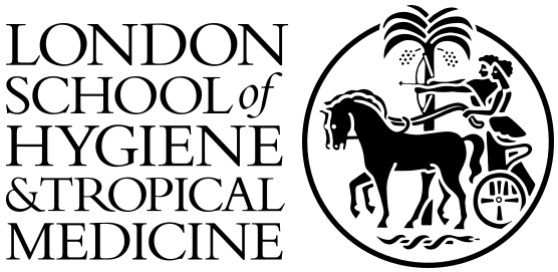

STUDIES ON HOST-PARASITE RELATIONSHIPS IN ANIMALS INFECTED WITH *BRUGIA PAHANGI*

A thesis submitted for the degree of Doctor of Philosophy

in the

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by

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I dedicate this work to the memory of my father and members of my family who have urged me on, this far.

"That the play is the 'Man',
And its hero the Conqueror Worm."

(Edgar Allan Poe)

ABSTRACT

Third stage larvae of Brugia pahangi irradiated with 10, 25 and 45 krads. of Co.60 were inhibited in their development beyond the juvenile adult stage, the fourth larval stage and third larval stage respectively. The higher two doses altered the migration pattern of most of the parasites, which were confined to the subcortical sinus of the infected lymph nodes. Male parasites were more susceptible to irradiation than were females.

Repeated infections with irradiated B.pahangi did not greatly change the architecture of the lymphatics of the e cats.

Cats were repeatedly vaccinated with irradiated B.pahangi to determine whether attenuated parasites protected against challenge infections. Cats immunized with parasites irradiated with 10 krads. resisted 60.3 - 98.5% of the homologous challenge infections; and cats immunized with parasites irradiated with 25 krads. resisted 61 - 93% of challenge infections. The resistance in the immunized animals was mounted against all the stages of the life cycle.

Two cats given heterologous challenges with Brugia patei resisted 78.6% and 65.3% of the challenge inoculations. One cat which was infected with normal parasites, and challenged after it had become amicrofilaraemic, also resisted challenge.

Jirds vaccinated with parasites irradiated with 45 krads. resisted challenges, whilst vaccination with non-irradiated worms and parasites irradiated with 25 krads. did not protect these animals.

Antibody responses to various homologous antigens were higher in cats given repeated infections than in cats given single infections. Antibodies against microfilariae were detected only when the animals had suppressed their microfilariae. No antibodies against adult stages could be detected in animals infected with irradiated larvae.

The only significant change in the white blood cell population was eosinophilia. The highest eosinophilia occurred at the time of the onset of microfilaraemia.

There were no significant changes in the serum components of cats infected with *B.pahangi*.

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CONTENTS

Chapter 1

INTRODUCTION

A. Brugian filariasis

Filariasis is a debilitating disease that adds to the burden of multitudes in underdeveloped tropical countries. At least 250 million people (WHO, 1974) are infected with Wuchereria bancrofti or Brugia malayi. Brugian filariasis is confined to South East Asia, parts of Japan, Korea, South India and China (Mawking, 1973). A reduction in the prevelence of filariasis has occurred in small parts of endemic areas, especially in Sri Lanka, where B. malayi has disappeared (WHO, 1974). However, there is evidence to suggest that elsewhere the disease has increased since 1954 both in prevalence, distribution and range in many parts of Asia and Africa. Factors such as population increase and unplanned urbanisation may have contributed to this increase (WHO, 1974). Improved methods of diagnosing circulating microfilariae in the blood, using the Millepore membrane filter (Desowitz and Southgate, 1973) and counting chamber (Denham et al., 1971) will further increase the number of cases recorded.

The causative agent of Brugian filariasis in man is a nematode parasite transmitted by mosquito species of the genera Mansonia, Anopheles and Aedes. Lichtenstein (1927) was the first to differentiate Brugian and Bancroftian filariasis on clinical grounds. Brug (1927) described microfilariae recovered from man in Indonesia, but it was not until 1940 that Rao and Maplestone isolated the adult worms and named the parasite Wuchereria malayi. Sixteen years later,

during a survey in West Malaysia, Buckley and Edeson (1956) found a microfilaria in the blood of dogs, cats and primates which resembled W. malayi in man. They isolated two distinct types of adults from the lymphatics of these animals. One resembled W. malayi, and the other was a new parasite, which they named Wuchereria pahangi (Buckley and Edeson, 1956). Buckley et al. (1958) reported another malayi-type microfilaria from the blood of cats and dogs on Pate Island, off the Kenyan coast. They too retrieved adults from the lymphatics and named the parasite Wuchereria patei. Buckley (1958b) detected substantial differences between these parasites and W. bancrofti, and erected a new genus, Brugia, to incorporate the spp. malayi, pahangi and patei. Later, other species of Brugia were described; B. buckleyi (Dissanaike and Paramananthan, 1962), B. ceylonensis (Jayawardene, 1962), B. beaveri (Ash and Little, 1964), B. guyanensis (Orihel, 1964), and B. tupaiae (Orihel, 1966). David and Edeson (1965) discovered another kind of microfilaria which they named "microfilaria Timor". Adults of these have recently been reared in the laboratory in the jird (Dennis, personal communication).

In West Malaysia, the transmission cycle of Brugia spp. involves man and other vertebrates (see Fig. 1). Under natural conditions, man is infected with both the periodic and subperiodic strains of B. malayi. The subperiodic strain is also found in a variety of other vertebrates, e.g. the domestic cat, civet cat and the primates Presbytis obscurus and Macaca irus (Laing et al., 1960). The transmission pattern is further complicated as the domestic cat (Felis catus) and the civet cat (Paradoxiurus hermaphroditus) are found infected with both subperiodic B. malayi and non-periodic B. pahangi.

Fig.1. NATURAL INFECTIONS of BRUGIA in MAN and VERTEBRATES in WEST MALAYSIA

B. pahangi is also widely found in many animals; slow loris (Nycticebus coucang), mouse rat (Echinosorex gymnurus) and others. Human infections of B. pahangi have not been recorded in surveys, but Edeson et al. (1960a) produced a patent infection in a human volunteer. Thus there is a zoonotic situation, where man is continually exposed to infections from animals. Clearly this situation is important.

Occasionally, the periodicity of the microfilariae of the parasite changed when in a different host. The subperiodic strain of B. malayi in man and cats, became nocturnally periodic in leaf eating monkeys and rhesus monkeys (Laing, 1961). A third strain of B. malayi that is diurnally subperiodic has also been described (Cabrera and Rosebooms, 1965). Many experimental animals have been used to maintain B. pahangi in the laboratory and to study its host-parasite relationship. The parasite has been maintained in cats (Edeson et al., 1960b; Schacher, 1962; Ewert and Singh, 1969; Denham [illegible], 1972a), dogs (Schacher and Sahyoun, 1967; Ash et al., 1974a) [illegible] 4s (Ash and Riley, 1970a; Suswillo, 1974). Ash (1973a) found [illegible] male jirds were more susceptible to B. pahangi infections than [illegible] ales but Denham (1974a) found that patency rates of cats infected [illegible] the parasite did not vary between the two sexes.

[illegible] fe-cycle

Fig. 2 shows the life-cycle of B. pahangi in the laboratory.

The sheathed microfilariae are ingested by mosquitoes feeding on blood of an infected host. The microfilariae exsheath in the midgut of the mosquito, pass through the wall of the gut and

Fig. 2. LIFE CYCLE OF BRUGIA PAHANGI IN THE LABORATORY

migrate to the flight muscles, where they moult twice to become infective larvae in about 11 days. These migrate to the haemocoel of the head. Ewert and Ho (1967) and McGreevy et al. (1974) demonstrated that when infected mosquitoes fed on vertebrate hosts, filariform larvae escaped from the labellae into a droplet of mosquito haemolymph over the wound in the skin made by the mosquitoes mouth parts. These larvae gained entry to the dermal lymphatics and migrated to the lymphatic vessels. Ewert and Singh (1971) closely imitated the natural method of infection by depositing infective larvae of B. pahangi on the skin of a cat limb punctured with numerous pin pricks. Others have infected animals orally (Gwadz and Chernin, 1973) or by ocular infusion (Ah et al., 1974b).

Infective larvae reach the nearest lymph node within 16 to 24 hours (Edeson and Buckley, 1959; Ewert and Bilhari, 1971; Denham et al., 1972a; and Sumwill[illegible], lodge within the subcapsular sinus of the nodes and later migrate back to the afferent vessels. The first moult occurs on day 8-9, followed by the second on day 23 (for males) and 33 (for females) (Schacher, 1962). The prepatent period in cats is 53-94 days (Schacher, 1962; Denham et al., 1972a).

C. Immunology and Pathology

Knowledge of the immunology of Bancroftian and Brugian filariasis in humans is sparse. Denham and McGreevy (1976) have reviewed immunity to Brugian filariasis. The paucity of experimentation in the field of filarial immunity is largely due to absence of readily available models for immunological manipulations, such as immunodeprivation, immuno potentiation and immunosuppression. However,

inbred hosts are now available for use in some host-parasite systems, e.g. Litomosoides carinii in albino rats, Brugia booliati in rats, Cardiofilaria nilasi in chickens, and Dipetalonema viteae in jirds. Lack of experimental models for W. bancrofti infections leaves an enormous gap in the understanding of Bancroftian filariasis.

In clinical-epidemiological surveys in endemic areas of filariasis, non-infected individuals are always found. This apparent resistance to infection in some persons cannot be correlated with experimental situations as the immune status of these persons is unknown. In some experimental systems, circulating microfilariae disappeared spontaneously, though the hosts harboured populations of adult worms. Albino rats infected with L. carinii became amicrofilaraemic after a brief period of circulating microfilaraemia (Bagai, Subrahmanyam and Singh, 1968). This active suppression of microfilariae appeared to be most active in the pleural cavity (Bagai and Subrahmanyam, 1970). The immunity was not transferred by passive transfer of serum from these "latent" rats to clean rats. Immunosuppression of these immune animals with cortisone resulted in the return of microfilariae to peripheral blood (Bagai and Subrahmanyam, 1970). Support for the view that immunity suppresses microfilaraemia in this system was obtained by Ramakrishnan et al (1962) who transferred adult L. carinii from immune to clean rats; the transferred worms released microfilariae into the circulating blood. It has now been shown that this active suppression of microfilariae is complement dependent, and due to macrophages, lymphocytes and polymorphs adhering to microfilariae in the pleural cavity (Subrahmanyam, personal communication). This adhesion phenomenon

occurred only in animals that became 'latent'. Subrahmanyam concluded that both humoral and cellular factors play a collaborative role against microfilaraemia in albino rats.

Denham et al. (1972b) found that in some cats which had received multiple infections with B. pahangi infective larvae, the established circulating microfilariae suddenly disappeared. The number of infections needed to produce this effect in these cats varied. These amicrofilaraemic cats remained immune to subsequent challenges with all stages of B. pahangi. The protection developed was extremely strong. Some cats which had decreasing microfilaraemia were also resistant to challenges. These cats were probably becoming amicrofilaraemic (Denham and McGreevy, 1976). During autopsies of these immune animals, live adult worms of the immunizing infections were always found, but less than 1% of the challenge doses, including the 24 hour challenge worms, were retrieved. Denham and McGreevy (1976) suggested that the response in these animals was probably due to acquired immunity. They do not, however, rule out the possibility of lymphatic damage forming a barrier to establishment of some of the challenge worms especially in the leg which had been repeatedly infected. A similar attempt to produce "immune" jirds by repeated infection with B. pahangi has been conducted by Kowalski and Ash (1975) and Suswillo (personal communication). Suswillo gave 5, 10 and 15 weekly repeat infections with 50 larvae of B. pahangi in each inoculation. However, there was no difference in the establishment of adult worms, suggesting that immunity could not be induced even with many repeat infections in jirds. Kowalski and Ash (1975) inoculated jirds either with single or repeat inoculations of 75 worms each time. Female worms

recovered from jirds repeatedly inoculated with B. pahangi were smaller than female worms retrieved after single infection.

The microfilaria is the important stage from the transmission viewpoint. Thus the immunogenic status of the microfilarial stage must be elucidated, so as to assess whether a microfilarial vaccine could be produced (WHO, workshop in Immunopathology of Filariasis). As early as 1935, Knott transfused live microfilariae of W. bancrofti into 3 volunteers; one non-filarial, one showing signs of elephantiasis and one with clinical filariasis. Whilst the transfused microfilariae continued to circulate in the blood of the non-filarial subject, they disappeared from the blood of the elephantiasis patient within 2½ days. In the patient showing clinical filariasis, the infused microfilariae did not appear at all in the circulation. Similar results were obtained by Hawking (1940). Cats which had become amicrofilaraemic (Denham et al., 1972b), transfused with microfilariae of B. pahangi, behaved in a similar fashion. The transfused microfilariae disappeared within one hour in these test animals, they remained in circulation in control animals for 3 weeks (Ponnudurai et al., 1975).

Smithers (1968) in his review of the immunogenicity of blood microfilariae suggested that this stage of the parasite evoked only weak responses in the host. Wong et al. (1964b) repeatedly injected dogs with microfilariae of Dirofilaria immitis and made these animals immune to challenge infections with the same microfilariae species. Sera from these animals agglutinated homologous living microfilariae and prevented the production of microfilariae by adult worms in vivo. Wong et al. (1964b) performed passive transfer experiments, inoculating [illegible] animals with circulating microfilariae of D. immitis

and recorded a decrease in the levels of circulating microfilariae. Dogs immunized with microfilariae of *D. immitis* showed specific immunity (Wong *et al.*, 1971). The animals were challenged with infective larvae of *D. immitis* and *D. pahangi*. No microfilariae occurred in the blood of the dogs challenged with *D. immitis*, whereas those animals challenged with *D. pahangi* became patent.

After patency had occurred, microfilariae persisted in circulation for a long period (Wilson and Ramachandran, 1971; Denham *et al.*, 1972a). Denham *et al.* (1972a) gave cats single and multiple infections of *D. pahangi*. They found that cats inoculated once, irrespective of the number of infective larvae used, showed a reasonably uniform pattern of microfilaraemia. Increasing the number of infective larvae used resulted in a greater number of adult worms being recovered, but the microfilarial levels remained basically similar. This pattern changed when the animals were given multiple infections (Denham *et al.*, 1972b).

