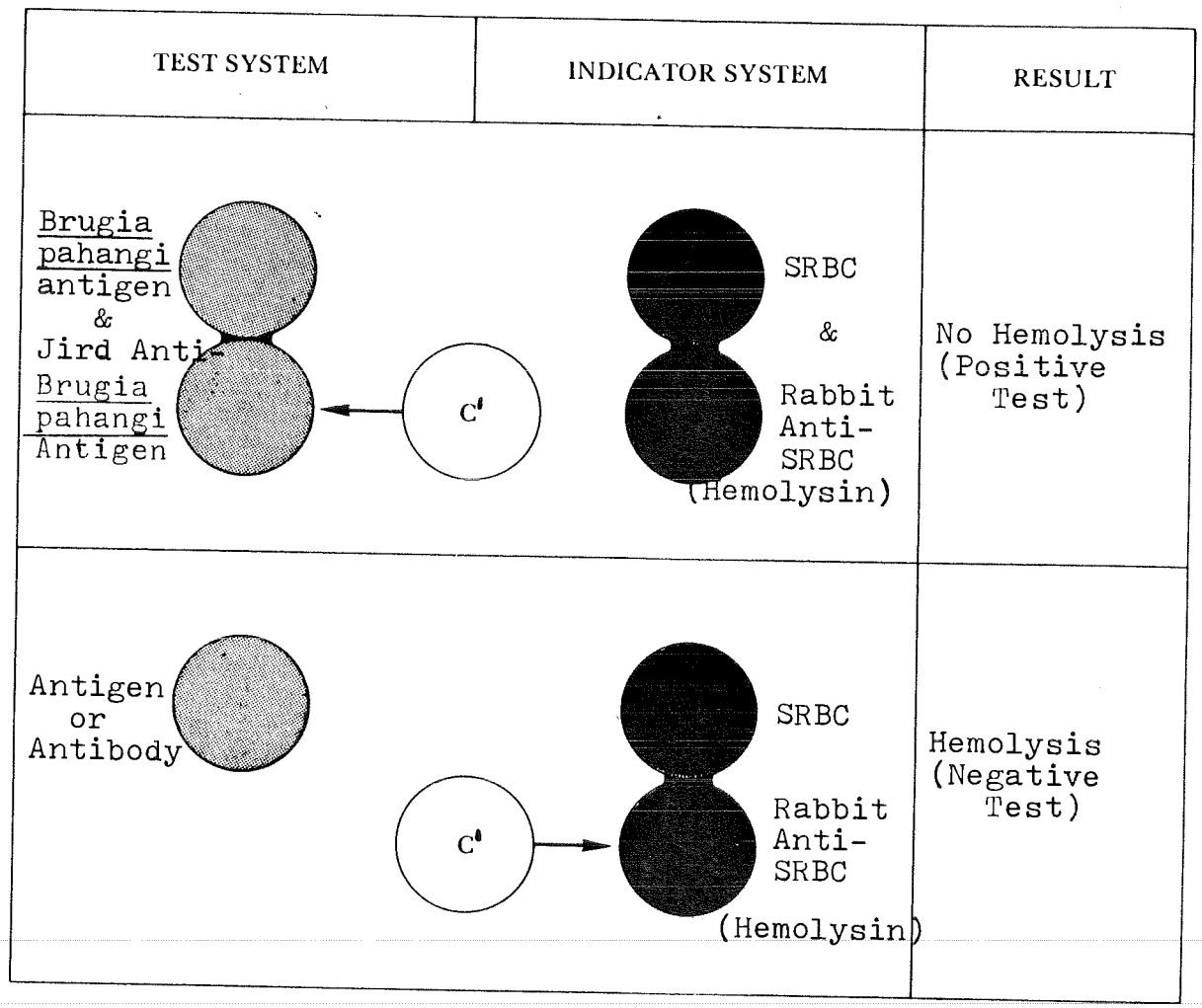


Figure 1; Diagrammatic representation of positive and negative complement fixation tests (Adapted and taken from: Methods in Immunology; Third Edition, Pg. 402; Garvey, Cremer & Sussdorf; 1977; W.A. Benjamin, Inc.)

Hemolysin Titration:

The hemolysin titration proceedure was used to determine the optimal amount of hemolysin required for adequate sensitization of the SRBC's (to effect a 50% end point).

One ml. of the 2.8% SRBC suspension was placed in tubes with 1.0 ml. of varying dilutions of hemolysin in VBD buffer (1:100 through 1:8000). The tubes were then incubated for 30 minutes in a 37°C. waterbath. The dilutions of sensitized cells (0.2 mls. of each dilution), were added to fixed amounts of VBD buffer (0.4 ml.) and Guinea Pig complement (0.4 mls. of a 1:400 dilution in VBD buffer). The suspensions were incubated for 1 hour in a 37°C. water bath, centrafuged and the percentage of hemolysis read against the color standards.

Complement Titration:

The titration of complement follows in much the same fashion as the hemolysin titration. Varying dilutions of Guinea Pig complement (C') were added to a fixed amount of optimally sensitized SRBC's and incubated for 1 hour, in a 37°C. water bath, centrifuged, and read against the color standards. The procedure for the C' titration is listed in Table 1.

Table 1.

Added in Order:	Tube No.: (1)	(2)	(3)	(4)
(1) VBD buffer:	0.8 ml.	0.6 ml.	0.55 ml.	0.4 ml.
(2) 1:400 C':	0.0 ml.	0.2 ml.	0.25 ml.	0.4 ml.
(3) Optimally Sensitized SRBC's:	0.2 ml.	0.2 ml.	0.2 ml.	0.2 ml.

The percentage of lysis (Y) was determined in each assay tube by direct comparison with the color standards. The (Y) value was used to determine the quantity of diluted complement (Y/1-Y), and plotted on log-log paper. (Fig. 2). Straight lines were drawn through the plotted points on both sides of the vertical "1" line. The midpoint of each straight line was then determined, followed by a line drawn connecting the 2 midpoints. The quantity of diluted complement producing 50% lysis was found from the intercept of the plotted line and the vertical "1" line. The intercept on the vertical axis was determined to be 0.26, therefore 0.26 ml. of diluted complement (1:267) equalled $1C'H_{50}$ unit. The complement fixation master test employs $5C'H_{50}$ units; therefore; 1.3 mls. of a 1:267 dilution equalled the $5C'H_{50}$ units. Due to the scarcity of homologous antigen, further titrations and the complement was adapted to a microplate system in "C" plates. In the microtest, 0.5 ml. of diluted complement containing $5C'H_{50}$ units were needed. The subsequent complement dilution was scaled down to accomplish this with a 1:10 dilution of complement in VBD buffer.

Antigen Titration:

The optimal dilution of antigen is defined as that dilution which gives the highest complement fixing antibody titer with a specific standard antisera. The optimal dilution is determined using a 30% lysis end point.

Due to the scarcity of B. pahangi antigen, the rest of the assay was adapted to a microtiter system. The microtiter system simply re-

duces the amounts of materials used by a factor of 10. The assay was performed in "U" shaped microplates.

The antigen titration was performed by adding varying antigen dilutions (in VBD buffer; with dilutions of 1:25 through 1:256,000) to varying antisera dilutions (in VBD buffer; with dilutions 1:4 through 1:256) with a constant amount of complement were allowed to react overnight before adding the optimally sensitized SRBC's. After the addition of the sensitized cells, the plates were incubated at 37°C. for 30 minutes, allowed to settle, and the percent hemolysis read against the color standards.

The Complement Fixation Test:

After successful completion of the preliminary titrations, the complement fixation assay was performed using the optimal dilutions of the titrated materials against the test seras.

The jird test sera was diluted in VBD buffer with the range of dilution being 1:4 through 1:512. After the dilutions of the test sera was made, the optimally diluted B. pahangi antigen was added and allowed to react for 15 minutes at room temperature before adding complement. After the addition of complement, the plates were allowed to react overnight before the addition of the sensitized SRBC's. Upon the addition of the SRBC's, the plates were sealed and incubated for 30 minutes at 37°C. and then allowed to settle overnight. The amount of hemolysis was read against the color standards. The last well showing 30% lysis was regarded as the highest antibody titer.

Results:

Infection with B. pahangi in M. unguiculatus:

Male outbred jirds (M. unguiculatus), were innoculated with doses of 100 or 200 infective B. pahangi larvae (l_3's). Animals were divided into 2 groups by l_3 dosage levels, 100* or 200**. Following innoculation, all test animals became positive for microfilaria in the peripheral blood. Seventeen of 20 test animals developed infections within or shortly after 60 days, with the longest prepatent period being 118 days. Readings of microfilarial densities were taken after the first month, through 12 months, with the final microfilaria reading between 12 and 21 months. Microfilarial densities vacillated through the course of infection (Table 2).