Duke (1960) and Wong (1964a) postulated that factors in the host blood maintain a delicate balance between the host immunity and microfilariae released by the adult female worms. Stabilised microfilaraemia also occurs in *D. immitis* infections in dogs (Wong, 1964a), and this continued despite the removal of large quantities of blood. In loiasis in monkeys, the spleen plays a major role in destroying the microfilariae (Duke, 1960). When monkeys with low level circulating microfilaraemia were splenectomised, the level of circulating microfilariae increased.

Primary infections of *L. carinii* in cotton rats inhibit the growth of subsequent infections with the same parasite (Scott and Macdonald, 1958; Bertram, 1966). Scott and Macdonald (1958) transplanted different stages of the worms to study the stage specific

immunity. Although the adult and fourth stages of the worm stimulated responses in the host to inhibit growth and development of the parasites of secondary infections, this effect was best manifested when late third stage worms were transplanted into cotton rats. The retarding effect of pre-existing worms acts primarily on worms during the first 7 days of the development in the host. However, this effect on secondary infections was manifested during the subsequent 14 days, even though these worms were transferred to a new host for the last 7 of the 14 days (Scott et al., 1958). Denham et al. (1972b) also reported that female worms retrieved from cats repeatedly infected with B. pahangi were smaller than those from single inoculations.

Attempts have been made to produce immunity to Brugian filariasis with excretory and secretory antigens (Fredericks and Ramachandran, 1969). They incubated infective larvae of B. malayi in culture medium, but monkeys vaccinated with this medium were not protected. It may be that infective larvae, being the exogenous stage and non-feeding, did not release antigenic excretory or secretory products.

McGreevy et al. (1975) demonstrated that B. pahangi do not use immunological disguise by incorporating host material to escape host defence mechanisms, as did Schistosoma mansoni (Smithers et al., 1969).

Immunity developed due to attenuated filarial parasites is discussed in the introduction to Chapter 4.

When cats are infected with B. pahangi, most pathology is produced by the adult stages (Rogers and Denham, 1974). Lymphatic damage in cats with single infections was not progressive and the main pathology occurred within 16 weeks. In cats which were repeatedly infected for long periods, the popliteal nodes were enormously

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enlarged and hard when palpated (Rogers and Denham, 1974). The lymphatics were dilated, hard and ropey. There was transient lymphoedema in some cats (Rogers and Denham, 1974), but no appreciable change in the rate of lymph flow in the infected legs of the cats (Rogers and Denham, 1975). Schacher et al. (1969, 1971) also demonstrated lymphoedema in dogs infected with Brugia spp. The pathology of the infected lymph vessels and nodes have been visualised using two roentgenographic techniques; lymphangiography (Goonaratne et al., 1971; Schacher et al., 1969; Ewert et al., 1972) and xeroradiography (Rogers et al., 1975a). Rogers et al. (1975b) discussed in detail the histological changes in the nodes of cats infected singly or repeatedly with B. pahangi.

The following aspects of the host-parasite relationships of cats infected with B. pahangi were studied.

a) The effects of irradiation on the growth rate, percentage recovery, motility, morphology and maturation of the parasite.

b) The resistance of cats repeatedly infected with irradiated and non-irradiated B. pahangi to challenge with infective larvae of homologous or heterologous species.

c) The changes in the lymphatics of cats infected with irradiated and non-irradiated parasites using the xeroradiographic method.

d) Haematological changes in infected cats, with special emphasis on the peripheral eosinophil levels.

e) Antibody responses of cats infected with irradiated and non-irradiated B. pahangi to various stages of the same parasite and some stages of other filarial parasites using the Indirect Fluorescent Antibody Test (IFAT).

f) A preliminary study was made of the resistance of jirds repeatedly infected with irradiated and non-irradiated [redacted].

Chapter 2

GENERAL MATERIALS AND METHODS

The Vector

The mosquito vector used to maintain experimental infection cycles of B. pahangi in cats was Aedes aegypti, a susceptible strain carrying the f^{m} gene (Macdonald, 1962). Its maintenance in the laboratory was found to be easier than most other mosquitoes. A stock strain of this vector is maintained in the Department of Medical Helminthology, London School of Hygiene and Tropical Medicine, and the eggs from these were used for raising colonies when required.

Maintenance of mosquitoes

Eggs of A. aegypti deposited on damp filter papers and stored in desiccators at 80% relative humidity, were transferred to plastic bowls 34 cm.in diameter and 10 cm. in depth, half filled with water at 28°C. The bowls were then left in an insectary at 28°C for 24 hours in order to obtain maximum hatching of the eggs. A little liver powder (desiccated liver powder, Armour Pharmaceutical Company Ltd.) was sprinkled on the surface of the water for the newly hatched larvae. Only a minimum amount of food was given at any one time to prevent scum formation on the water surface in the bowls(usually the mosquito larvae were fed twice a day to keep the amount of food given at any one time to a minimum. Three days after they had hatched, larvae were transferred into bath tubs measuring 4 ft. x

2 ft. x 2 ft., containing warm water. More liver powder was sprinkled on to the water daily to replenish the food, but a constant check was made to avoid scum formation due to excess food. The whole tub was covered with nylon netting to prevent the escape of mosquitoes developing from precocious larvae. The freshly emerged larvae went through three more instar stages and started to pupate on the 6th day after hatching.

As soon as pupae were seen in the tub, the water was run off and the larvae and pupae collected on a sieve. The larvae, male pupae and female pupae were separated in the apparatus shown on Plate 1. This apparatus worked on the principle of allowing objects of one size to pass through a variable gap between two glass plates. The size of this gap was controlled by a threaded screw so that as it was gradually opened larger and larger objects could get through (i.e. larvae, then male pupae then female pupae). The mixture of larvae to be separated was poured through the gap at the top of the separator and water flushed through continuously from a hose pipe. The screw was adjusted to allow the larvae to pass through first, these were returned to the bath tubs or to bowls. The larvae were followed by small male pupae and finally the large female pupae. A device, designed by the author, was used to measure a volume that contained approximately 1,000 female and 100 male pupae. This device consists of a perforated, central glass column with a funnel at the top end. Water poured in, spurts out through the pores and passes through an outer adjoining chamber with an outlet. When measuring pupae the bottom outlet of the main column was blocked with a thumb and water containing pupae poured through the top end (see Plate 2). When the accumulated pupae reached a predetermined

Plate 1: Pupae separator

Plate 2: Apparatus for measuring pupae

level they were collected into a tube. These were then transferred to plastic petri dishes in nylon cages measuring 1 x 1 x 1 ft. and kept in an insectary at a temperature of 28°C and 80% relative humidity. All the pupae developed into adults within 2 to 3 days. Currants or slices of apple were placed on top of the cages as a source of food. Small plastic bowls containing water and filter paper cone were placed in the cages to provide oviposition sites for the female mosquitoes and a source of drinking water.

Four days after their emergence, the adult mosquitoes were given their first blood meal from an uninfected guinea pig. After anaesthetisation with Nembutal (Abbot Laboratories Ltd.) fur from a flank was clipped off, and the animal placed on top of the cages. This ensured that the first batch of eggs laid, were from mosquitoes not subjected to selection pressure by pathogenic filarid worms.

Infecting mosquitoes with B. pahangi and B. patei

One day before the infective feed, the currants were removed from the top of the cages to obtain better engorgement of blood by the starved mosquitoes during feeding. A cat infected with the required parasite and having approximately 3 microfilariae per cu. mm. in the peripheral blood, was used to infect the mosquitoes. The cat was anaesthetised with Nembutal and fur from one side was removed with electric clippers. The animal was then placed across the cage with the shaved region facing the mosquitoes (see Plate 3). The mosquitoes were allowed to feed for 15 minutes. A second feed was given on the following day to ensure that all female mosquitoes had

a blood meal. After removing the cat from the cages, the currants were replaced. After the infective feed the mosquitoes were kept for 11 days at 28°C and 80% relative humidity. During this time they were given currants or raisins every day and water in their egg bowls. Any dead mosquitoes were removed from the cages.

Mass dissection of mosquitoes and collection of larvae

The infected mosquitoes in the cages were immobilised in a -20°C deep freeze for approximately 30 seconds. The mosquitoes were shaken into corners of the cages, large test tubes introduced into the cages and the mosquitoes were collected into these. The mosquitoes were killed by rapidly tapping the tubes against the palm of the hand.

A modified Baermann apparatus was set up (see Plate 5). This consisted of a glass funnel into which was placed a sieve with a pore size of 75 microns. A short length of rubber tubing was attached to the column of the funnel and clamped with a pair of artery forceps. Medium 199 was poured into the funnel, to reach half way to the level of the sieve, and air bubbles carefully removed from under the sieve.

A little 199 Medium was placed on a 3 x 6 inch glass plate and 30 - 40 mosquitoes arranged in a single layer over the fluid. They were then lightly crushed to break open the heads, thoraces and abdomens, by gently applying pressure with a centrifuge tube. A quick examination of the contents of the glass plate ensured that all the mosquitoes were sufficiently ruptured. The crushed mosquitoes on the glass plate were flushed into the Baermann sieve with more 199 Medium. Larvae emerged from the mosquito tissues, migrated through

Plate 3: Infecting Aedes aegypti with Brugia pahangi on an infected cat

Plate 4: Modified Lawrence cat restraining box

the pores of the sieve and collected at the lower end of the funnel. The preparation was left about 30 minutes before the infective larvae were removed from the bottom of the apparatus in a little medium.

Infective larvae of *B. pahangi* were counted out into lots of the required number (which in most cases was 100). Only actively moving undamaged larvae were included. These were taken into separate 1 ml. syringes with 0.5 ml. of 199 medium.

Cats

Cats used in the following experiment were purchased from dealers and caged individually or allowed to run free in a room. Food for these animals consisted of milk and canned pet food ('Whiskas'). The cats were infected subcutaneously, using a 21G 1½ inch needle, into the volar surface of the foot. Occasionally cats were unmanageable or a restraining box was used (see Plate 4). The syringes which had been used to inoculate larvae, were flushed out with medium, and the number of larvae remaining noted. Thus it was possible to calculate exactly how many larvae had been inoculated. The animals were infected in different legs as required in the particular experiment.

Autopsy of cats

The animal to be autopsied was anaesthetised by intraperitoneal administration of Nembutal. A sample of 5 ml. of heparinised cardiac blood was taken and passed through a 5 μ Nuclepore filter (Nuclepore

Plate 5: Baermann apparatus for obtaining infective larvae from crushed mosquitoes.

Plate 6: Lymphatic vessels of the hind limb of an infected cat, outlined with Evans Blue.

Inc., Pleasanton, California, U.S.A.) 25 mm. in diameter, held in a Swinnex filter holder (Dennis and Kaan, 1971). The syringe was then loaded with distilled water, reattached to the holder and the water pushed through. This procedure was repeated until the filtrate was colourless. The holder was then dismantled, the filter membrane removed and placed onto a glass slide with the blood side upwards. The membrane was stained with Giemsa stain (Revector microscopic stain) and examined for microfilariae. Immediately after the blood sample had been taken about 0.3 ml. of 1% Evans Blue (EB) in phosphate buffered saline (PBS) was inoculated into the interdigital areas of each foot to outline the lymphatic vessels and the lymphatic nodes. Fur from the limbs and the adjoining areas was removed with an electric clipper. About 15 minutes after inoculating the dye, the animal was exsanguinated by puncturing the inferior vena cava. Removal of all blood allowed easier visualization of the, now blued, lymphatic system.

The lymphatic system was carefully dissected (see Plate 6) from other tissues and transferred into marked petri dishes containing 199 medium. Separate containers were used to hold the afferent and efferent lymphatics, and the various nodes. (Figure 3 shows the relevant lymphatic vessels and nodes of cats.) Pelt, leg, lung and heart were also soaked in warm saline. The lymphatic tissues were carefully teased, to enable the worms present to migrate into the medium. The petri dishes were examined for worms under a x 40 dissecting microscope. The contents were inoculated at room temperature overnight before a final examination. Finally the tissues were pressed under two glass plates to locate any dead and calcified worms.

On recovery the worms were sexed and the state of their motility

Fig. 3. LYMPHATIC DRAINAGE SYSTEM OF THE FORE-LIMBS AND HIND LIMBS OF CATS

A- parotid nodes
B- medial retropharyngeal node
C- cervical nodes
D- axillary nodes
E- afferent vessels to C&D
F- afferent vessels to G
G- popliteal node
H- interfemoral node
I- inguinal nodes
J- iliac nodes
K- sacral nodes
L- afferent vessels to K

noted before transferring them into a solution of alcohol (70%) and glycerine mixed in equal volumes. The female worms were examined microscopically under high power for the presence of microfilariae and embryonated eggs in their uteri. The alcohol evaporated within 2 to 3 days leaving the worms in pure glycerine. These worms were then mounted in hanging drops of dehydrated glycerine in cavity slides, for measurements and detailed morphological observations, both of which were accomplished by using a camera lucida. Indian ink drawings were made with the tracings obtained from the camera lucida.

Counting microfilariae

When a blood sample was required to count microfilariae in the peripheral blood, a gold-line, graduated pipette was used to measure out 10-100 cu. mm. of blood. The blood was transferred into a counting chamber which contained distilled water to lyse the red blood cells. A dissection microscope (at x 30 magnification) was used to count the easily visible, moving microfilariae strip by strip in a counting chamber (Fig. 4).

The specialized techniques used in these experiments, for example, irradiation of infective larvae, procedure for performing the Indirect Fluorescent Antibody Test, blood cell counts, etc. are dealt with in the individual chapters.

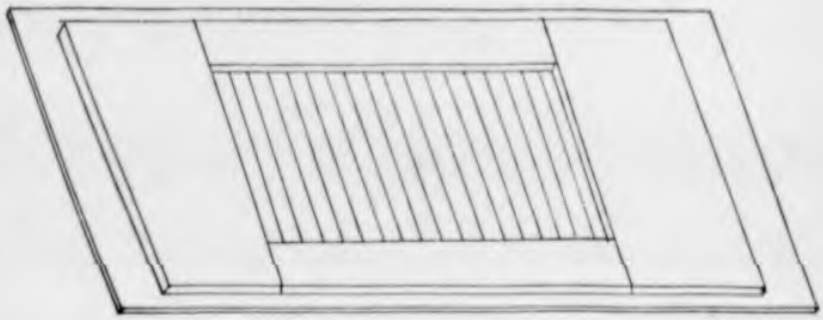


Fig.4. THE COUNTING CHAMBER

Chapter 3

THE EFFECTS OF IRRADIATION ON H. PAHANGI

General Introduction

The biological processes of parasites undergo regressive, mutilating and irreversible changes when subjected to irradiation. An irradiated organism can escape death only if its powers of regeneration are good (Bacq and Alexander, 1961). Attenuation is a process by which the virulence and pathogenicity of an organism is modified by chemical, physical or biological means and is rendered less pathogenic. Both gamma rays and x-rays have been frequently used to irradiate parasites; they are electromagnetic radiations of short wave length, and differ from each other in their intensity of energy emission. The biological effects produced on organisms are similar (Smith, Jones and Hunt, 1974). Irradiation of parasites often results in reduced infection rates. Jarrett et al. (1958b) recovered 0.05% of the original inoculum of irradiated Dictyocaulus viviparus in cattle, compared with 22.7%, after infection with normal worms. Reduced recoveries have also been observed in infections with irradiated parasites, by Dow et al. (1958) with Uncinaria stenocephala and by Miller (1964) with Ancylostoma caninum.

Worms suffered greater damage if the irradiation level was increased. In normal infections of Dictyocaulus filaria in sheep, 4.24% of the worms reached the lung, causing parasitic bronchitis. When the parasites were irradiated with 40 krads. of Co.60, only 0.22% of the original inoculum reached the lung (Jovanovic et al., 1961). No parasites reached the lung when the irradiation dose was

increased to 60 krads. Jovanovic et al. (1961), who used x-ray in place of Co60 to irradiate D. filaria, also found that fewer adults reached the lung.

The most obvious change caused by irradiation is the inhibition of growth and development. Trichinella spiralis larvae exposed to 4 krads. of Co60 showed retarded growth 96 hours after infection (Gould et al., 1957). The average length of irradiated worms was 1.5 mm., whilst the average length of non-irradiated worms was 2.4 mm. Worms were even smaller if the level of irradiation was increased.

The reproductive capacity of the parasite is also altered due to irradiation. Jarrett et al., (1958b) found that fewer irradiated Trichostrongylus colubriformis infective larvae reached maturity in lambs. Lambs given T. colubriformis third stage larvae irradiated with 40 krads. of Co60 produced 420 eggs per gram of faeces compared with 2,400 eggs per gram of faeces from lambs given normal larvae.

Tyzzer and Honeiji (1916) wholebody irradiated rats infected with T. spiralis and altered the development of different stages of the parasite to varying degrees. Alicata (1951) exposed T. spiralis to 15 and 20 krads. of Co60 and found that the cuticle of the adult worms, especially the female, was wrinkled and had abnormal thickenings. The ovaries were shrunken and malformed. Gould et al. (1957) were able to detect morphological changes in irradiated T. spiralis larvae recovered as early as 12-18 hours after infection. (Their publication includes many elegant plates that illustrate the morphological changes in the parasite due to irradiation.) The normal development of microfilariae of D. immitis in mosquitoes was inhibited by irradiation (Dunbury and Sadun, 1968). Irradiated mosquitoes which had ingested microfilariae produced only early

sausage stage larvae even 10 days after infection.

Exposure to irradiation changes the rate of maturation of parasites and consequently, the duration and migratory patterns. By subjecting them to optimal doses of irradiation, it is possible to stop the migration of worms at specific sites. Infective larvae of A. caninum exposed to cobalt irradiation and inoculated into pups were arrested in the lungs where the host was able to mount a better resistance (Miller, 1963). Attenuation could also prevent parasites from arriving at the sites where they cause most pathology. Jovanovic et al. (1961) found that few irradiated D. filaria reached the lung. Von Lichtenberg and Sadun (1963) however, reported that some cercariae of Schistosoma mansoni, irradiated with up to 50 krads. of Co60 continued to migrate normally to the lung and liver.

Parasites of both sexes are affected differently by irradiation. Risk and Keith (1960) were the first to note that male worms were more susceptible to ionizing radiation. They found that in sheep infected with Oesophagostomum radiatum treated with 2 krads. of x-ray, the ratio of male to female worms was 1:7 whereas in infections with the untreated parasite, they recovered equal numbers of males and females. The increased susceptibility of male parasites to irradiation have been documented by Ciocordia and Bisseli (1960) working with T. colubriformis. Jovanovic et al. (1961) with D. filaria. Miller (1963) with A. caninum and Guest et al (1971) with D. immitis.

Although female worms are generally more resistant to attenuation, irreversible damage to the reproductive system occurs, resulting in sexual sterility. T. spiralis larvae needed an exposure of 400 rads. of Co60 to cause sterility (Evans et al., 1941).

Ciocordia and Bisell (1960) reported that when they exposed Trichostrongylus axei to 5 krads. of x-ray, the larvae became more infective. However, this increased infectivity, was not noticed when the irradiation level was increased. Jovanovic et al. (1961) studied the viability of irradiated and control larvae of D. filaria in vivo and in vitro. Viability and motility of irradiated infective larvae was decreased compared with that of normal larvae.

The primary aim of the experiments reported in this chapter was to determine the level of gamma irradiation with Co60 required to inhibit the development of D. pahangi beyond the third, fourth, and adult stages. During this calibration study the effects of gamma rays on growth rate, percentage recovery, motility, morphology and maturation was studied. Particular attention was paid to the effects of irradiation on the morphology of the reproductive system.

Method of Irradiating Larvae

Larvae to be irradiated were loaded into 1 ml. syringes, using a 21G 1½ inch needle, with 0.5 ml. of medium 199. Air gaps of about 0.1 ml. were introduced between the needle and the medium to ensure that all worms were uniformly exposed to the source of irradiation. The larvae were irradiated in a cobalt unit (Vickers-Armstrong) at the Middlesex Hospital Medical School, London. The unit had an output of 2,000 rads (2 krads.) per minute . The syringes, loaded with the infective stages of the parasites,were placed vertically in the unit and irradiated with 10, 25, 45, 50, 75, or 100 krads. of Co.60.

Cats were infected with irradiated and non-irradiated infective stages of B. pahangi as described in the chapter on materials and methods. Initially only the hind legs of the animals were infected, but later all four legs were inoculated. In each experiment, one leg was infected with normal, non-irradiated worms, and the other legs with parasites exposed to different levels of Co.60. This allowed the effects of irradiation to be evaluated in a single host as it has been shown that larvae inoculated into one limb seldom migrate beyond that limb.

Experiment 1.

The purpose of this experiment was to evaluate the effects of irradiation with 25 krads. on B. pahangi.

Three cats were inoculated in the Lhl (left hind leg) with infective larvae irradiated with 25 krads. and in the Rhl (right hind leg) with non-irradiated worms.

The cats were killed and autopsied 7, 12 and 18 days after infection. Table 1 shows the number of worms recovered, their mean lengths and the motility of worms in medium 199 after recovery.

There was a decrease in the percentage recovery of irradiated worms, compared with normal worms, on days 7, 12 and 18. There was no difference in the length of the two groups of worms recovered on day 7. However, on day 12 and 18 male and female worms that had been irradiated were significantly smaller than untreated worms. All the worms were active in medium 199.

TABLE 1. PERCENTAGE RECOVERY AND MEAN LENGTH OF IRRADIATED AND NON-IRRADIATED B.PAHANGI FROM INFECTED CATS

* larvae could not be sexed.

cat No.	cat limb	level of irradiation (krads.)	day after infection	% of inoculated worms recovered	mean length male in mm.	mean length female in mm.	motility
P58	Rh1	0	7	45.8		2.25* (0.06)	normal
	Lh1	25	7	18.0		1.95* (0.06)	"
P60	Rh1	0	12	46.0	4.32 (0.3)	5.57 (0.2)	"
	Lh1	25	12	45.0	3.89 (0.1)	4.61 (0.2)	"
P59	Rh1	0	18	47.0	5.56 (0.01)	7.26 (0.8)	"
	Lh1	25	18	21.0	3.67 (0.06)	5.17 (0.2)	"

Experiment 2

The purpose of this experiment was to determine the effect of irradiation with 45 krads. on B. pahangi.

Three cats were infected in the Lhl with parasites exposed to 45 krads. and in their Rhl with non-irradiated worms. The cats were autopsied on days 7, 12 and 18. Table 2 shows the results obtained. There was no difference in the percentages of worms recovered on day 7, but on subsequent occasions fewer worms were recovered from the legs inoculated with irradiated worms. The irradiated worms retrieved 18 days after infection could not be sexed, as their reproductive system was either deformed or distorted. The difference in the mean lengths of the normal and irradiated worms was significant on day 12 and 18 days post-infection. All the worms were active in medium 199.

Experiment 3

In this experiment a study was made of the effects of higher levels of irradiation in order to evaluate the minimum dose of irradiation required to prevent development of infective stages of B. pahangi.

Three cats were infected in the Lhl with parasites exposed to irradiation dosages of 50, 75, or 100 krads. Their Rhls were inoculated with normal worms. The cats were killed on different days from the previous experiments (Experiments 1&2), in order to observe the earliest signs of lack of motility and growth in the irradiated worms.

TABLE 2. PERCENTAGE RECOVERY AND MEAN LENGTH OF IRRADIATED AND NON-IRRADIATED *B.PAHANGI* FROM INFECTED CATS

* larvae could nor be sexed

cat No.	cat limb	level of irradiation (krads.)	day after infection	% of inoculated worms recovered	mean length male	mean length female	motility
					in mm.		
P61	Rh1	0	7	34.0	1.84* (0.06)		normal
	Lh1	45	7	50.5	1.96* (0.06)		"
P62	Rh1	0	12	39.8	4.34 (0.3)	5.56 (0.2)	"
	Lh1	45	12	4.1	1.67 (0.3)	3.25 (0.2)	"
P63	Rh1	0	18	40.0	5.53 (0.02)	7.28 (0.08)	"
	Lh1	45	18	18.0	3.78*		"

Table 3 shows the results. All the non-irradiated worms were active on recovery. 81.8% of the parasites exposed to 50 krads. were also motile. Only one worm was recovered from the cat infected with parasites irradiated with 75 krads. and this was non-motile. However, the recovery from the control leg (9%) was also poor in this animal. All these worms were active. In the cat inoculated with parasites exposed to 100 krads., 10% of the irradiated worms were recovered as compared to 62.1% recovery of non-irradiated worms. None of the worms exposed to 100 krads. showed normal motility although some of them moved feebly.

No attempt was made to repeat the effect of 75 krads. on the parasite as 100 krads. completely prevented development of the parasite, and worms irradiated with 50 krads. remained active till day 15. There was no difference in the size of non-irradiated worms and parasites exposed to 100 krads. 4 days after infection. There was a significant difference between the normal worms and worms irradiated with 50 and 75 krads. on [illegible] respectively.

Experiment 4

The purpose of this experiment was to determine long term effects of irradiation with 10, 25 and 45 krads. 10 cats were infected with larvae irradiated with 10 krads. (L10), 25 krads. (Rh1), and 45 krads. (L41) and normal larvae (Rh1). The cats were killed on days 4, 7, 14, 24, 36, 38, 77 and 97 after infection.

Lymphatic vessels and nodes from the limbs inoculated with normal worms or worms irradiated with 10 krads. were varicosed, enlarged and fibrosed in cats autopsied 30 or more days after

TABLE 3. PERCENTAGE RECOVERY AND MEAN LENGTH OF IRRADIATED AND NON-IRRADIATED *B.PAHANGI* FROM INFECTED CATS

* larvae could not be sexed

cat No.	cat limb	level of irradiation (krads.)	day after infection	% of inoculated worms recovered	mean length in mm. male	mean length in mm. female	% larvae motile
P100	Rhl	0	4	62.1	1.81* (0.06)		100
	Lhl	100	4	10.0	1.79* (0.05)		0
P73	Rhl	0	11	9.0	3.29 (0.2)	6.72 (0.2)	100
	Lhl	75	11	1.0	2.22*		0
P72	Rhl	0	15	24.5	3.78 (0.1)	4.64 (0.07)	100
	Lhl	50	15	29.7	1.6 (0.05)	2.68 (0.1)	81.8

infection. However, few or no gross changes were visible in the lymphatics and nodes removed from limbs inoculated with worms exposed to 25 or 45 krads.

In limbs inoculated with normal larvae, the majority of larvae migrated to the nearest lymph nodes via the lymphatics within the first 24 hours. After 10-14 days in the perinodal sinus, the fourth stage larvae returned to the lymphatics afferent to the node and developed into adult stages (see Plate 7). This pattern was closely paralleled by worms exposed to 10 krad. Larvae that had been exposed to 25 and 45 krads. migrated to the node, but failed to return to the efferent vessels (see Plate 8). Fig. 5 shows the migration pattern of non-irradiated and irradiated worms.

After infection with normal larvae there was no decline in the recovery rate until after 30-40 days post infection, but in rats inoculated with worms subjected to irradiation with 10 or 25 krads., there was a steady decrease in the number of worms recovered (see Fig. 6). When worms were irradiated with 45 krads. a low, decreasing, rate of worm recovery was found.

All non-irradiated worms recovered were active in 199 medium and there was no detectable loss of activity in worms exposed to 10 krads. Exposure to both 25 and 45 krads. reduced the motility of the parasites. All worms irradiated with 45 krads. recovered 36 days after infection, showed only feeble motility.