At the time of necropsy, adult worms were recovered from the testes, heart and pulmonary regions, pelt and carcus, and viscera. The adults recovered were first divided by sex, then counted for the particular area. The majority of the recovered adults were found in either the testicular region or the pulmonary region. The percentage of males to females vacillated between necropsied regions and from animal to animal (Table 3). These results do not show definative ratios between males and females recovered, either from the individual area or total animal. In most cases, animals which received 200 l_3 dosages as opposed to those which received 100 l_3 dosages, show a higher total worm recovery.

In both sets of data (Tables 2 and 3), control subjects were shown to be negative for microfilaria and adult male and female worms.

Hemolysin Titration:

Varying amounts of rabbit anti-sheep red blood cell antibody (hemolysin) were titrated against constant amounts of complement and SRBC's. The optimal amount of hemolysin required for the adequate sensitization of the SRBC's was determined (Figure 2). Several hemolysin titrations were performed to determine consistency in testing and results. Reading from left to right in Figure 2, the greatest percentage of SRBC hemolysis (85%), was observed with the 1:1000 dilution followed by consistent 70% hemolysis for dilutions 1:1500; 1:2000; and 1:2500. After 1:2500, resulting hemolysis percentages for 1:3000; 1:4000; and 1:8000 dropped sharply to as low as 35% hemolysis. The optimal dilution as determined in Figure 2, was taken as the second point on the plateau (graphically) where varying hemolysin dilutions showed a consistent percentage of hemolysis of SRBC's. The 1:2000 dilutions were found to be the optimal amount of hemolysin needed for adequate sensitization of the SRBC's.

Complement Titration:

In complement titration a 50% hemolytic endpoint was used to determine the potency of the complement. The protocol for complement titration has previously been described (Table 1), and performed in triplicate. Varying volumes of a constant dilution of complement plotted against increasing hemolytic endpoints (Y/1-Y) show that the midpoint of the intercept line coincides with a .26 ml. volume of complement on the vertical axis. As previously described (Materials and Methods: Complement Titration), the complement master test em-

ploys 5C'H_{50} units. Therefore, 1.3 mls. of the 1:267 dilution of stock complement was 5 times the amount needed to lyse 50% of the sensitized SRBC's in a 2.8% suspension with VBD buffer.

Antigen Titration:

Varying antigen dilutions were compared with varying standard antisera dilutions (Table 4). A curve drawn around the 1:25 and 1:50 dilutions is shown, without being anti-complementary. The dilution of 1:50 was chosen over that of 1:25 due to the scarcity of homologous antigen. The complement-antigen controls show adequate hemolysis corresponding directly with the amount of C'H_{50} units used with any sign of anticomplementary activity.

Complement Fixation Testing:

Test animals were divided into 2 groups: those with consistantly low microfilaremias throughout the course of infection (Group A); and those with consistantly high microfilaremias throughout the course of infection (Group B) (Table 5). The dividing point for each group was made in correspondence with term microfilarial densities (Table 2), and a microfilarial count taken at the time of necropsy. Animals with densities below 500 microfilarie per 20 mm^3 of peripheral blood were considered low (Group A), and those greater than 500 were considered high (Group B).

Reciprocal antibody titers for complement fixation testing are shown for samples taken the day before infection, at 1.5 months, to 3 month intervals with post 12 month samples ranging from 12 to 21

months. Complement fixation testing between groups A and B for each sample and month were performed on the same day to establish consistency in testing. Both groups, A and B, showed patterns of consistent increases in complement fixing circulating antibody to the homologous B. pahangi antigen. Eighty percent of the animals in group A showed positive antibody titers at 6 weeks post-innoculation with 100% of the animals in group B positive during the same period. Antibody titers, after 6 weeks, in both groups, were shown to be positive 90% to 100% throughout the course of infection, with increasing means with time (Table 5). The mean and standard error are shown for each group (Table 5). The Mann-Whitney U test for non-parametric statistical analysis between means was employed (Sokal and Rohlf, 1969). Significant differences between means were demonstrated at 3, 6, and post 12 month periods. The P values for months, 3 and 6 were shown to be less than 0.05 (P= .05) with the post 12 month P value being less than 0.01 (P= .01). Antibody titer means for group A and B are depicted graphically in Figures 3 and 4, respectively, with the differences between means demonstrated in Figure 5. The reciprocal antibody trends for both groups combined with complement fixing circulating antibody titers ranging from 1:4 to 1:256 were obtained (Figure 6).

All control animals tested negative for antibody to B. pahangi antigen in complement fixation testing.

Table 2: B. pahangi microfilarial densities per month post-immoculation is shown. Test animals are divided into 3 groups by the number of infected larvae each recieved (100*, 200**, or none***) Adult worms recovered at necropsy are also demonstrated.

Table 2

B. pahangi Microfilarial Densities per Month Post-Innoculation

animal Number	Date Infected	Adult worms Recovered	Months Post Innoculation/Microfilariae per 20 mm^3 of Blood 1	2	3	4	5	6	7	8	9	10	11	12	Final Mf.
288*	11-11-78	17	0	2	36	16	3	39	12	34	38	44	62	47	47
289*	11-11-78	37	0	2	5	160	131	123	194	210	333	593	284	499	401
290*	11-11-78	34	0	1	11	15	7	54	31	86	212	166	200	168	297
291*	11-11-78	34	0	1	41	298	146	113	17	16	18	83	75	12	12
293*	11-11-78	18	0	0	27	189	311	487	434	525	675	469	802	+500	+500
333*	3-2-79	29	0	19	24	17	18	42	25	58	44	1	23	9	0
334*	3-2-79	39	0	0	0	0	15	4	2	1	0	0	3	6	1
335*	3-2-79	30	0	37	36	0	0	0	0	0	0	0	1	0	1
336*	3-2-79	32	0	27	136	234	280	509	309	232	104	191	571	493	+500
338*	3-2-79	29	0	34	149	178	289	429	484	801	807	626	+1000	+2000	+500
339*	3-2-79	32	0	5	14	1	11	0	0	0	0	-	-	-	-
296**	11-15-78	34	0	13	313	466	992	709	523	301	438	639	1076	438	+500
297**	11-15-78	41	0	1	40	91	247	410	377	275	225	394	248	235	+500
301**	11-15-78	59	0	2	99	136	394	515	473	932	338	435	679	617	+500
302**	11-15-78	38	0	6	22	0	35	16	49	37	52	40	63	61	61
345**	3-28-79	30	0	0	30	60	77	26	117	20	253	222	7500	406	+500
349**	4-22-79	21	0	1	23	6	46	16	4	0	12	21	3	173	2
350**	4-22-79	58	0	12	67	214	472	569	211	260	494	352	+500	+1000	+500
351**	4-22-79	22	0	5	62	128	80	113	176	46	380	227	457	+800	+500
Controls															
340***		0	0	0	0	0	0	0	0	0	0	0	0	0	0
341***		0	0	0	0	0	0	0	0	0	0	0	0	0	0
353***		0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Infected with 100 l_3 B. pahangi
** Infectec with 200 l_3 B. pahangi
*** NO l_3's
-- Dead
+ Greater than

Table 3: Adult B. pahangi recovered at necropsy are shown. Males and females from 4 anatomical areas of residence is shown, with totals for males and females, and total worm burden recoveries

Table 3

Adult B. pahangi Recovered at Necropsy

Animal Number	B. pahangi I_3 Number	Testes Males	Testes Females	Heart & Lungs Males	Heart & Lungs Females	Pelt & Carcus Males	Pelt & Carcus Females	Viscera Males	Viscera Females	Total Males	Total Females	Adult Worms Recovered (Total)
288	100	6	7	3	1	0	0	0	0	9	8	17
289	100	8	8	8	6	3	3	0	0	19	17	37
290	100	7	14	3	4	0	1	0	0	10	19	29
291	100	8	4	16	6	0	0	0	0	24	10	34
293	100	5	8	1	0	3	1	0	0	9	9	18
311	100	11	7	7	0	0	1	0	0	18	8	26
333	100	11	13	1	1	1	1	1	0	14	15	29
334	100	19	14	4	2	0	0	0	0	23	16	39
335	100	1	0	8	9	2	9	0	1	11	19	30
336	100	3	1	16	12	0	0	0	0	19	13	32
338	100	7	4	7	5	3	3	0	0	17	12	29
339	100	7	4	8	8	4	1	0	0	19	13	32
296	200	11	17	3	5	3	1	0	0	17	23	41
297	200	5	10	6	9	0	2	1	1	12	21	34
301	200	30	20	3	6	0	0	0	0	33	26	59
302	200	7	21	6	4	0	0	0	0	13	25	38
345	200	18	8	3	1	0	0	0	0	21	9	30
349	200	2	1	10	8	0	0	0	0	12	9	21
350	200	14	4	16	13	0	1	0	0	30	28	58
351	200	4	3	6	8	0	0	0	0	10	12	22
Controls												
340	0	0	0	0	0	0	0	0	0	0	0	0
341	0	0	0	0	0	0	0	0	0	0	0	0
353	0	0	0	0	0	0	0	0	0	0	0	0