During autopsy of an animal with a normal infection of *H. pahangi*, approximately equal numbers of male and female adult stages were recovered. After inoculation of worms receiving 10 and 25 krads., no significant difference in the sexes was noticed up to day 38, from when fewer male worms were found. On occasions, all

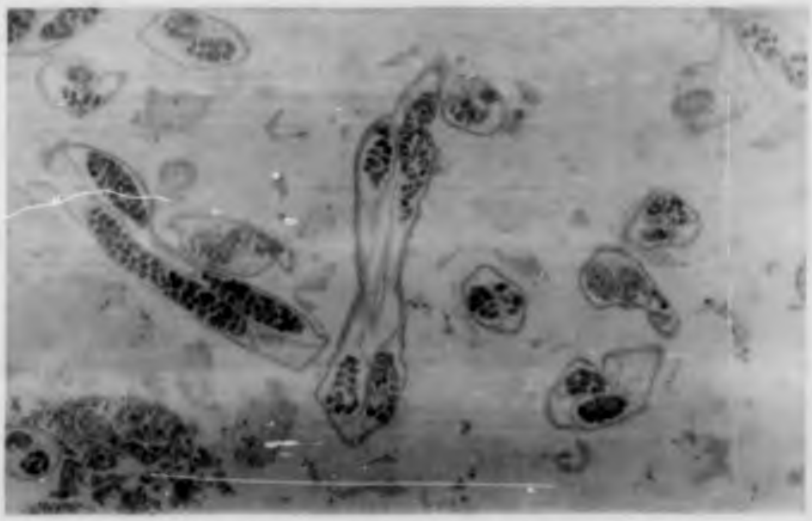


Plate 7. Non-irradiated adult B.pahangi in the afferent lymphatics of an infected cat. (x875) (HE)

Plate 8. Irradiated B.pahangi larvae in the subcortical sinus of popliteal lymph node of a cat. (x220) (HE)

Fig. 5 LOCATION OF IRRADIATED AND NON-IRRADIATED B. PAHANGI IN CATS

RECOVERY OF BRUGIA PAHANGI FROM INFECTED CATS

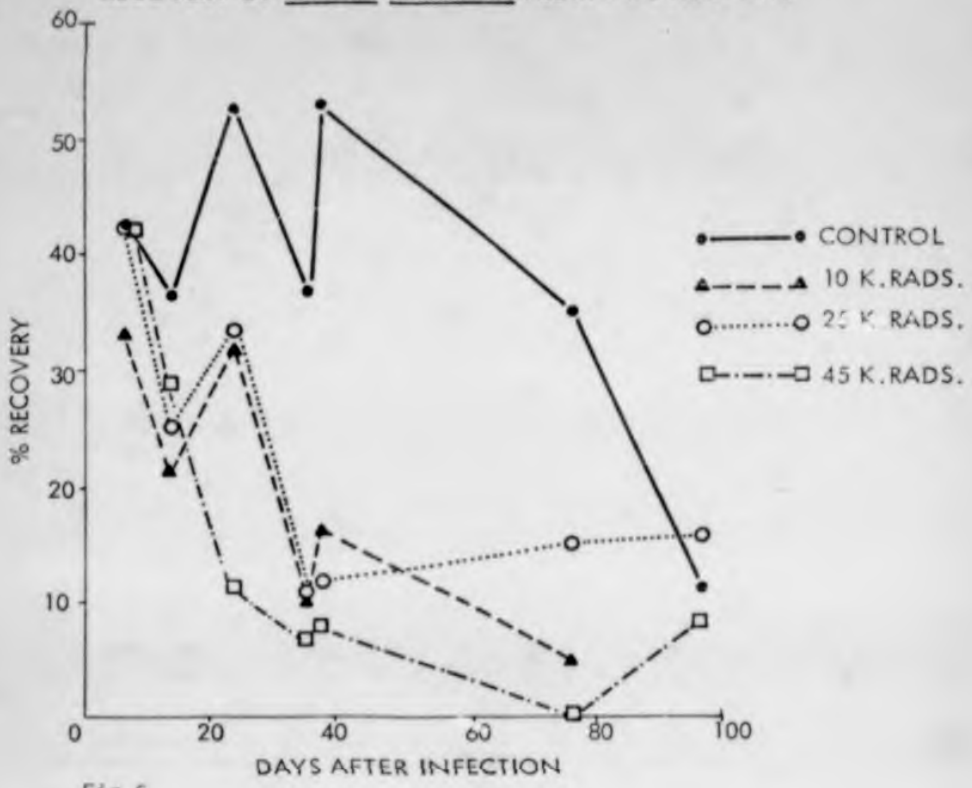


Fig.6.

GROWTH OF BRUGIA PAHANGI IN CATS

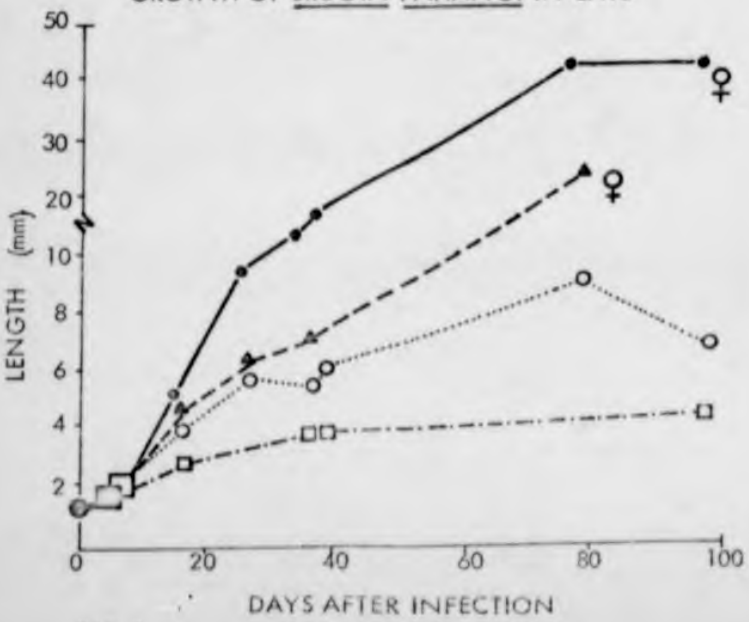


Fig.7.

the worms recovered were females. It was difficult to decide the sex of worms exposed to 45 krads. as their genital primordia did not differentiate.

There was a steady increase in the mean length of both male and female non-irradiated worms. The female worms recovered on day 97 had attained a mean length of 4.3 cm. and the males a mean length of 1.75 cm. Worms subjected to irradiation grew less rapidly and failed to reach the length of normal worms. Their sizes on day 77 showed a reduction with increasing level of irradiation. Female worms exposed to 10 krads. attained a mean length of 2.48 cm. by day 77 (no male worms were recovered). Worms irradiated with 25 krads. reached a mean length of 0.62 cm. on day 97, and those irradiated with 45 krads. reached a mean length of 0.41 cm. on the same day. In neither case was it possible to ascertain the sex of the worms. Fig. 7 compares the mean lengths of irradiated and non-irradiated worms recovered on different days (details in Table 4).

Hanging drop mounts of all recovered worms were prepared and examined under x 30 magnification. The uterus of female worms recovered on day 77 and 97 from limbs of cats receiving infections with non-irradiated D. pahangi was filled with microfilariae and embryonated eggs. Worms exposed to 10 krads. of Co.60 developed a reproductive system, but the uterus was small and damaged and did not have microfilariae. Complete sterility was only achieved when worms were exposed to irradiation levels of 25 and 45 krads. The parasites irradiated with 25 krads. grew only to the stage of development seen in late fourth stage of the normal life-cycle. The few worms recovered 97 days after inoculation did not contain eggs. Severe damage to the reproductive system was caused when worms were

TABLE 4. MEAN LENGTH OF IRRADIATED AND NON-IRRADIATED B.PAHANGI (female worms) in MM. RECOVERED OVER VARIOUS DAYS AFTER INOCULATION. (se)

DAYS AFTER INFECTION	4	7		14		24	36	38	77	97
Non-irradiated infection	2.04 (0.06)	1.92 (0.1)	2.40 (0.1)	4.67 (0.2)	5.62 (0.2)	9.25 (0.3)	12.48 (0.5)	15.43 (0.6)	42.30 (2.7)	43.0 (0.4)
Irradiated with 10 krads.	-	1.84 (0.06)	2.05 (0.06)	5.16 (0.1)	4.92 (0.3)	6.51 (0.2)	6.97 (0.8)	6.46 (0.8)	24.75 (6.4)	-
Irradiated with 25 krads.	1.71 (0.03)	2.06 (0.07)	2.01 (0.07)	4.07 (0.3)	4.35 (0.3)	6.42 (0.1)	5.43 (0.4)	6.30 (0.4)	9.23 (1.3)	6.27 (0.4)
Irradiated with 45 krads.	1.76 (0.07)	1.86 (0.05)	2.18 (0.07)	2.24 (0.2)	3.19 (0.2)	3.12 (0.3)	3.68 (0.3)	4.07 (0.2)	-	4.12 (0.2)

exposed to 45 krads.

A more detailed study of the effect of irradiation on the development of the reproductive system of B. pahangi

Buckley and Edeson (1958) published the earliest detailed description of male and female reproductive organs of B. pahangi. Schacher (1962) described the changes in the reproductive system of B. pahangi from the earliest third stage genital primordium of the male and female worm, and the spicule primordium of the male, through to the mature adult structures of both sexes. Others have described genital regions of mature or developing filarial parasites.

Normal development of reproductive organs in B. pahangi in cats

Schacher (1962) described the early structure of genital primordia of male and female worms, 48 hours after inoculation into cats.

Male

The male genitalia primordia in the early third stage larvae lie free, just behind the base of the oesophagus. The development is rapid, and within 48 hours the chain of cells of the genital primordia form a shape resembling an inverted 'V' or 'U' and by the 3rd. day this shape looks like a 'shepherd's crook'. At the time of the third moult, the distal tip is not easily discernible. After 2 to 3 days of development in the cat, a cluster of hyaline cells

appears near the region of the rectum, which subsequently forms the spicule primordia. The moult to 4th. stage larvae takes place on day 8-9.

At about 10-11 days after inoculation, the spicule primordia differentiate anteriorly, forming clear tubular spicules and then grow posteriorly uniting with the rectum to form the cloaca. The gubernaculum appears on about day 14. By day 23 after initial infection, adult male features of spicules, gubernaculum and adanal papillae appear. The tail begins to coil.

The adult male spicules are unequal and dissimilar; the left spicule longer than the right (Buckley and Edeson, 1958; Schacher, 1962). This consists of three parts. The first is a tubular proximal part, open and slightly expanded at its proximal end. Then follows the short and non-tubular region. The third part is coiled in a sinistral fashion and, thus, the right spicule is not easily observed.

Female

The third stage female genital primordium attaches to the ventral body wall at about the mid-point of the oesophagus, and grows posteriorly. By the time of the third moult, the genitalia are divided distally into the characteristic double uterine branches terminating in large cap-cells. In the late third stage, the vaginal portion has a central core, surrounded by a cleft of cells extending from the vulvular anlage almost to the level of the uterine bifurcation. The lumen of the uterus begins to appear by day 8-9.

In the fourth stage larvae, the female genitalia extend to the hindermost region of the worm. The vulvular region during this stage is constant, at or just behind the mid-point of the oesophagus. At

about 20 to 23 days, the vagina assumes the adult features with dilation of the atrium, and the appearance of muscle fibres and the larvejector apparatus. The vulvular aperture is round and leads into an atrium lined with hyaline epithelium. Behind this atrium bulb, a stricture forms a small terminal chamber. Later, as the ovijector gradually twists on its axis, the connection of the vagina uterina is shifted first to the ventral, then to the right lateral surface of the ovijector. This exaggerated growth, principally of the bulbular region, reduces the terminal chamber to an elongated bulb on its posteroventral or posterolateral side. The vulva and ovijector are cuticle-lined; the bulb contains epithelial cells which reduce its lumen (Schacher, 1962).

Mature ova can be observed in the uterus or seminal receptacle after about day 33. Intra-uterine cleavage of ova is first seen at about 38 days. By the 45th. day, 'tadpole' or more advanced embryos appear in the upper uterus. Fertile eggs in the lower uterus are covered by thin membraneous shells and the ova are surrounded by a clear perivitelline space. Infertile eggs are granular, lacking both the shells and the clear space around the ovum. Later, microfilariae are present in the upper uterine branches. The oviducts are thick walled, with narrow lumens and are convoluted. This posterior region acts as a seminal receptacle. The uterine branches are straight, sometimes slightly twisted due to torsion of the body. Bifurcation of the uterus occurs behind the oesophagus. The vagina vera is modified into a muscular, pyriform ovijector. The female B. pahangi continue to produce microfilariae for several years (Wilson and Ramachandran, 1971; Denham et al., 1972a).

Normal and irradiated worms recovered on day 7

(Illustrations on page 53)

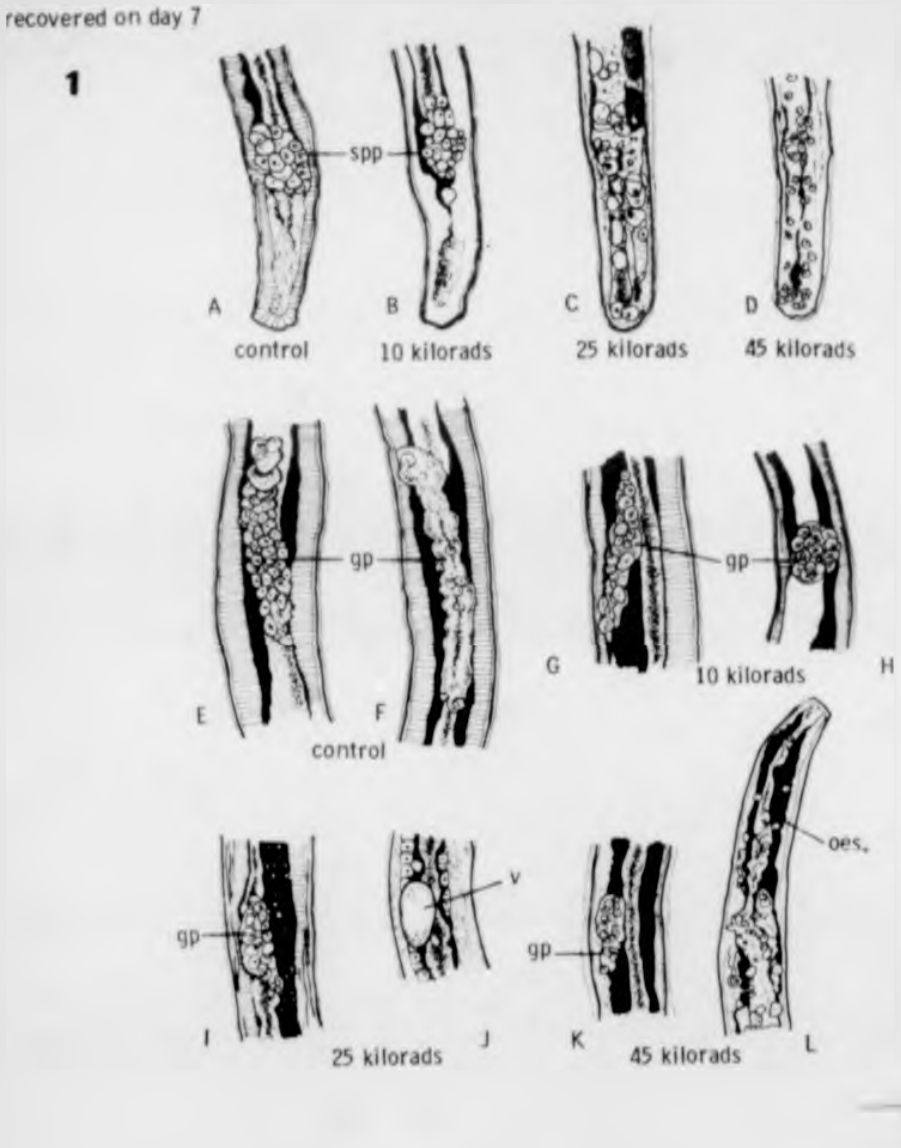
The tail region of normal male worms (1A) showed a clearly defined mass of cells, the spicule primordium. In larvae that had been irradiated with 10 krads. (1B) the spicule primordial cells were smaller. Total disorganisation of the primordial cells occurred in worms irradiated with 25 krads. (1C) and 45 krads. (1D). In larvae receiving 45 krads. the cells were arranged abnormally.

By day 7 post-infection, the genital primordia of normal female larvae had differentiated, the cells had grown posteriorly (1E and 1F) after attaching to the ventral wall of the epithelial layer of the worm. In parasites that had been irradiated with 10 krads. (1G and 1H) the genital primordia showed grades of disorganisation. Some developed normally, though not to so advanced a stage as in the non-irradiated worms. Others were in their early stage of organization, indicated by the primitive mass of cells. Larvae irradiated with 25 krads. (1I) and 45 krads. (1K) appeared not to have developed beyond very early third stages. Vacuoles appeared in some larvae (1J) and most of the larvae exposed to 45 krads. had a malformed oesophagus.

Normal and irradiated larvae recovered on day 14

(Illustrations on page 54)

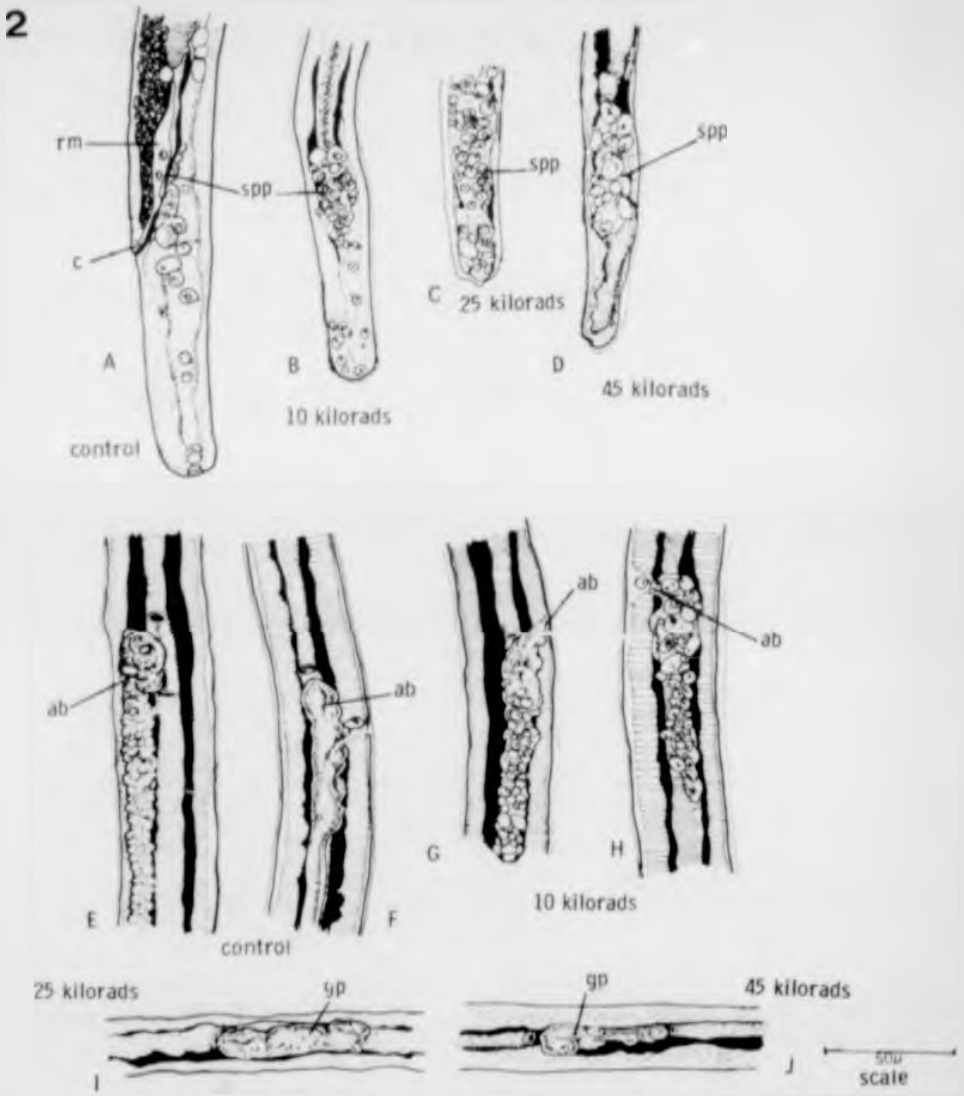
The spicules were well developed in the normal male worm (2A). The distal portion of the spicules advanced anteriorly and formed the cloaca, and the protractor muscles made their appearance. The region posterior to the spicule complex had an orderly array of cells. In larvae irradiated with 10 krads. (2B) the spicule primordium cells



1A-D posterior region of Brugia pahangi. A-B late third stage male worm showing spicule primordia (spp). - C-D undifferentiated tail region. E-F genital primordium of normal female worm (gp). G, H, I, K- show primordia, irradiated worms. J- larva with a vacuole (v). L- anterior region showing disorganised oesophagus (oes.)

EFFECT OF IRRADIATION ON THE REPRODUCTIVE SYSTEM OF BRUGIA PAHANGI

recovered on day 14



2 A-D posterior region of male worms. A - normal larva with spicule (spp), retractor muscles (rm) cloaca (c), B - larva with primordium of spicule. C-D male larvae with spicule primordia disorganised. E-F atrial bulb forming in normal worms. G-H atrial bulb (ab) primordia forming I-J larvae with genital primordium (gp).

were as in earlier, day 7 stages of normal worms, whilst those larvae irradiated with 25 krads. (2C) and 45 krads. (2D) showed total disarray of spicule primordial cells. The caudal papillae of infective larvae of B. pahangi were still present although the non-irradiated larvae had by this time moulted into 4th. stage worms.

The cells of the genital primordia of female worms had advanced into the mid-region of the body. The atrial bulb (2E) and the vaginal passage were clearly visible (2F). The reproductive parts of female worms irradiated with 10 krads. (2G and 2H) had not differentiated as in the normal worms, but the initial attachment of the primordia to the ventral wall had occurred. However, in larvae irradiated with 25 krads. (2I) and 45 krads. (2J), the structure of the primordia remained primitive, as in the early third stage larvae.

Normal and irradiated worms recovered on day 24

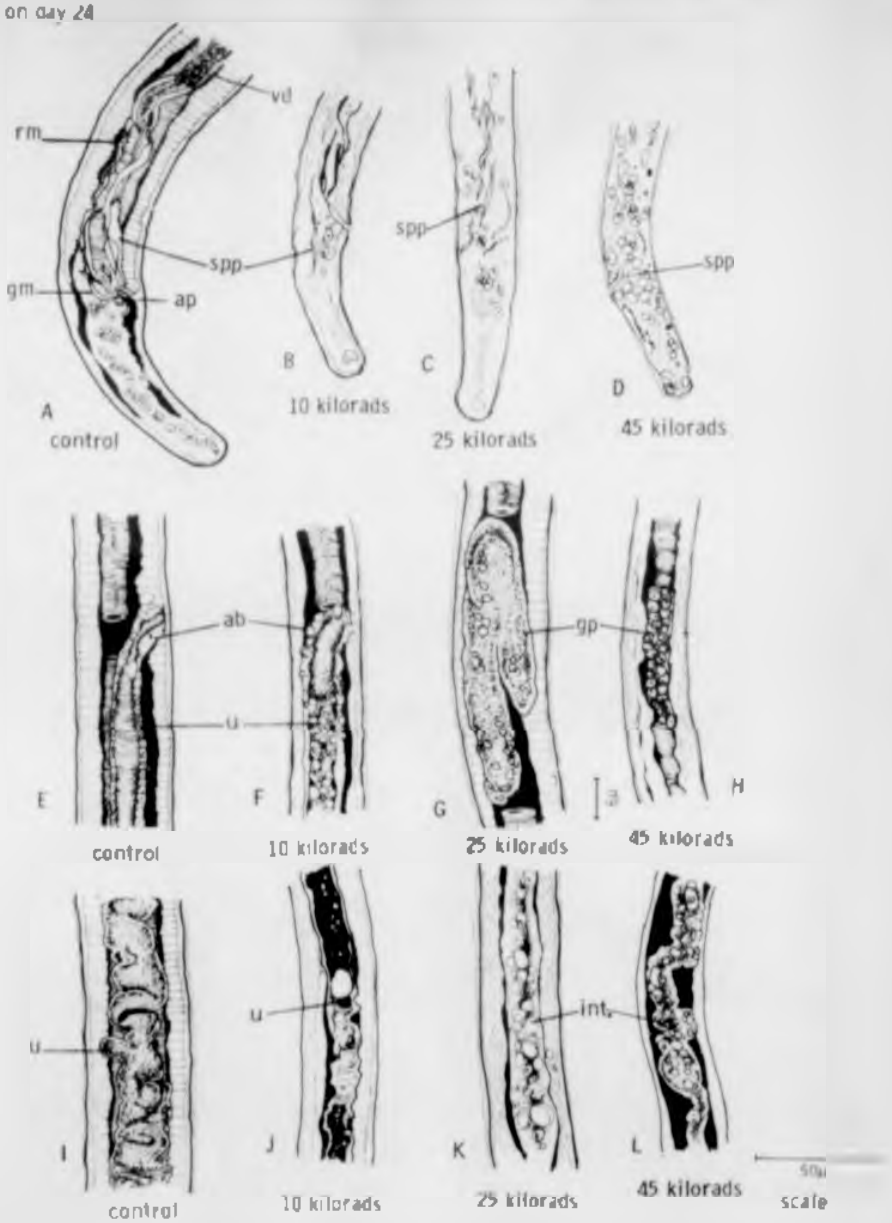
(Illustrations on page 56)

The basic pattern of the adult male spicule complex was clearly established by day 24 with the formation of spicules, vas deferens, protractor muscles, gubernaculum and cloaca (3A). In worms irradiated with 10 krads. (3B) and 25 krads. (3C), malformed spicule structures, totally disorganized, could be seen. Those larvae irradiated with 45 krads. only showed spicular thickenings (3D).

The early fourth stage female genitalia showed adult characteristics. The vaginal passage joined the uterus, which was lined with hyaline epithelial cells. The atrial bulb had formed (3E). Female worms irradiated with 10 krads. (3F) showed all the parts present in

recovery on day 28

3



3 A-D posterior region of male worms, A well developed normal larva with spicules (spp) vas deferens (vd), adanal papilla (ap), and sclerotized gubernaculum (gm). B D show degrees of spicule formation. F-L female worms E-F show atrial bulb (ab) and uterus (u), G-H with genital primordium (gp). I- show uterus convoluting J- with vacuolated uterus, (u), K-L show disorganized intestine (int.)

the normal larvae, but with irregularities in the epithelial cells of the vagina and the uterus. The genital primordia of those larvae irradiated with 25 krads. (3G) and 45 krads. (3H) retained the primitive structure of third stage larvae.

The uterus of the normal female worm had bifurcated and showed a convoluted appearance. The uteri of worms irradiated with 10 krads. were irregular, had large vacuoles and debris-like particles. The mid-regions of larvae that had been exposed to 25 krads. (3K) and 45 krads. (3L) had no uterus. Their intestines were irregular, degenerative and appeared to be non-functional.