Table 4 B. pahangi Antigen Titration (Block Titration Table)

Antigen Dilution	Antisera Dilutions in VBD Buffer 1:2	1:4	1:8	1:16	1:32	1:64	1:128	Complement-Antigen Controls $5C'H_{50}$	$2.5C'H_{50}$	$1.25C'H_{50}$
1:12.5	0	0	40	60	95	100	100	100	95	70
1:25	0	20	40	60	100	100	100	100	95	70
1:50	10	20	50	60	100	100	100	100	95	70
1:100	10	20	50	75	100	100	100	100	95	70
1:200	10	20	50	80	100	100	100	100	95	70
1:400	10	20	75	90	100	100	100	100	95	70
1:800	10	20	70	90	100	100	100	100	95	70
1:1600	10	20	70	90	100	100	100	100	95	70
1:3200	10	20	70	90	100	100	100	100	95	70
1:6400	10	20	70	90	100	100	100	100	100	70
1:12800	10	20	70	90	100	100	100	100	95	70

Percentage of Hemolysis of SRBC's

Complement-VBD Buffer Controls		
100	100	80

Table 4 shows the block titration of B. pahangi against a standard jird antisera (anti-B. pahangi) B. pahangi antigen dilutions (vertical), are titrated against jird anti-B. pahangi antisera (horizontal), in the presence of C' and sensitized SRBC's. Percentage of hemolysis of the sensitized cells are shown. The optimal dilution for testing is shown in the curve.

* No sample
+ Greater than
- Less than
NS Not significant

Table 5: Reciprocal antibody titers to B. pahangi seen during complement fixation testing of infected M. unguiculatus sera. Test animals were divided into 2 groups by microfilarial densities. Group A being less than 500 and group B being greater than 500. Mean and standard error is shown for; recovered adult B. pahangi at necropsy; and the reciprocal antibody titers, for both groups and total. The Mann-Whitney U test for non-parametric analysis between means is shown with significant differences during the third, sixth and post 12 month periods.

Table 5

M. unguiculatus Reciprocal Antibody Titers to B. pahangi

	Animal Number	Adult Worms Recovered	Microfilariae At Necropsy	Months Post-Innoculation/Reciprocal Antibody Titers 0 Mo.	1.5 Mo.	3 Mo.	6 Mo.	9 Mo.	12 Mo.	+12 Mo.
Group A	333	29	0	0	32	32	32	32	32	64
	339	32	0	0	64	32	32	32	*	*
	334	39	1	0	16	32	32	64	64	*
	335	30	1	0	64	16	64	128	128	256
	349	21	2	0	8	0	8	8	16	64
	291	34	12	*	32	64	64	64	128	128
	288	17	47	*	0	0	0	4	4	*
	302	38	61	*	32	16	64	64	8	128
	290	34	297	*	8	32	32	32	128	128
	289	37	401	*	0	16	16	16	32	64
	Mean	31.100	82.200	0.000	25.600	26.667	34.400	44.400	60.000	118.857
	Standard Error:			0.000	7.520	5.963	7.356	11.720	17.960	25.850
Group B	293	18	+500	*	16	8	16	16	16	8
	336	32	+500	0	32	0	0	16	16	64
	338	29	+500	0	64	16	8	16	8	16
	296	34	+500	*	16	16	16	32	32	32
	297	41	+500	*	16	8	8	8	32	32
	301	59	+500	*	16	8	8	32	32	32
	345	30	+500	0	16	8	16	32	16	32
	350	58	+500	0	8	4	8	16	64	32
	351	22	+500	0	8	0	16	8	32	32
	Mean:	35.888	+500.000	0.000	21.333	7.556	10.667	19.556	27.556	31.111
	Standard Error:			0.000	5.810	1.837	1.885	3.296	5.515	5.067
	Total Mean:			0.000	24.000	17.111	23.158	32.632	43.778	69.500
	Total Standard Error:			0.000	4.242	3.823	4.773	6.855	9.927	15.844
	Mann-Whitney Test:				NS	NS	P=-.05	P=-.05	NS	NS	P=-.01
	Controls									
	340			0	0	0	0	0	0	0
	341			0	0	0	0	0	0	0
	353			0	0	0	0	0	0	0

Figure 2: Varying dilutions of hemolysin, in the presence of a constant amount of complement produces a "plateau" with increasing dilutions (left to right). The optimal dilution (from the figure), is defined as the second point in the plateau. The hemolysin titration preformed indicates the optimal dilution to be 1:2000.

Figure: 2

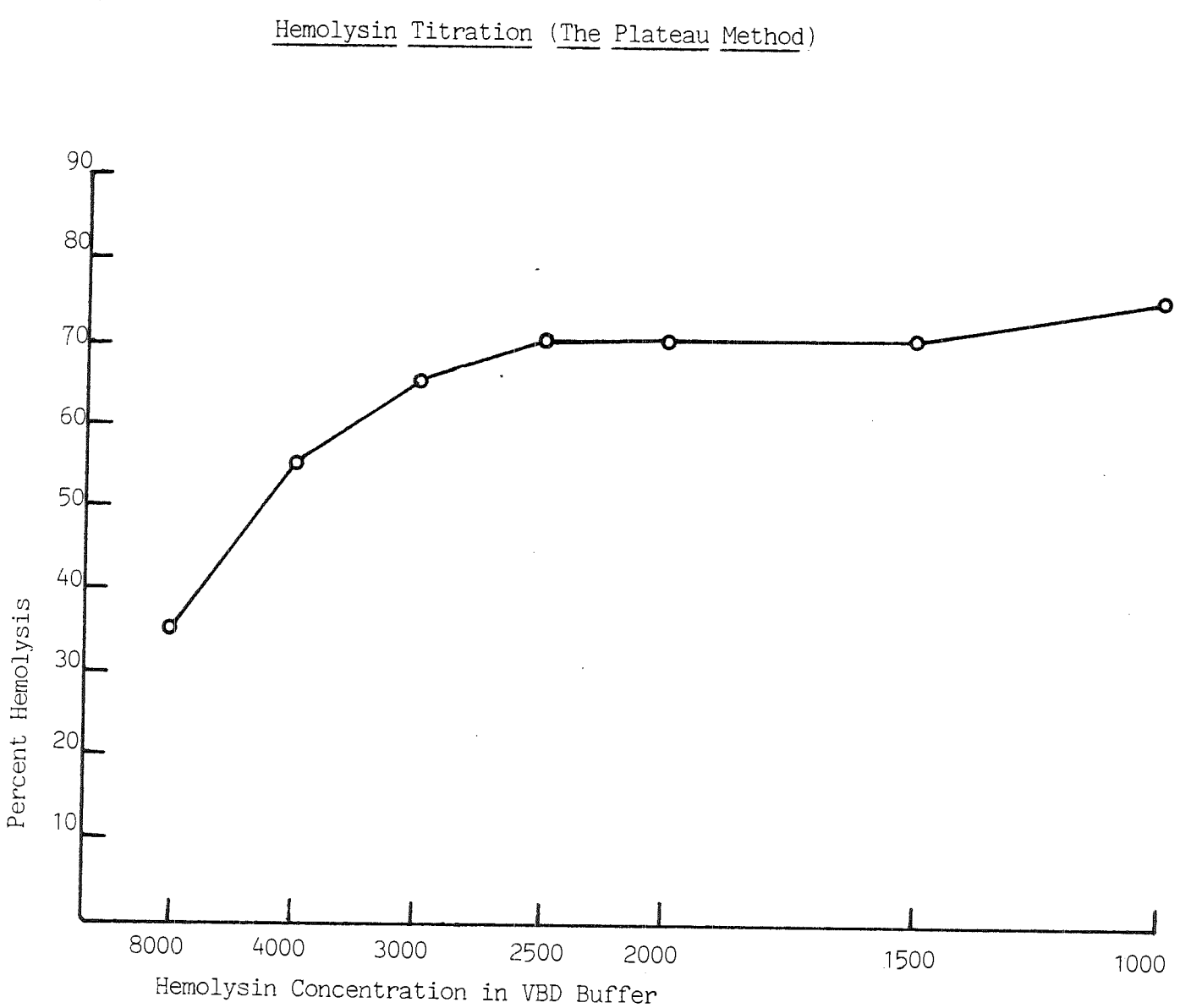

Hemolysin Titration (The Plateau Method)
90
80
70
60
50
40
30
20
10
Percent Hemolysis
8000
4000
3000
2500
2000
1500
1000
Hemolysin Concentration in VBD Buffer

Figure 3: "A, B, C, and D are experimently derived percentages of hemolysis (= Y) converted to Y/1-Y and the latter plotted against corresponding milliter to complement dilution". The solid circle represents the midpoint indicating the intercept for finding milliter of complement dilution producing 50% hemolysis. The figure shows .26 as being the intercept point, therefore: .26 mls. of the 1/267 ml. dilution = 1 $C'H_{50}$ unit (HEW Report, 1965).