Normal and irradiated worms recovered on day 36

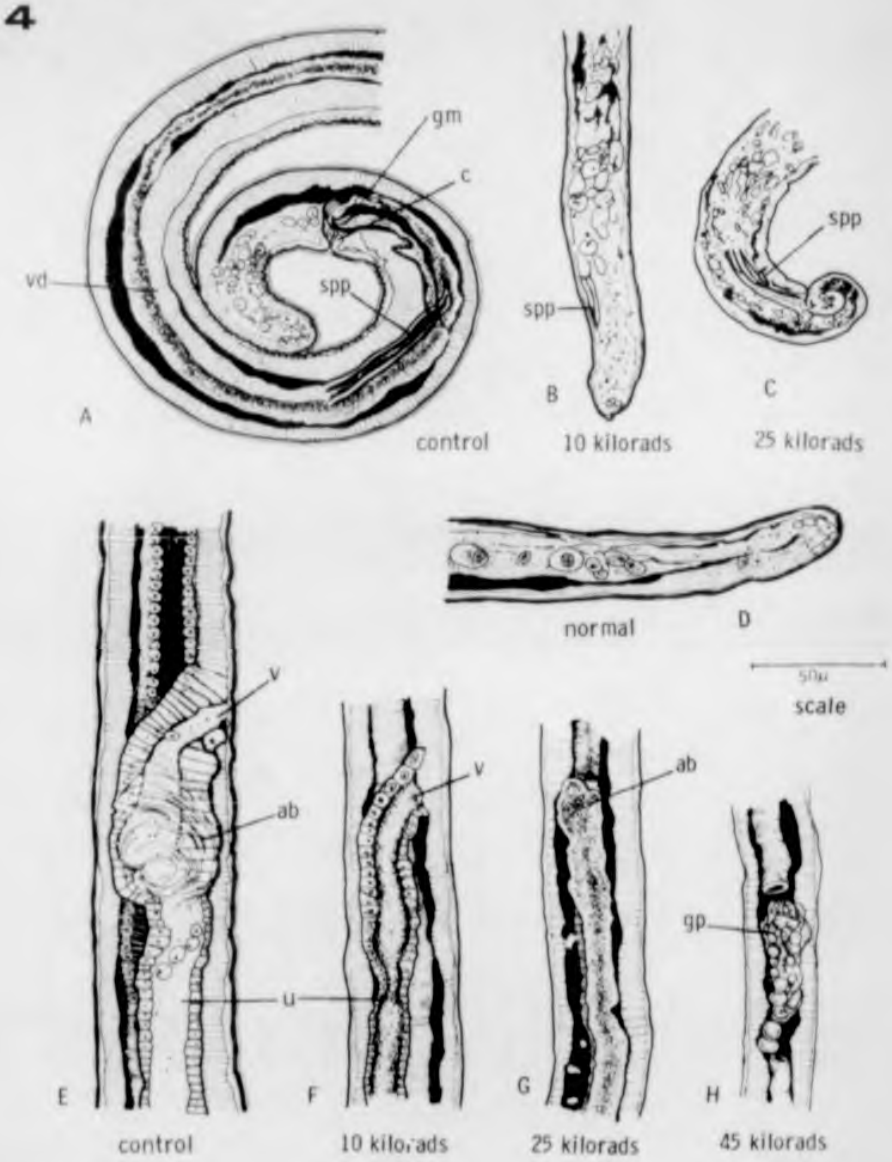
(Illustrations on page 58)

The normal, and non-irradiated male larvae (4A) had developed all the adult structures. Male worms that had been irradiated with 10 krads. (4B) showed spicule organisation but appeared to be non-functional. One male worm irradiated with 25 krads. (4C) at this time had traces of male spicules, but disproportionate growth had resulted in a small tight coiling of the tail. No distinguishable male worms were recovered after irradiation with 45 krads.

Figure 4D shows the tail region of normal female worms recovered on day 36. By day 36 normal female worms had fully developed reproductive organs. The vagina and uteri were lined by regular epithelial cells (4E). Fibrous muscles appeared in the region of the atrial bulb. Retarded growth was seen in the female worms exposed to irradiation levels of 10 krads. (4F). Though the primitive structures of the female genital organs were recognisable, cells were non-uniform in these worms. The vaginal passage was delimited in its

recovery on day 36

4



4 A-C tail region of male worms A- normal male with spicules, vas deferens, (vd), gubernaculum (gm), and cloaca (c). B-C show disorganized spicules (spp). D- posterior region of normal female worm. E-H female worms E-normal worm with atrial bulb (ab), vagina (v), uterus (u). F- slightly underdeveloped genitalia G-H early genital primordia (gp).

growth by the cuticle and epithelial linings of the worm. The female reproductive parts of worms irradiated with 25 krads. (4G) had only developed to the structure seen in the normal worms on day 14. Growth of male worms exposed to the highest irradiation dosage (4H) was arrested, as they still retained genital primordial characters similar to early 3rd. stage larvae.

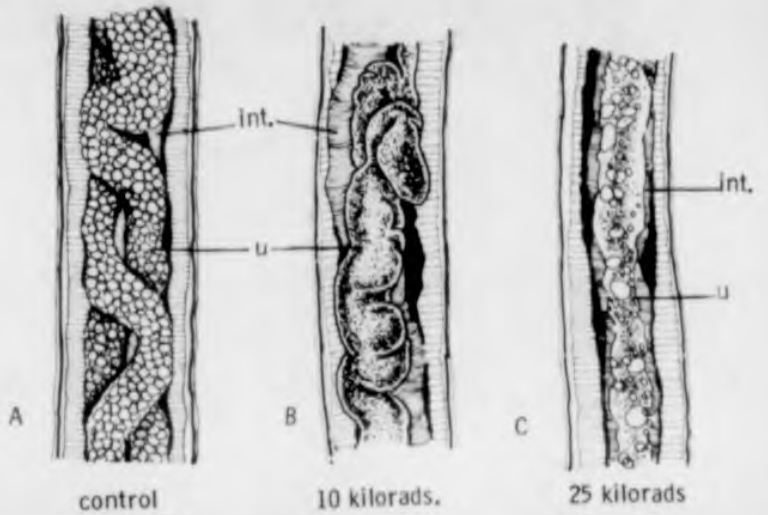
Mid-region of normal and irradiated worms recovered on days 36 and 77. (Illustrations on page 60)

The mid-region of normal female worms (5A) recovered 36 days after infection had intertwining, double uteri filled with unfertilized eggs. By day 77 their uteri were packed with embryonated eggs and microfilariae. The development of uteri in the worms irradiated with 10 krads. (5B) was inhibited. The convolutions of the uteri, due to bifurcation were seen. This stage was comparable to those larvae observed in normal 24 day old female worms. There were no normal eggs within these worms on day 77 (5E). Their vacuolated uteri contained deformed eggs and particles of 'debris'. Worms exposed to 25 krads. (5C) had distinguishable uteri, but these were irregular, warty and vacuolated. By day 77 (5F), the uterus was a mere bag of cavities and partitions. B. pahangi infective larvae previously irradiated with 45 krads. suffered the greatest damage. Their intestines were filled with irregular protuberances and thickenings.

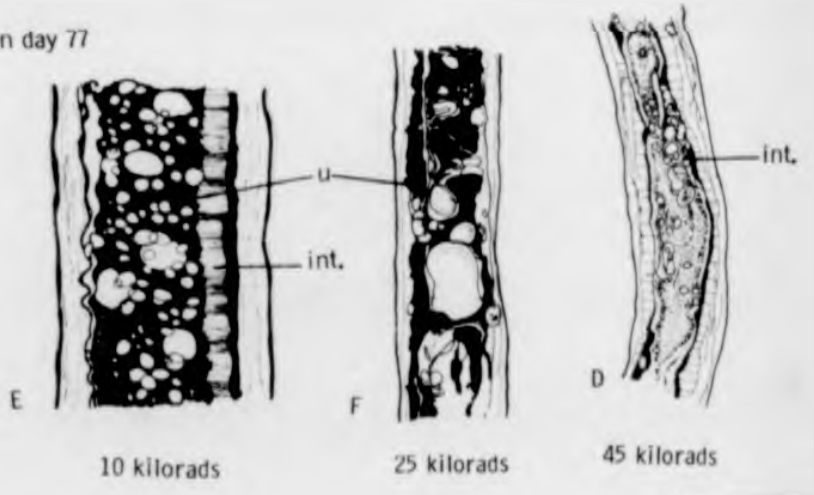
EFFECT OF IRRADIATION ON THE REPRODUCTIVE SYSTEM OF BRUGIA PAHANGI

recovery on day 36

5



recovered on day 77



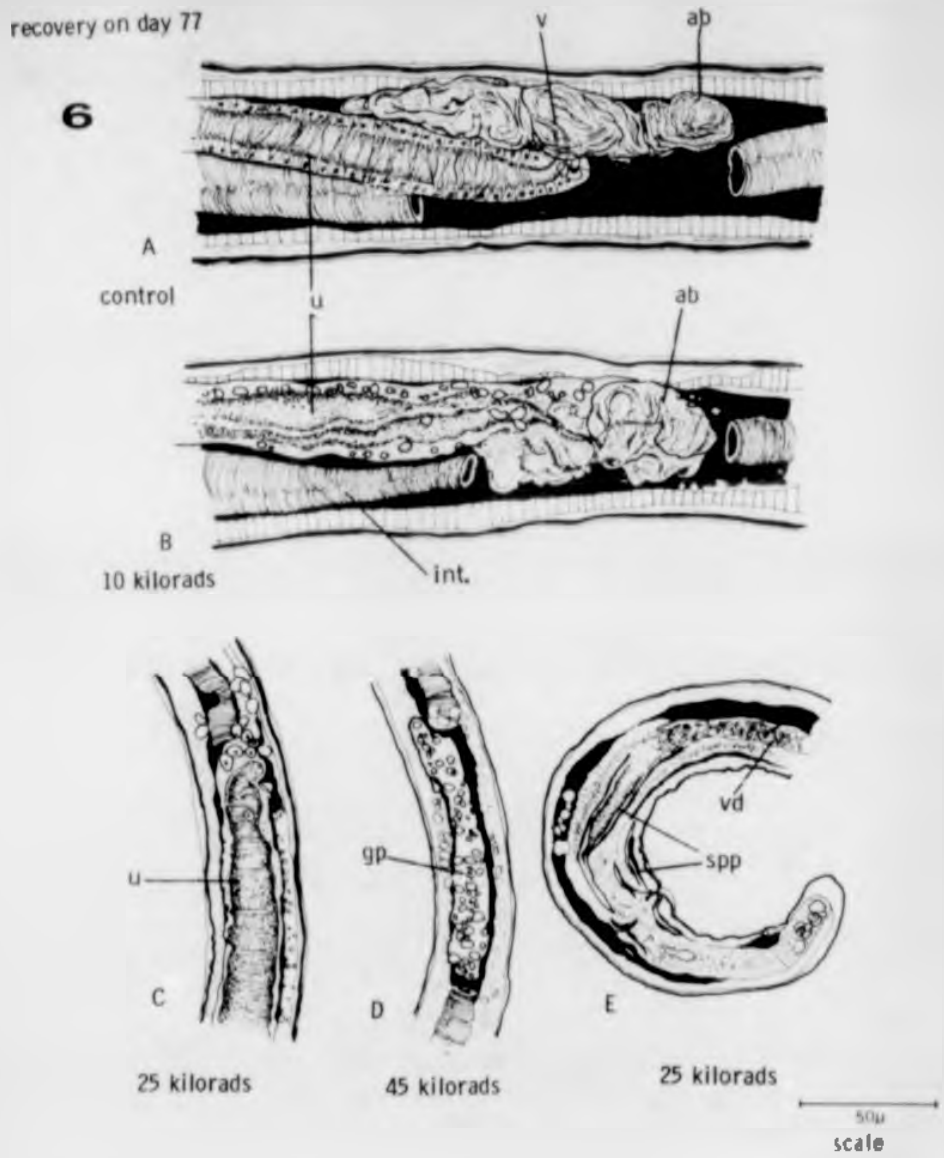
5 A-F uterus or mid-region of worms. A - normal female with double uterus (u). B-C show deformed uterus. D - with disorganised intestine (int.). E - uterus of 77 day old worm with undifferentiated eggs in uterus. F - uterus of 77 day old worm.

Normal and irradiated worms recovered on day 77

(Illustrations on page 62)

By day 77 the female worms (6A) were fully developed. The narrow passage of the vagina led to the exterior. The atrial bulb had grown and its torsion could be seen. The upper region of the uteri contained free microfilariae. Some of the worms irradiated with 10 krads. had reproductive systems where all the adult features were distinguishable (6B), but the atrial bulb and the fibrous muscles appeared to have collapsed. Cells lining the uteri were irregular, with no differentiated nuclei visible. Female worms subjected to irradiation with 25 krads. (6C) had miniature uteri. Anteriorly, a few disorganised cells showed the inhibited formation of the atrial bulb. The features recognisable compared with those of normal female worms recovered on day 24 (3E). The cuticular layer of these worms was very thick. Worms exposed to 45 krads. and recovered on day 77 (6D) had not developed beyond the third stage. The primitive genital primordia could still be seen in most of the worms.

One male worm (6E) recovered on day 77 had a partially developed spicule. However, there was no regular organization of cells as seen in a normal larva. Vacuoles were present in several regions of the tail of this larva.



6 A-C show female genitalia. A - normal worm with atrial bulb (ab), vagina (v), uterus (u). B - deformed genitalia. C - worm with primordium of atrial bulb (ab). D - with undifferentiated genital primordium (gp). E - tail region of male larva with spicule primordium (spp) and vas deferens (vd).

Discussion

It has long been established that gamma irradiation has adverse effects on the biological potentials of various organisms. The primary objective in irradiating parasites has been either to produce sexual sterility or to prevent development beyond certain stages so that non-pathogenic vaccines can be produced. Research with this objective has been so successfully conducted with D. viviparus (Jarrett et al., 1958a), D. filaria (Jovanovic et al., 1961) and A. caninum (Miller, 1964) that commercial vaccines were produced.

In the experiments reported above different levels of Co.60 irradiation were used to prevent infective larvae of D. pahangi developing into sexually mature adults, or to prevent them developing beyond early developmental stages.

100 krads. of Co.60 completely inhibited development of the infective larvae and reduced their motility within a few days. Irradiation with 45 and 75 krads. virtually prevented development and few of the recovered worms were active. The recovery of the irradiated parasites was generally lower.

The results indicated that infective larvae of D. pahangi exposed to 10 krads. failed to mature sexually. Their reproductive organs showed adult structures, but the damage observed and the absence of eggs showed that these worms were sterile. Development was terminated at the 4th. stage of the life-cycle, when the infective stages were exposed to 25 krads. They grew only to the length of a normal 4th. stage larva (about 24 days old). This view was confirmed on examining the reproductive parts, where the disorganised structures showed the basic pattern of juvenile adults.

Irradiation with 45 krads. completely prevented infective larvae developing beyond the third stage. The primitive organisation of the genital primordia remained unaltered in these worms. In some of these larvae, the caudal papillae of infective *B. pahangi* larvae were still present. Wong *et al.* (1974) found that irradiated *D. immitis* died before moulting to the adult stages.

Irradiation also altered the pattern of migration of the worms. They behaved as third and very early fourth stage worms, as they did not migrate out of the perinodal lymphatic sinus to the afferent lymphatic.

The general effect of irradiation was to stretch the duration of each stage in the life-cycle of the parasites. With the higher levels used, this prolongation was so great that the later stages were not represented. It is possible that these larvae that behave as third stage worms could be immunogenic due to their extended, strategic location, close to the lymph nodes. Miller (1963) showed that *A. caninum* larvae irradiated so that they could not migrate past the lung stages were much more immunogenic than the equivalent number of normal worms.

It has been found by many workers that female parasites can survive higher dosage levels than can male worms. This phenomenon of resistance in the female has also been found in higher animals (Bacq and Alexander, 1961). A product similar to oestrogen in females of higher animals may also be found in lower organisms making them more resistant to irradiation. A dose level of 10 krads. killed male worms more quickly than females, whilst dosages of 25 and 45 krads. were completely lethal to the male worms. Gordon *et al.* (1960) found that male *Trichostrongylus colubriformis* were similarly susceptible to irradiation. Their note that absence of mature females

with eggs may be due to the absence of male worms, must be borne in mind. Resistant females were obtained by Miller (1963) in A.caninum where the ratio of male and female worms changed from 1:2 in non-irradiated infections to 1:34 in infections exposed to 60 krads. Jovanovic et al. (1961) also found that female D. filaria were more resistant to irradiation than were males.

Chapter 4

RESISTANCE OF CATS REPEATEDLY IMMUNIZED WITH IRRADIATED AND NON-IRRADIATED D. PALANGI

Introduction

Review of the development of radiation-attenuated vaccines against helminthic infections

Pasteur first used an attenuated, live vaccine against fowl cholera, Pasteurella multicoda, in 1891. In attenuation, physical (irradiation by x-ray, gamma ray, heat, etc.), biological (repeated inoculation, successive culture), and chemical methods are used to reduce the pathogenicity of organisms without loss of immunogenicity. Indeed, in some diseases that induce poor immunity, as in many helminthiases, attenuation by irradiation has actually increased the immunogenicity of the parasite.

In the field of parasitology, greatest success has been achieved by veterinarians, with the successful production of irradiated vaccines against bovine and ovine parasitic bronchitis, and canine hookworm infection. Despite extensive research on human parasitic diseases, no major breakthrough has yet been made.

Resistance against T. spiralis in mice has been demonstrated by many workers using irradiated larvae (Levin and Evans, 1942; Alicata, 1951; Gould, 1955; and Evans, 1970), but as there was no viable commercial market, a vaccine did not emerge from such studies. Jarrett et al. (1950a)were the first to produce a commercial vaccine against D. viviparus infections in cattle. This product was initially

tested on two commercial farms and in both instances the irradiated inoculum gave the animals 90% protection against challenge (Jarrett et al., 1958a, 1958b). The commercial vaccine in use now, consists of 2 doses of 1,000 infective larvae irradiated with 40 krads of Co.60, given at one month intervals. This vaccine has proved to be an outstanding success (Soulsby, 1972).

This breakthrough was quickly followed by a vaccine against D. filaria infections in sheep (Sokolic et al., 1963). This product was as successful and effective as that against H. vivipamus. This vaccine gave very good protection when animals were challenged 15 days after a schedule of double immunization (Sokolic et al., 1961) but if only a single dose of vaccine were used, only 50% of the sheep resisted challenge. Sokolic et al. (1963) also tested the efficacy of the vaccine on existing infections of D. filaria and found that the number of infected animals dropped by 60%.

Jarrett et al. (1960) gave two doses of an irradiated vaccine of Trichostrongylus colubriformis larvae to sheep and these animals showed a high degree of resistance to challenges with normal T. colubriformis worms. Mulligan et al. (1961) was not able to obtain similar success and suggested that the failure in their case may have been been due to individual variation of the parasites.

The most recent success in parasitic vaccine research was against canine hookworm infections (Miller, 1973). This product consisted of irradiated A. caninum infective larvae and was most effective when two vaccine doses were administered orally to 2 month old puppies. As in many helminth infections, the immunity developed was not absolute. The hookworm vaccine, also induced resistance against interspecific and intergeneric infections of Ancylostoma braziliensis and Uncinaria stenocephala. Dogs that were given irradiated U. stenocephala

resisted further challenges with that parasite (Dow et al., 1958).

Milligan et al. (1961) immunized sheep with irradiated Haemonchus contortus larvae and demonstrated resistance against challenges with the normal parasite. Such encouraging results were obtained only when the animals were 6-9 months old. Ross et al.(1962) infected sheep with H. contortus irradiated with 45 krads. and obtained 60% reduction in worm burdens of the immunized animals. They were of the opinion that the irradiation dose used by Milligan et al. (1961) may have been too high.

Villella et al. (1961) immunized mice with Schistosoma mansoni irradiated with 3 krads. of x-ray and produced immune animals. However, when they increased the dose level to 7 krads. no immunity resulted. More encouraging results were obtained by Hsu et al. (1962) who demonstrated [illegible] rhesus monkeys [illegible] inoculated with irradiated cercariae of a non-human strain of Schistosoma japonicum. they were protected against subsequent challenges with the human strain. Similar experiments conducted with albino mice (1965) and chimpanzees (1970) as hosts did not yield the same results. Radke and Sadun (1961) infected mice with irradiated cercariae of S. mansoni and the resulting immunity enabled the mice to resist a massive challenge with normal cercariae, which would usually have killed them. Sheep immunized with Schistosoma mattheei irradiated with 6 krads. of Co.60 gave the animals 75% protection against subsequent challenges with S. mattheei (Taylor, 1975). Varga (1968) vaccinated chickens with attenuated Syngamus trachea larvae and demonstrated protection ranging from 80%-100% in these animals against further challenges with the normal parasite.

Irradiation experiments in filariasis

Research on Immunoprophylaxis of Bancroftian and Brugian filariasis is difficult due to the absence of strong demonstrable immunity. However, the fact that dogs vaccinated with irradiated larvae produce stronger immunity against A. caninum than dogs infected with normal larvae leads one to hope that irradiated filarial parasites may produce stronger immunity than seen in normal infections.

Fredericks and Ramachandran (1969) in their exploratory experiments inoculated monkeys with x-irradiated B. malayi larvae. Encouraging results were obtained. Wong et al. (1969) continued these experiments by immunizing monkeys and cats with infective larvae of B. malayi irradiated with 10-40 krads. The monkeys inoculated with larvae irradiated with 10 krads. were not protected. However, of the 7 monkeys inoculated with larvae exposed to 20 krads., 5 resisted challenge and did not become microfilaraemic. Wong et al. (1969) also challenged 3 monkeys, 12 months after the scheduled immunization with irradiated parasites and of these two were resistant. However, 79% of cats immunized in a similar fashion remained unprotected, and became microfilaraemic due to the challenge worms reaching sexual maturity (Ramachandran, 1970). It was suggested that cats may not mount as good a defence as rhesus monkeys against B. malayi.

Ah et al. (1972) infected dogs with irradiated D. immitis and found that these animals had reduced challenge infections, and suppressed microfilaraemia completely. Wong et al. (1974) reported on the preliminary investigations on producing a vaccine against D. immitis infections. Best protection was obtained when the

challenge was given 3 months after vaccination. Dogs inoculated with irradiated B. pahangi resisted 57% of the challenge worms (Ah et al., 1974a).

Preparation of Irradiated Vaccines

The following points must be considered when attempting to produce a live, attenuated vaccine.

The choice of the method of irradiation often depends on the accessibility of the source, but when using gamma rays, the only variable factor is time (Mulligan, 1975). The rate of the delivery is not critical, and various workers have stressed the fact that this did not alter the effect produced by the total dose.

It is necessary to have sterile conditions when culturing and irradiating parasites. Impurities in the medium can prevent thorough irradiation (Mulligan, 1963). When culturing parasites in great numbers, the medium must be free of viruses, bacteria, mycoplasma, etc. (Miller, 1975). Mulligan (1975) stressed that when irradiating parasites, the oxygen content (oxygen effect) and the temperature of the suspending liquid must be carefully monitored. Mass production of infective material is facilitated if no intermediate hosts are involved (Miller, 1975).

It must be borne in mind that all actively moving larvae may not be invasive (Poynter and Terry, 1963). Thus after irradiation the invasiveness of the infective worms must be tested. Miller (1975) indicated that the invasiveness of the bulk of the attenuated larvae may be drastically affected without any apparent change in their viability or motility.

Parasites irradiated with the optimal dose should produce little or none of the pathology caused by the non-irradiated infections (Jennings, 1963). This was indicated clearly in irradiated and non-irradiated infections with D. viviparus (Jarrett et al., 1958b). Immunization with under-irradiated parasites results in the inoculum causing pathology, whereas an over-irradiated vaccine may not be immunogenic. Parasite development should be altered so that they go through at least one moult phase, thus providing the host with functional antigens to stimulate immunity (Soulsby, 1961). It would also be advantageous if irradiation were to arrest the development of parasites at their most immunogenic stage. Sometimes, the final location of attenuated parasites differs from that of non-irradiated parasites. This also can be beneficial to the host, if better immunity is elicited. Stoll (1961) suggested that if non-migratory parasites were introduced in abnormal sites, the metabolic products of these worms could be recognised by the host as foreign materials.

Procedures for mass production of a vaccine must be standardised. This is important because the biotic potencies of parasites can vary, resulting in unequal potency of different batches of a vaccine (Miller, 1975).

Perhaps, the most important factor of all is the biotic viability, or shelf life, of the product. The vaccine should also be easily available (Prochazka and Twanek, 1968). Infective larvae are non-feeders and thus optimal storage temperature should be calibrated, to prevent loss of metabolic energy of the parasites (Prochazka and Twanek, 1968). The product can be universally useful only if it does not require highly sophisticated storage methods. Also, the methods

of administering the vaccine by personnel in endemic areas must be direct and simple.

The vaccine should, preferably, give protection to young vaccinates, before exposure to natural infections. There is often a latent period between administering the vaccine and the ability of the vaccinate to successfully resist challenges (Wong et al., 1969; Miller, 1975). Thus the vaccine must be administered when the host is immunologically mature.

In the work reported in Chapter 3, it was found that B. pahangi infective larvae irradiated with 10 krads. and inoculated into cats developed into juvenile adults; those irradiated with 25 krads. developed into late fourth stage larvae; and those irradiated with 45 krads. did not develop beyond the third stage. In the experiments reported below, the aim was to repeatedly immunize cats with:-

a) infective larvae irradiated with 10 krads.

b) infective larvae irradiated with 25 krads.

and test the resistance of these animals against challenges with B. malayi.

Materials and Methods

The methods of harvesting infective larvae from infected mosquitoes, counting them, and inoculating cats are described in Chapter 2, and the method of irradiating the parasites is detailed in Chapter 3.

The normal procedure adopted for challenging immunized animals unless stated, was as follows. The immunized animals were challenged on 3 occasions. The first challenge (to be recovered 28 days from

the time of challenge) was inoculated into the Lhl, Rhl and Lfl; the second challenge (to be recovered 14 days after challenge) into the Lhl, Rhl and Rfl; and the final challenge was inoculated into each leg one day before autopsy. The challenge schedule can be summarised thus:-

Challenge no.	Lhl	Rhl	Lfl	Rfl	Day prior to autopsy
1	x	x	x	-	28
2	x	x	-	x	14
3	x	x	x	x	1

This challenge schedule was followed as it was possible to differentiate each batch of challenge worms based on their size differences. It has been used in all challenge schedules of cats in the Filariasis Unit of the Department of Medical Helminthology, London School of Hygiene and Tropical Medicine and this work provides a useful baseline.

Whenever an immunised animal was challenged, an uninfected cat was similarly challenged using larvae from the same batch. In this way, the number of challenge worms retrieved from immunized animals during autopsy could be compared with the challenge control.

The cats were autopsied as described in Chapter 2.

Worms retrieved from immunised and challenge control animals were sexed and transferred into 70% alcohol mixed with 10% glycerine in equal volumes. The percentage recovery of worms from each limb was then calculated. The degree of resistance in the "immunised" animals was calculated using the following formula:-

$\frac{x-y}{x}$ where

x is the percentage of worms recovered from limbs of control animal

and y is the percentage of worms recovered from limbs of the immunised animal. Resistance of immunised cats was calculated for each limb separately, using the percentage recovery from the corresponding limb of the corresponding control cat in the formula. Resistance shown by the immunized animals to each stage of the challenge parasite was also worked out, to determine whether the immunity developed was complete, or was localised at the site of immunization.

The experimental cats were immunised in three legs; the Rhl being left unimmunised.