Figure 3:

Complement Titration (The Determination of $C'H_{50}$ Units)

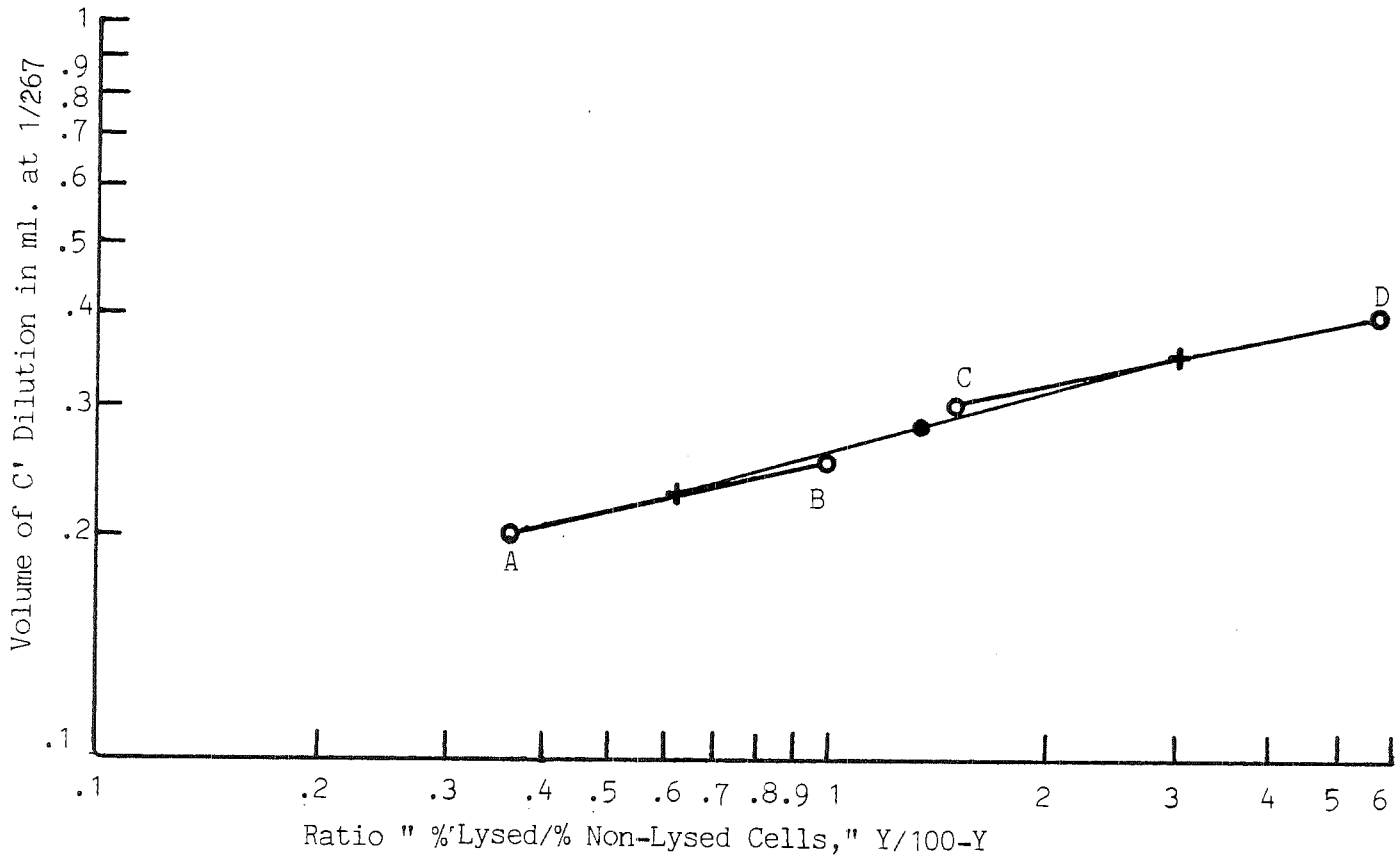


Figure 4: The mean reciprocal antibody titers for group A is represented through the course of infection with B. pahangi. The standard error is also represented for each point plotted.

Figure 4: Group A Animals with Microfilaremias Less than 500: Mean Reciprocal Antibody Titers

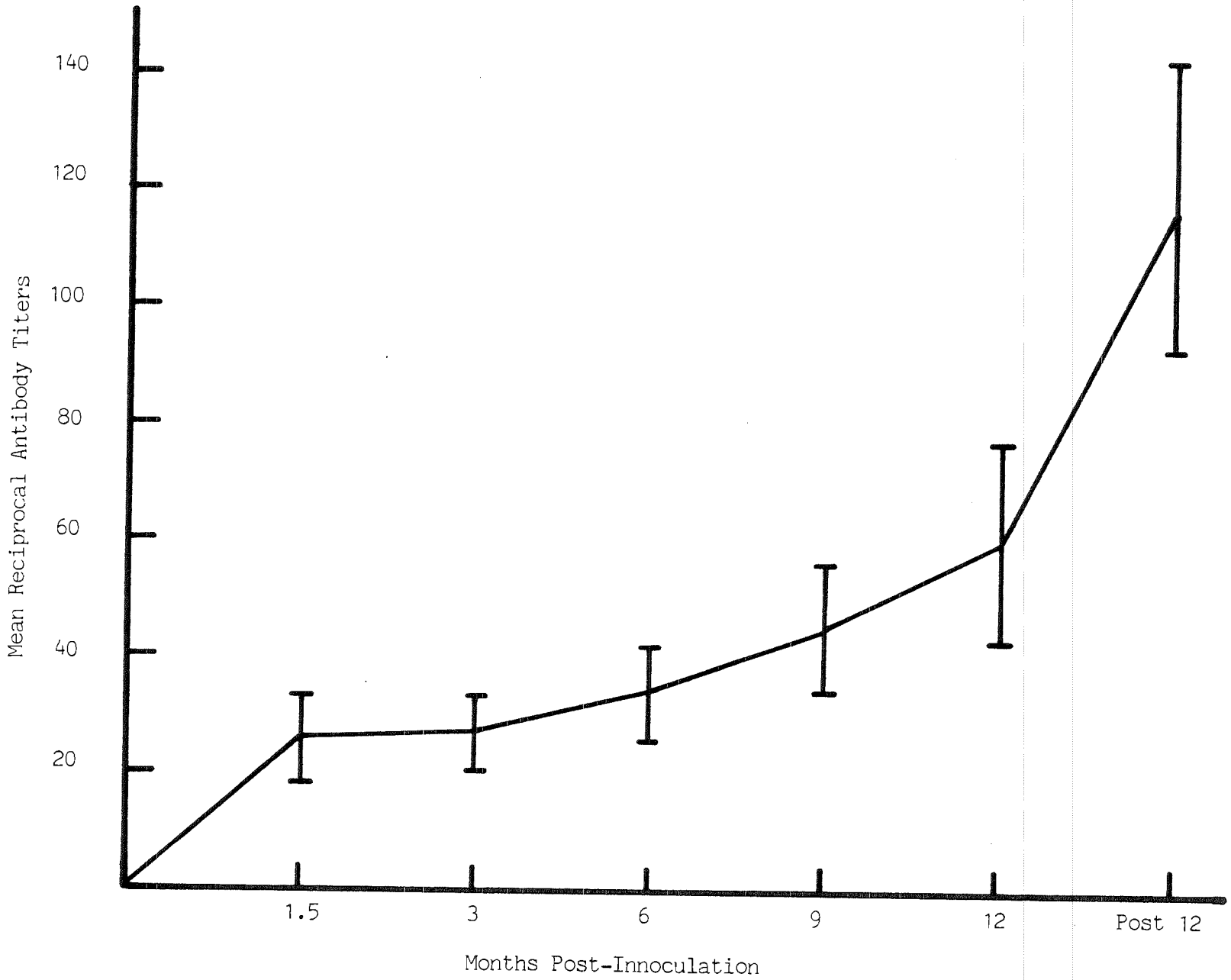


Figure 5: The mean reciprocal antibody titers for group B is represented through the course of infection with B. pahangi. The standard error is also represented for each point plotted.

Figure 5; Group B Animals with Microfilaremias Greater than 500: Mean Reciprocal Antibody Titers

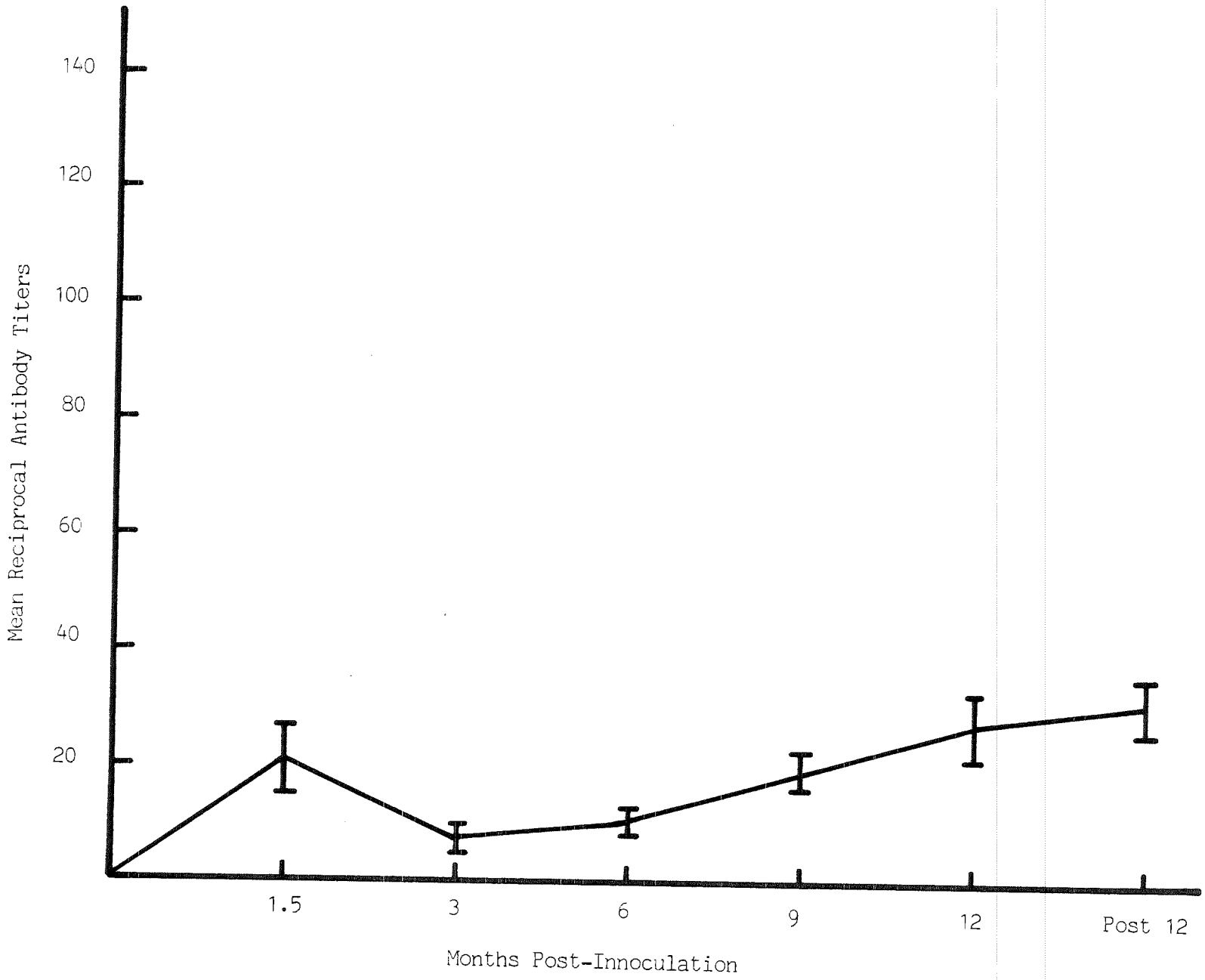


Figure 6: The mean reciprocal antibody titers for groups A and B is represented comparitively through the course of infection with B. pahangi. The standard errors are also represented for each group.

Figure 6: Mean Comparison Between Animals of Group A and Group B: Mean Reciprocal Antibody Titers

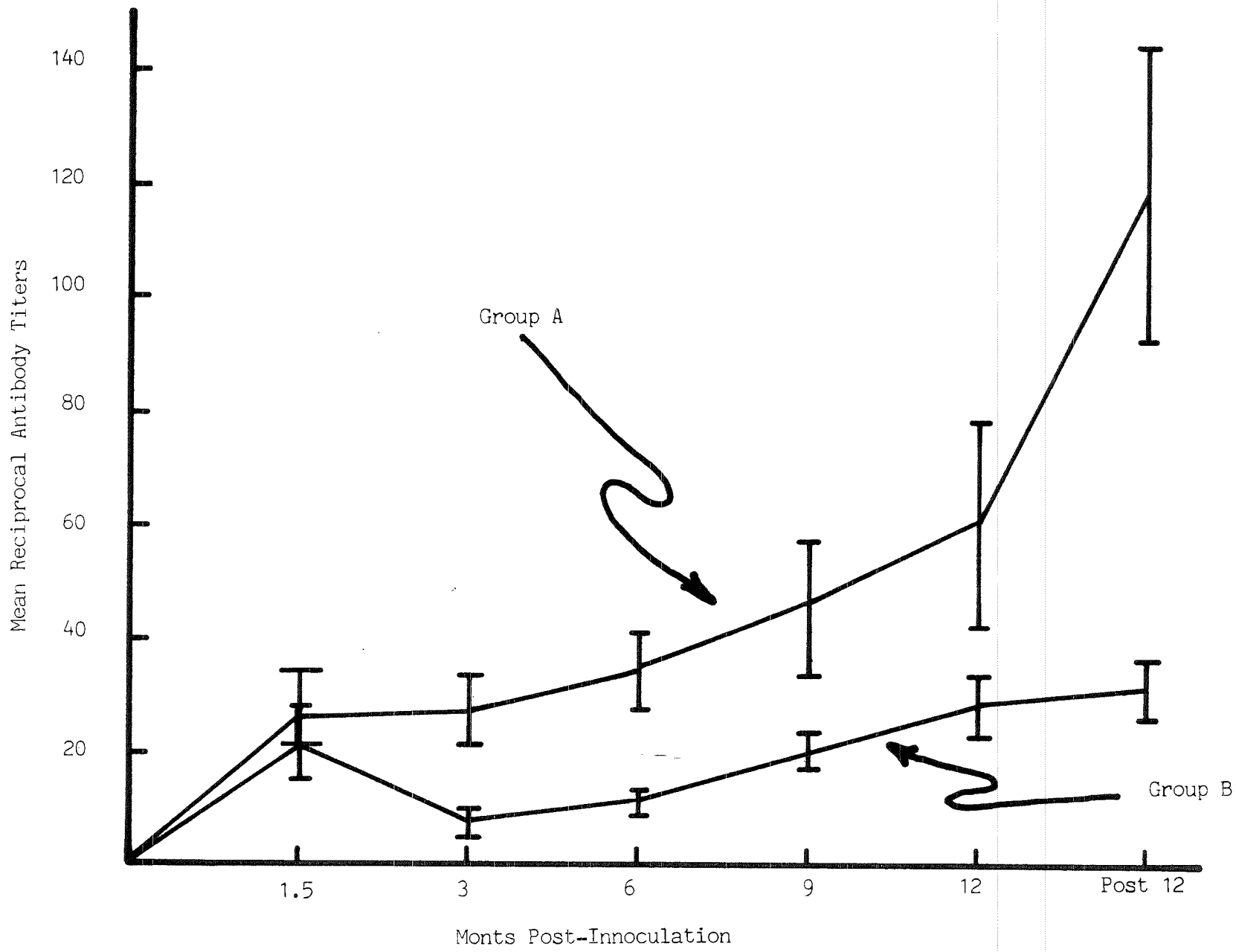
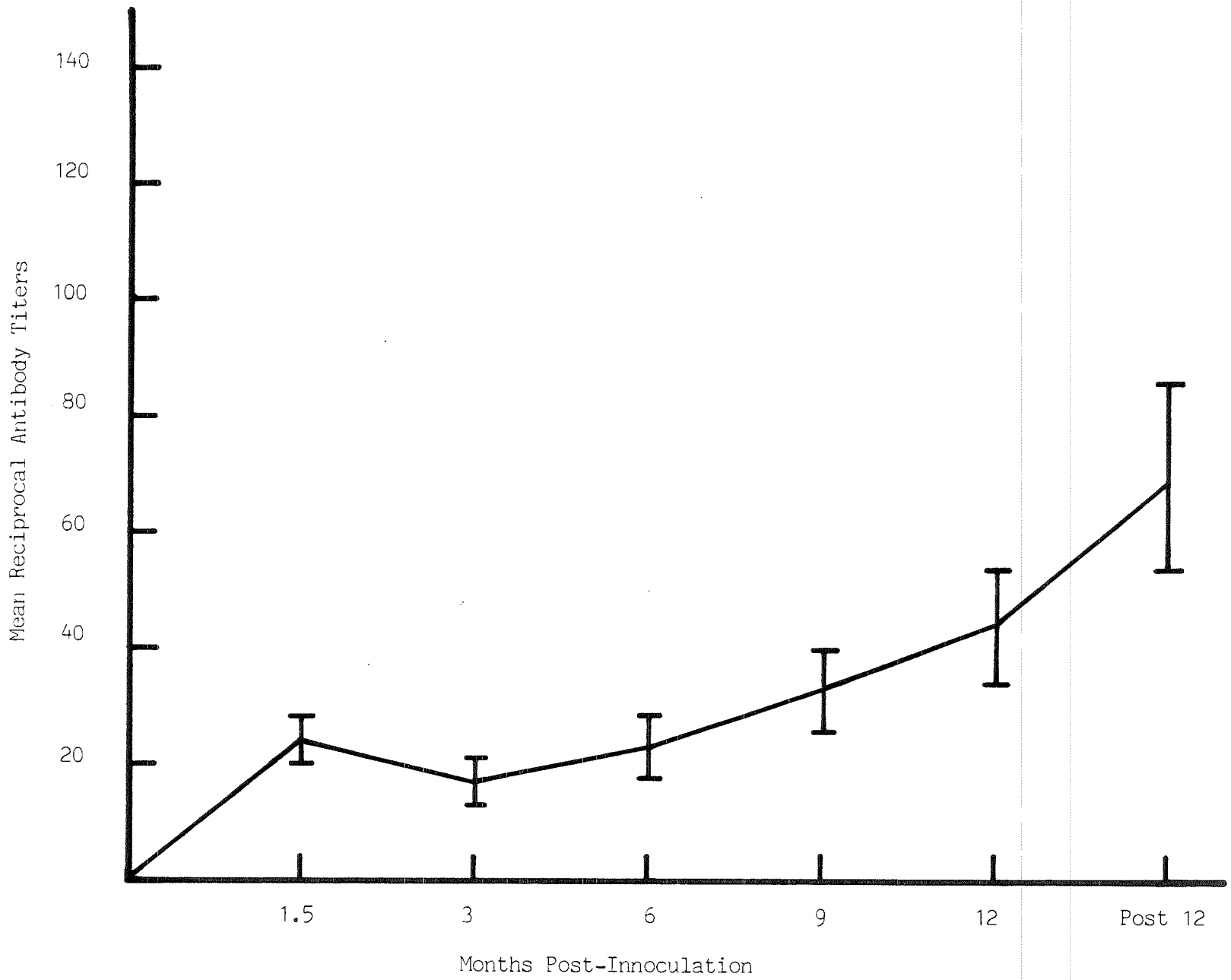


Figure 7: The average mean reciprocal antibody titers for both groups A and B is represented through the course of infection with B. pahangi. The standard error is also represented for each point plotted.

Figure 7: Average of the Means for both Groups A and B: Mean Reciprocal Antibody Titers



Discussion:

Infection with B. pahangi in M. unguiculatus

In the presented study, all 20 test animals developed patent, sustained infections. Seventeen test animals developed patent infections within, or shortly after 60 days, with the longest prepatent period being 118 days. These findings are consistent with those observed by Ash and Riley (1970). Microfilaria densities vacillated throughout the course of infection in all animals (Table 3), with variable adult worm recoveries made at necropsy (Table 4). It must be noted that throughout the course of infection, approximately 50% of the animals sampled showed consistently low microfilaria densities, regardless of l_3 number innoculated. These animals were separated from those of consistently high microfilarial densities to form 2 groups during testing to determine possible differences in antibody titers, if any. Worm recoveries differed slightly with respect to the ratio of males to females from those observed by Ash and Riley (1970) and Ash (1971). In the presented study, no clear ratio could be drawn from the total sample for the ratio of males to females as contrasted to Ash and Riley's findings of 2 females to 1 male ratio. However, this study is in agreement with total adult worm recovery being approximately 25% of the total infective larval dosage innoculated. The majority of the recovered worms were found in the testicles and their associated lymphatics, with significant numbers recovered in the heart and pulmonary arteries. These findings are also consistent with those

of Ash and Riley (1970).

In general, this study substantiates the findings of previous researchers, (Ash and Riley, 1970; Ash, 1971; McCall et. al. 1974; Ash and Schacher, 1970), with respect to areas of adult residence, worm burden percentages, and the beneficial use of the jird as an alternative host for B. pahangi.

Hemolysin Titration:

The amount of hemolysis produced by several dilutions of rabbit hemolysin in VBD buffer, in the presence of a constant amount of complement, was measured against a predetermined set of color standards.

"When the Laboratory Bench Complement Fixation (LBCF; HEW Technical Report #74; 1965) titration results as read against color standards are plotted on arithmetic graph paper, the curve will show, if a sufficient concentration of hemolysin is present, that increasing amounts of hemolysin then produce no significantly greater percent hemolysis; therefore a plateau results". (HEW Technical Report #74, 1965)

The plateau method employed in this study (represented in Figure 2) was found to be very reliable for the determination of an optimal hemolysin concentration. The use of color standards as opposed to spectrophotometry proved more useful with ease of identification between individual tubes of hemolysates. With the color standards, percentages of hemolysis could be made within ± 5%. This margin of error, through several trials, was found to be insignificant.

By using color standards, much more time was saved.

Several problems were encountered in the course of this titration and the following complement titration. In the preliminary titrations, percentages of hemolysis did not correspond with either the color standards or the standardized method. Lysates were found to be far too low, (percentage hemolysis) with minor adjustments producing 100% hemolysis. Through several trials, it was found that either the complement stocks or hemolysin stocks were either too old or spoiled. New stocks were obtained from Grand Island Biological Co., and resultant titrations proved successful. All tests and titrations were performed several times over an extended period in order to demonstrate reliability in testing and consistency in results.

Complement Titration:

In complement titration, a 50% hemolytic endpoint was employed to determine the potency of the stock complement. The 50% endpoint was defined as the volume of complement needed to lyse 50% of a sensitized 2.8% SRBC suspension in the test system. In terms of the 50% endpoint, the potency of the stock complement was described in $C'H_{50}$ units. One $C'H_{50}$ unit was enough complement to lyse 50% of the sensitized 2.8% SRBC suspension. The potency was determined to be 1:267, therefore 1.0 mls. of this dilution would lyse 50% of the cell suspension. The $C'H_{50}$ unit was derived from the reciprocal of the complement dilution which lysed 50% of the cell suspension (HEW Technical Report #74, 1965).

Several trials were performed as previously described (Table 1),

with the potency of the stock at 267 $C'H_{50}$. The complement fixation master test employed $5C'H_{50}$ units; therefore 1.3 mls. of the 1:267 complement dilution equalled the $5C'H_{50}$ units needed. The lysates produced during titration were read against the color standards with the results graphically depicted (Figure 3). This method produced accurate results with 0.26 mls. of the 1:267 complement dilution being 1 $C'H_{50}$ unit. After the establishment of the stock complement potency, with the corresponding $C'H_{50}$ units, scaling dilutions to accommodate microtest conditions with "U" plates was easily accomplished. The resulatant dilution to achieve the $5C'H_{50}$ units in the "U" plate was a 1:9 of stock complement to VBD buffer. Therefore, 50 microliters equalled the amount needed for the master test.

As with the hemolysin titration, several titrations were performed in order to establish validity in testing. After the previous problems encountered with poor stocks, the addition of new stocks proved fruitful with relative ease in establishing the optimal dilutions necessary.

Antigen Titration:

In subsequent titrations, the key factor was in the reproducability of the results obtained in each test. Once this framework had been established, the antigen titration was performed. The titration of antigen was termed a "block" titration due to the varying dilutions of antigen and antisera used (Table 4). Varying dilutions of antigen and standardized antisera (in VBD buffer), were titrated against each other. The concentration of antigen, unlike

that of complement, was not based on a "unit" system, rather by the optimal dilution which gave reproducable results. The optimal dilution of antigen was defined as the dilution which gave the highest complement fixing activity (without being anticomplementary), with the standard antisera and control sera. Anticomplementary dilutions of antigen were determined when less than 85% hemolysis resulted in the antigen-complement control wells containing 2.5 $C'H_{50}$ units (Table 4). The optimal dilution was found to be between 1:25 and 1:50, without being anticomplementary (Table 4). Due to the scarcity of homologous antigen, the 1:50 dilution was chosen. Through repeated testing, the 1:50 dilution proved to be adequate for the purposes of this framework.

The Complement Fixation Assay:

In testing, 93.5% of the test animals innoculated showed positive titers of curculating antibodies with complement fixing properties as high as 1:64, 6 weeks post-innoculation. Antibody titers were shown to vacillate during the course of infection (Table 5), with titers ranging from 1:4 to 1:256. The complement fixing antibody titers were expected to be lower than those observed in indirect hemagglutination as reported by Tanaka, 1969 and Tanaka et. al., 1970, however, drew close correlation with B. pahangi in M. unguiculatus (Peak IHA titer; 1:512, Benjamin and Soulsby, 1976; Kowalski, unpublished). Antibody titers were shown to increase up to 6 weeks post infection with a marked decrease by 3 months post-innoculation. By the sixth month the amount of circulating complement

fixing antibody was shown to steadily increase starting a second peak 12 months (Between 12 and 21 months) post-innoculation, to the time of necropsy. These finding are in partial agreement with those of Kowalski (unpublished), using indirect hemagglutination. Kowalski found antibody appearing in the blood as early as 3 to 4 weeks post-innoculation, with peak titers showing 2 to 3 months post-innoculation. The circulating antibody would then drop off slightly at 3.5 to 4 months post-innoculation but remain steady thereafter. The presented study draws exception in that, circulating antibody was shown to steadily increase 6 months post-innoculation, also peak antibody titers were not demonstrated until late in the infection (Post 12 months).

Tanaka (1969) tested cotton rats infected with Litomosoides using complement fixation with homologous antigen. Testing occured 11 weeks post-innoculation at a comparatively early stage of infection with good results. The reliability of complement fixation testing for long term filarial infections has been demonstrated (Tanaka, 1969; Tanaka et. al., 1970). Several Litomosoides studies have been performed using complement fixation and homologous antigen with good results. However, to date, no studies have employed complement fixation with homologous B. pahangi antigen.

Tanaka et. al. (1970) performed cross-reactions in complement fixation tests among filarial species. Brugia malayi and Wuchereria bancrofti were both tested for cross-reactivity with L. carinii, D. immitus, and Setaria cervi. W. bancrofti showed a fairly good response with titers as high as 1:64, however, no response was seen with B. malayi for cross-reactivity with heterologous filarial antigens.

Other studies done using complement fixation with O. volvulus and homologous antigen showed low peak titers at 1:64.

The scarcity of homologous antigen for Brugia spp. has often hindered immunologic studies, with homologous antigen for Wuchereria being almost impossible to obtain.

Test Animals

The test animals were divided into 2 groups; group A, which showed consistantly low microfilaremias throughout the course of infection, and group B, with consistantly high microfilarial densities throughout. Group A showed greater mean reciprocal antibody titers throughout the course of testing, with steady increases each period. Group B displayed low mean titers throughout the sampling (as opposed to group A. Antibody titers peaked at 6 weeks post innoculation followed by a 3 fold decrease between the means 12 weeks post infection, with steady increases thereafter to necropsy where the mean titers were at their highest level. The Mann-Whitney "U" test, (Sokal and Rohlf, 1969), showed significant statistical variations (between mean reciprocal antibody titers of groups A and B), during the third and sixth month with P values = $<.05$, and at the twelth month, where the greatest despairity existed with P = $<.01$ (Table 5; Figure 6). Significant statistical variations between the means using the Mann-Whitney "U" test indicate a definite correlation between low microfilarial densities and high antibody titers. This correlation indicates possible circulating antibody directed against microfilaria, gravid females, and/or metabolic wastes produced by the adult worms.

The data demonstrates, however, that the circulating antibody has specific complement fixing activity in the presence of homologous antigen.

Both groups showed good stage specificity and reliability in complement fixation testing. Relatively high mean antibody titers were observed 6 weeks post innoculation, before patency and detectable microfilaremias. Antibody activity at this early stage of infection indicates an immunological response (humoral and possibly cellular) directed against immature and mature establishing adults. Much more data are needed to make any firm statements in regard to these postulations concerning the jirds immunological response. However, complement fixing circulating antibody directed against B. pahangi is present with definite correlations to microfilaremias. The homologous antigen used was a combination of adult male, female, and microfilaria. Further tests with isolated specific antigen will be more effective for the further characterization of circulating antibody.

The results of this study show good stage specificity in complement fixation testing with homologous antigen. The complement fixation test should be considered a valuable diagnostic tool for further filarial studies both with humans and experimental animals.

References

Ah, H.S. and Thompson, P.E. (1973). Brugia pahangi: infections and their effect on the lymphatic system of Mongolian jirds (Meriones unguiculatus). Expl. Parasit. 34, P. 393-411.

Ah, H.S., McCall, J.W., and Thompson, P.E. (1974). Vaccination against experimental Brugia pahangi infections in dogs. Proc. 3rd. Int. Congr. Parasit. 3, p. 1236-1237.

Ahmed, S.S. (1966). Location of developing and adult worms of Brugia sp. in naturally and experimentally infected animals. J. Trop. Med. Hyg. 69, p. 291-293.

Ahmed, S.S. (1967). Studies on the laboratory transmission of sub-periodic Brugia malayi and B. pahangi. I. The resistance of guinea pigs, rabbits, and white mice to infection. Ann. Trop. Med. Parasit. 61, p. 93-100.

Ash, L.R. (1971). Preferential susceptibility of male jirds (Meriones unguiculatus) to infection with Brugia pahangi. J. Parasit. 57, p. 777-780.

Ash, L.R. and Riley, J.M. (1970a). Development of subperiodic Brugia malayi in the Jird, Meriones unguiculatus, with notes on infections in other rodents. J. Parasit. 56, p. 969-973.

Ash, L.R. and Riley, J.M. (1970b). Development of Brugia pahangi in the Jirds, Meriones unguiculatus. with notes on infections in other rodents." J. Parasit. 56, p. 962-968.

Ash, L.R. and Scharcher, J.F.; (1971). Early life cycle and larval morphogenesis of Wuchereria bancrofti: in the jird, Meriones unguiculatus. J. Parasit. U. 57 No. 5, p. 1031-1043.

Ash, L.R. (1973). Chronic Brugia pahangi and Brugia malayi infections in Meriones unguiculatus. J. Parasit. 59, p. 442-447.

Barbosa, Rombert, P.C., and Rochce, R.P.M. (1972). (From Sasa, 1976). Revta. Patol. Trop. Vol. p. 93.

Barr, A.R. (1964). Notes on the colonization and biology of Armigeres Subalbatus (Diptera, Culicidae). Ann. Trop. Med. Parasit. 58, p. 171-179.

Benjamin, D.B. and Soulsby, E.S.L. (1974). The homocytropic antibody response to Brugia pahangi in the Multimammate rat. (Mastomys natalensis). Proc. 3rd. Int. Congr. Parasit. 2, p. 643.

Buckley, J.J.C. and Edeson, J.F.B. (1956). On the adult morphology of Wuchereria sp. (malayi?) from a monkey (Macacairus) and from cats in Malaya and on Wuchereria pahangi n. sp. from a dog and a cat. J. Helm. 30, p. 1-20.

Buckley, J.J.C. (1958). A new genus, Brugia, for Wuchereria spp. of the malayi group. Proc. 6th int. Congr. Trop. Med. Malaria 2, p. 385-391.

Buckley, J.J.C. (1960). Ann. Trop. Med. Parasit. 54:75 (Taken from Sasa, M. (1976) Human Filariasis p. 34).

Burg, S.L. (1927). "Een nieuwe Filaria-soort (Filaria Malayi), parasiteerende bij den meêsch (Voorloopige Mededeeling)." Geneesk. Tijdschr. Ned-Iondin 67, p. 750-754.

Cheong, W.H., Sivanandam, S., and Hassan, A. Gino. (1967). A note on an attempt at producing a rat strain of sub-periodic Brugia malayi from a natural simian host. Med. J. Malaya 21, p. 378.

Chong, L.K. and Wong, M.M. (1967). Experimental infection of laboratory mice with Brugia pahangi. Med. J. Malaya 21, p. 382.

Cross, J.H., Portono, F., Hus, M-Y.K., Ash, L.R., and Demigati, S. (1979). Experimental transmission of Wuchereria bancrofti to monkeys. Am. J. Trop. Med. Hgg. 28 (1), p. 56-66.

Denham, D.A., Ponnudurai, T., Nelson, G.S., Rogers, R., and Guy, F. (1972). Studies with Brugia pahangi. II. The effect of repeated infection on parasite levels in cats. Int. J. Parasit. 2, p. 401-407.

Denham, D.A. and McGreevy, P.B. (1977). Brugian Filariasis: Epidemiological and experimental studies. Adv. Parasit. 15, p. 243-309.

Deverelx, D. and Ash, L.R. (1978). Increased susceptibility to infection with Brugia pahangi in aged female jirds (Meriones unguiculatus). J. Parasit. 64 (1), p. 115-118.

Dissanaike, A.S. and Mak, J.W. (1978). Experimental infection of the long-tailed macaque, Macaca fasicularis with Wuchereria bancrofti (rural strain). S.E. Asian J. Trop. Med. Pub. Hlth. 9 (3), p. 451-2.

Edeson, J.F.B. and Wharton, R.H. (1958). The experimental transmission of Wuchereria malayi from man to various animals in Malaya. Trans. R. Soc. Trop. Med. Hyg. 52, p. 25-45.

Edeson, J.F.B. (1962). The epidemiology and treatment of infection due to Brugia malayi. Bull. Wld. Hlth. Organ. 27, p. 529-541.

El Bihari, S. and Ewert, A. (1971). Distribution of developing and mature Brugia malayi in rhesus monkeys and in the jird, Meriones unguiculatus, after a single infection. J. Parasit. 57, p. 1170-1174.

Faust, E.C.; Russel, P.F.; and Jung, R.C.; (1970). "Clinical Parasitology"; 8th Ed. 890 pp. Lea and Febiger, Philadelphia.

Garvey, J.S., Cremer, N.E., and Sussdorf, D.H. (1977). Methods in Immunology 3rd. Edition. W.A. Benjamin, Inc. Reading, MA. p. 545.

Grove, D.I. and Davis, R.S. (1978). Serological diagnosis of Bancroftian and Malayan filariasis. Am. J. Trop. Med. Hyg. 27 (3), p. 508-513.

Gubler, D.J. and Bhattacharya, N.C. (1974). A quantitative approach to the study of Bancroftian filariasis. Am. J. Trop. Med. Hyg. 23 (6), p. 1027-1036.

Harbut, C.L. (1973). The white rat and golden hamster as experimental hosts for Brugia pahangi and subperiodic Brugia malayi. S.E. Asian J. Trop. Med. Publ. Hlth. 4, p. 487-491.

Health, Education, and Welfare Report, (1965). Standardized diagnostic complement fixation method and adaption to micro test.

Hewitt, R.I.; Kushner, S.; Stewart, H.W.; White, E.; Wallace, W.S.; and Suggarow, Y. (1947). Experimental chemotherapy of filariasis III. Effect of 1-Diethylcarbamy 1-4- methylpiperazine hydrochloride against naturally acquired filarial infections in cotton rats and dogs. J. Lab. Clin. Med. 32, p. 1314-1329.

Kowalski, J.C. and Ash, L.R. (1975). Repeated infections of Brugia pahangi in the jird, Meriones unguiculatus. S.E. Asian J. Trop. Med. Publ. Hlth. 6, p. 195-198.

Laing, A.B.G.; Edeson, J.F.B.; and Warton, R.H. (1961). Studies on filariasis in Malaya: further experiments on the transmission of Brugia Malayi and Wuchereria bancrofti. Ann. Trop. Med. Parasit. 55, p. 86-92.

Lichtenstein, A. (1927); Filariaonderzock te Bireuen. Geneesk. Tijdschr. Ned-Indie 67, p. 742-749.

MacDonald, W.W. (1962). The selection of a strain of Aedes aegypti susceptible to infection with semi-periodic Brugia malayi. Ann. Trop. Med. Parasit. 56, p. 368-382.

Malone, J.B., Leininger, J.R., and Thompson, P.E. (1974). Brugia pahangi in golden hamsters. Trans. R. Soc. Trop. Med. Hyg. 68, p. 170-171.

Malone, J.B., Taylor, H.W., and Van Brackle, M. (1979). Greater susceptibility of PD-4 inbred hamsters to Brugia pahangi. Trans. Royal. Soc. Trop. Med. Hyg. 73 (4), p. 475.

McCall, J.W., Malone, J.B., Ah, H.S., and Thompson, P.E. (1973). Mongolian jirds (Meriones unguiculatus) infected with Brugia pahangi by the intraperitoneal route: a rich source of developing larvae, adult filariae, and microfilariae. J. Parasit. 59, p. 436.

McGreevy, P.B., Ismail, M.M., Phillips, T.M. and Denham, D.A. (1975). Studies with Brugia pahangi 10. An attempt to demonstrate the sharing of antigenic determinants between the worm and its hosts. J. Helminth. 49, p. 107-113.

McLaren, D.J., Clegg, J.A. and Smithers, S.R. (1975). Acquisition of host antigens by young Schistosoma mansoni in mice: correlation with failure to bind antibody in vitro. Parasitology 70, p. 65-67.

Nelson, G.S. (1959). The identification of infective Filarial larvae in mosquitoes: with a note on the species found in "wild" mosquitoes on the Kenya coast. J. Helminth. 32, p. 233-256.

Petramyi, G. and Mieth, H. (1974). Experimental infections of male Mastomys Matalensis with Brugia malayi, Proc. 3rd. int. Congr. Parasit. 2, p. 616.

Piessens, W.F., Ratiwayanto, S., Tuti, S., Palmiera, J.H., Piessens, P., Koiman, I. and Dennis, D.T. (1980). Antigen-specific suppressor factors in human filariasis with Brugia malayi. N. Eng. J. Med. 302 (15), p. 833-837.

Portaro, J.K., Kowalski, J.C., Howell, C. and Ash, L.R. (1977). Use of Lymphocyte Transformation for the detection of filarial infections, J. Parasit. 63 (1), p. 172-174.

Ramachandran, C.P.; Sanddosham, A.; and Sivanandan, S. (1966). Development of Wuchereria bancrofti in the domestic cat. Med. J. Malaya (Lab Meeting) 20: 333.

Ramachandran, C.P.; and Sivanandan, S. (1969). Studies on the transmission of Wuchereria bancrofti to animals in the Laboratory. Proc. Seminar on Filariasis and Immunology of Parasitic Infections, Singapore, p. 136-138.

Ramachandran, C.P. (1970). Attempts to immunize domestic cats with X-irradiated infective larvae of sub-periodic Brugia malayi. 1. parasitological aspects. S.E. Asian J. Trop. Med. Publ. Hlth. 1, p. 78-92.

Rao, S.S. and Maplestone, P.A. (1940). The adult of Microfilaria Malayi Burg, 1927 . Indian Med. Gez. 75, p. 159-160.

Ridley, D.S. and Hedge, E.C. (1977). Immunofluorescent reactions with microfilariae; Z. Bearing on host-parasite relations. Trans. Roy. Soc. Trop. Med. Hyg. 71, p. 522-525.

Sasa, M. (1976). Human Filariasis; University of Tokyo Press (University Park Press)

Sasa, M.; Shirasaka, R.; Joesoef, A.; Abdulwahas, R.; and Yamaura, H. (1976). A study of the Microfilariae periodicity at Bireuen, The type locality of Brugia malayi. S.E. Asian J. Trop. Med. Pub. Hlth. 7, 3,; p. 367-369.

Schacher, J.F. (1962). Developmental stages of Brugia pahangi in the final host. J. Parasit. 48, p. 693-706.

Smithers, S.R.; Terry, R.J.; and Hockley, D.J. (1969). Host antigens in schistosomiasis. Proc. R. Soc. B171, p. 483-494.

Sokal, R.R. and Rohlf, F.J. (1969). Biometry. W.H. Freeman, San Francisco, p. 776.

Sucharit, S. and MacDonald, W.W. (1973). Brugia pahangi in small laboratory animals: attempts to increase susceptibility of white rats to Brugia pahangi by host selection. S.E. Asian J. Trop. Med. Pub. Hlth. 4, p. 71-77.

Tanaka, H.; Kobayashi, J.; Ishii, A.; and Sasa, M. (1969). Complement fixation test with adult Litomosoides antigen in the cotton rat filariasis. Japan. J. Exp. Med. 39, p. 393-398.

Tanaka, H., Fujita, K., Sasa, M.; Tagawa, M., Naito, M., and Kurokawa, K. (1970). Cross-reactions in complement fixation test among filarial species. Japan. J. Exp. Med. 40 (1), p. 47-58.

Turner, L.H. and Edeson, J.F.B. (1957). Studies on filariasis in Malaya: the periodicity of the microfilaria of Wuchereria malayi. Ann. Trop. Med. Parasit. 51, p. 271-277.

Vincent, A.L., Frommes, S.P., and Ash, L.R. (1976). Brugia malayi, Brugia pahangi and Brugia patei: Pulmonary pathology in jirds, Meriones unguiculatus. Exp. Parasit. 40, p. 330-354.

Vincent, A.L. and Ash, L.R. (1978). Splenomegaly in jirds (Meriones unguiculatus) infected with Brugia malayi. (Nematoda: Filarioidea) and related species. Am. J. Trop. Med. Hyg. 27, (3), p. 514-520.

Vincent, A.L. and Sodeman, W.A. Jr. (1980). Development of Brugia pahangi in normal and nude mice. J. Parasit. 66 (3), p. 448.

Weller, P.F. (1978). Cell-mediated immunity in experimental filariasis: Lymphocyte Reactivity to filarial stage-specific antigens during acute and chronic infection. Cell. Immunol. 37, p. 369-382.

Wesley, I.V. (1973). Sex hormones and their influence on preferential susceptibility of the male jird (Meriones unguiculatus) to infection by Brugia pahangi (Nematoda: Filariodea). Dr. P.H. dissertation, Univ. California at Los Angeles. Wesley, I,V. and Ash, L.R. (1973). Ibid.

World Health Organization; (1967). WHO Expert Committee on Filariasis (Wuchereria and Brugia Infections) Second Report. WHO Techn. Report Ser. No. 359, p. 1-47.

WHO Technical Report, (1974). "Expert Committee on Filariasis, 3rd Report". Tech. Rep. Ser. Wld. Hhh. Org. 542, p. 54.

Wong, M. M., Fredericks, H.J. and Ramachandran, C.P. (1969). Studies on immunization against Brugia malayi in the reesus monkey. Bull. Wld. Hlth. Org. 40, p. 493-501.

Yokogawa, S. (1938). Investigation on the mode of transmission of Wuchereria bancrofti. (Second Report). Jap. J. Med. Sci. V. Path. 4: p. 197-204.