[illegible]

In this experiment 11 cats were inoculated repeatedly with [illegible] irradiated with 70 krads, as described in Chapter 3. The immunised cats were challenged with normal parasites along with a control animal, and left until past the prepatent period. The second and third challenges were inoculated after this period as described before, 14 and 1 days before autopsy. This was to determine if the parasites used to challenge the immunised animals developed to maturity and produced microfilariae. When this occurred, 20-30 mosquitoes were fed on these animals, dissected 11 days later to see if the microfilariae developed into infective larvae. In two such cases, the infective larvae were inoculated into 2 jirds, the animals killed 50 days later, and any adult worms retrieved. Full details of recoveries of larvae from each leg of the immunised and control cats are included in the Appendix. Overall resistance of cats is shown in Table 5; resistance against the adult stage in Table 6; resistance against the fourth larval stage in Table 7; and resistance

TABLE 5. TOTAL WORM RECOVERIES IN CATS IMMUNIZED WITH B. PAHANGI IRRADIATED WITH 10 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No. of larvae in immunization	No. of immun. infections	No. of larvae in challenge		% Recovery		% Protection	time from last immunization to challenge
			exptl.	control	exptl.	control		
A53	2328	10	888	1064	6.54	23.2	71.9	14
A60	2356	10	641	641	9.81	26.84	63.5	14
A42	2465	10	686	686	0.65	42.5	98.5	20
A50	2516	10	630	621	4.38	44.18	90.1	20
A44	2348	10	538	532	12.28	31.0	60.3	27
A43	2494	10	539	539	8.98	32.42	72.3	27
						mean protection	78.6	

TABLE 6. TOTAL ADULT WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 10 KRADS. AND CHALLENGED NORMAL LARVAE.

cat No.	No. of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
A53	294	287	1.67	19.87	91.0
A60	296	294	12.67	24.8	48.9
A42	425	435	0.3	36.0	99.2
A50	394	384	1.77	29.33	94.0
A43	193	196	11.16	30.3	63.2
A44	196	189	3.37	24.5	84.8

TABLE 7. TOTAL FOURTH STAGE WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 10 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No. of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
A53	199	194	5.70	28.2	79.8
A60	147	147	15.37	30.23	49.2
A43	192	194	12.7	29.1	56.4
A44	199	194	4.87	29.1	83.3

TABLE 8. TOTAL THIRD STAGE WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 10 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No. of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
A53	395	391	5.63	27.1	79.2
A60	198	200	3.5	26.0	86.5
A42	187	182	1.0	48.0	97.9
A50	188	194	6.35	59.7	89.0
A43	199	197	4.55	36.5	87.5
A44	194	197	24.25	33.75	28.2

against the third larval stage in Table 8.

Cats A57 and A58 were not challenged and were autopsied after the third and fifth immunizing doses, respectively. Lymphatics of these animals were fixed for histological examination. Another cat A55 was autopsied after the scheduled 10 immunizing doses. Only 7 worms of the total of 741 inoculated into the Lhl and 2 worms of the total of 744 inoculated into the Lfl were recovered. The lymphatics of the Rfl and unimmunized Rhl were fixed for histological examination. The immunized cats and their controls were given the first challenge 14-27 days after the last immunizing infection. The mean resistance in these cats was 78.6% with a range from 60.3 to 98.5%. There was no significant difference in the recovery of worms from the immunized and non-immunized limbs (see Table 9).

I shall now consider the detailed results obtained in each of the vaccinated cats.

A42 (female)

The vaccinated animal had not become patent by the time of autopsy when 10 ml. of blood was filtered through a Nuclepore membrane, and no microfilariae were detected. The challenge control became patent 56 days after the challenge and at the time of autopsy had 31 microfilariae in 10 mm^3 of blood. The autopsy was performed 151 days after the first challenge. The overall protection of this animal against challenge was 98.5%. Resistance against the adult stage was 99.2%, and against the third larval stage was 97.9%. The two adult worms recovered from the experimental animal did not have mature eggs

TABLE 9. COMPARISONS OF RECOVERIES FROM IMMUNIZED AND NON-IMMUNIZED LIMBS OF CATS INOCULATED WITH B.PAHANGI IRRADIATED WITH 10 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	Difference of adult worm recovery (Rhl-Lhl)	Difference of fourth stage worm recovery (Rhl-Lhl)	Difference of third stage worm recovey (Rhl-Lhl)
A42	0.0	-	0.0
A43	17.3	-11.6	0.2
A44	1.0	-2.4	11.0
A50	-0.1	-	-10.3
A53	-3.0	-6.1	1.1
A60	0.4	-26.0	6.0
	NS	NS	NS

or microfilariae in their uteri.

A43 (male)

The control animal became patent 75 days after infection and the vaccinated cat became patent 14 days later.

Mosquitoes fed on A43 contained infective larvae 11 days later. 23 infective larvae were inoculated into a jird, and 8 adult worms were recovered 50 days later. At the time of autopsy, the experimental cat had a microfilarial count of 24 in 10 mm^3 of blood. The overall resistance against challenge infection was 72.3%. Resistance against the adult stage was 63.2%| against the fourth larval stage was 56.4% and against the third larval stage was 87.5%. Adult worms recovered from A43 were normal and the female worms had microfilariae in the uteri.

A44 (female)

This cat became patent 108 days after the first challenge, but the challenge control became patent 59 days after first challenge inoculation. At the time of autopsy, the experimental cat had a microfilarial count of 6 in 20 mm^3 of blood whilst the control animal had 92 microfilariae in 20 mm^3 of blood. The overall resistance against challenge infections was 60.3%. The highest resistance was against the adult stage (84.8%) and the resistance against the fourth larval stage was 83.3% whereas the resistance against the third larval stage was poor (28.2%). The adult worms recovered from the experimental cat were normal and the females had microfilariae in the uteri.

A50 (male)

At the time of autopsy this cat had received 2 long term challenges and a 1 day challenge with normal B. pahangi.

The experimental cat became patent 94 days after the first challenge, and the control animal became patent 73 days after initial infection. At the time of autopsy the experimental cat had a microfilarial count of 2.5 in 10 mm^3 of blood, whilst the control animal had 88 microfilariae in 10 mm^3 of blood. Resistance against all stages was 90.1%, against the adult stage was 94.0%, and against the third larval stage 89.0%. The female worms recovered from the experimental animal were normal and microfilariae were detected in their uteri.

A53 (female)

The challenge control animal became patent 66 days after infection. Microfilariae could not be detected in the blood of the experimental animal throughout the period of observation. At the time of autopsy, however, microfilariae were found when 10 ml. of blood was run through a Nuclepore filter. The overall resistance mounted by this animal against challenge infection was 71.9%. Resistance against the adult stage was 91.0%; against the fourth larval stage was 79.8% and resistance against the third larval stage was 79.2%. The adult worms recovered from the experimental animal were normal.

A60 (female)

The challenge control became patent 87 days after infection and at the time of autopsy the microfilarial count was 9 in 10 mm^3 of blood. Microfilariae could not be detected in the blood of the experimental animal throughout the period of observation; at the time of autopsy microfilariae were found using the Nuclepore filter. The overall resistance of this animal to challenge was 63.5%. Resistance mounted by this animal against the adult stage was 48.9%, against the fourth larval stage was 49.2% and against the third larval stage was 86.5%. Adult worms recovered were all normal.

A56 (female)

This animal was challenged with infective larvae of B. pairi. Overall resistance and resistance against the different stages is shown in Fig. 10. Details of recovery from each limb are included in the Appendix.

The experimental animal became patent 104 days after the first challenge infection. The challenge control (004) became patent 114 days after infection. At the time of autopsy the microfilarial count in the experimental cat was 19 in 10 mm^3 of blood, and the control animal had 9 microfilariae in 10 mm^3 of blood. The overall resistance of this cat to heterologous challenge was 78.6%. Resistance to the adult stage of B. patei was 51.1%; to the fourth larval stage was 63.4% and to the third larval stage was 97.3%.

Resistance against challenges in the immunised leg was higher than in the non-immunised leg. This was found to be true for both the adult stage and the fourth larval stage. Resistance against the

TABLE 10. TOTAL WORM RECOVERIES IN CATS IMMUNIZED 10 TIMES WITH IRRADIATED B.PAHANGI (10 KRADS.) AND CHALLENGED WITH NORMAL B.PATEI.

cat No.	No.of larvae in immunization	No.of larvae in challenge exptl.	control	% Recovery exptl	control	% Protection
A56	2220	935	908	4.8	22.43	78.6
A59	2356	946	903	10.72	30.86	65.3

TOTAL ADULT WORM RECOVERIES IN CATS IMMUNIZED WITH IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL B.PATEI.

cat No.	No.of larvae in challenge exptl.	control	% Recovery exptl.	control	% Protection
A56	287	291	5.67	11.59	51.1
A59	296	291	4.07	8.25	50.7

TOTAL FOURTH STAGE WORM RECOVERIES IN CATS IMMUNIZED WITH IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL B.PATEI.

cat No	No.of larvae in challenge exptl.	control	% Recovery exptl.	control	% Protection
A56	249	223	9.33	25.5	63.4
A59	250	219	14.7	34.2	57.1

TOTAL THIRD STAGE WORM RECOVERIES IN CATS IMMUNIZED WITH IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL B.PATEI.

cat No	No. of larvae in challenge exptl.	control	% Recovery exptl.	control	% Protection
A56	399	394	0.75	28.25	97.3
A59	400	393	12.75	45.38	71.9

third larval stage did not differ greatly in the immunized and non-immunized legs.

The adult B. patei worms recovered from the experimental animal were normal.

A59 (male)

This animal was also given a heterologous challenge with B. patei. Overall resistance and resistance against the different stages is shown in Fig. 10. Details of recovery from each limb were included in the Appendix.

The control animal used as challenge died 30 days after the first challenge and was autopsied. The recoveries from the [illegible] limbs from this cat were Lhl - 34%, Rhl - 32%, and Lfl - 28%. Cat 004 was inoculated at the same time with the same batch of larvae, and this animal was used as challenge control for the first infection. Another kitten, 017, was used for the second and final challenges.

The experimental cat became patent 100 days after initial challenge with B. patei (cf. 004, control for cat A56). At the time of autopsy the microfilarial count of the experimental cat was 3 in 10 mm^3 of blood. The overall resistance mounted by A59 against challenges was 65.3%. Resistance against the adult stage was 50.7%; against the fourth larval stage was 57.1% and against the third larval stage was 71.9%. The non-immunized leg of the experimental animal was significantly more susceptible to infection with B. patei than the immunized leg. Adult B. patei worms recovered from cat A59 were normal.

Experiment 6

In this experiment 7 cats were immunized with 5 or 6 lots of infective larvae of *D. pahangi* which had been irradiated with 25 krads. Full details of recoveries from each leg of immunized and control cats are included in the Appendix. Tables 11, 12, 13 and 14 summarise details of infections and recoveries of different stages of the challenge worms. None of the experimental cats became patent during the immunization schedule, confirming that the parasites did not mature sexually. One cat (M72) was autopsied after the second "immunizing" infection. Worm recoveries were Lhl - 24%, Lfl - 27% and Rfl - 31%. Another cat (M76) was autopsied after its sixth immunizing infection. The lymphatics from the Lhl were fixed for histological examination. No worms were found in the Rfl and only 8 very small worms were found in the Lfl. The immunized cats and their individual controls were given their first challenge infection 12 - 79 days after the last immunizing infection. The mean resistance of these cats was 79.2%. The range of resistance was from 61 to 93%. There was no consistent difference associated with the time between the end of immunization and the time of challenge.

M77 (male)

This animal was immunized with 2,031 irradiated infective larvae and challenged 12 days after the last immunization dose. The total resistance mounted by this animal against all stages was 61.0%; the resistance against the adult stage was 85.7%, against the fourth stage 59.2%, and against the third stage 64.6%.

TABLE 11. TOTAL WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No. of larvae in immunization	No. of immun. infections	No. of larvae in challenge		% Recovery		% Protection	day from last immunization to challenge
			exptl.	control	exptl.	control		
M77	2031	6	847	846	10.56	27.3	61.0	12
M74	1426	5	792	786	1.70	26.4	93.0	17
M73	2003	6	298	292	10.97	35.36	69.0	35
M75	1990	6	146	150	3.17	18.33	8.27	63
M86	1459	5	998	988	3.0	28.75	90.0	79

mean protection 79.2

TABLE 12. TOTAL ADULT WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No. of larvae in challenge		%Recovery		% Protection
	exptl.	control	exptl.	control	
M77	149	149	2.20	15.33	85.7
M74	299	298	0.67	28.67	97.7
M73	298	292	10.97	35.36	69.0
M86	498	497	3.0	23.75	88.5

TABLE 13. TOTAL FOURTH STAGE WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No. of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
M77	298	298	12.5	30.6	59.2
M74	297	298	3.67	30.7	88.0

TABLE 14. TOTAL THIRD STAGE WORM RECOVERIES IN CATS IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL WORMS.

cat No.	No. of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
M77	400	399	10.63	29.0	64.6
M74	196	196	1.0	21.5	95.3
M75	146	150	3.17	18.33	82.7
M86	400	393	3.0	33.75	91.1

M74 (male)

This animal was immunized with 1,462 irradiated infective larvae. Immunization with irradiated larvae conferred a protection of 93.0% against all the challenge worms. The resistance against the three different stages was high; against adults it was 97.7%, against fourth stage larvae it was 88.0% and against third stage larvae it was 95.3%. The challenge worms retrieved from experimental cat were normal.

M73 (female)

This cat was challenged 35 days after the last immunization. The animal and the control were autopsied 28 days later, after a single challenge. Resistance mounted by this experimental cat against the challenge was 69.0%. Adult worms recovered were normal.

M75 (male)

This cat was challenged 75 days after immunization. After challenge, the animal was killed one day later. The total resistance in this animal against this challenge was 82.7%.

M86 (female)

This cat was immunized with a total of 1,459 irradiated N. americanus and challenged 79 days later. The experimental cat and its control were autopsied 108 days after the first challenge; the second challenge was given 54 days before autopsy and the final challenge 1 day before autopsy. This was to determine if the

parasites used as challenge dose grew to maturity.

The challenge control became patent 68 days after initial infection (2 microfilariae in 100 mm^3 of blood), and the count at the time of autopsy was 21 microfilariae in 20 mm^3 of blood. Microfilariae could not be detected in circulation in the experimental animal. During autopsy, however, microfilariae were found when 10 ml. of blood was run through a Nuclepore filter. The overall resistance mounted by this animal against challenges was 90%; resistance against the adult stage was 88.5% and against the third stage 91.1%. Adult worms recovered from the experimental animal were normal and the females had microfilariae in their uteri.

Experiment 7

In this experiment 4 cats were infected with normal (non-irradiated) infective larvae as described in Chapter 2. Full details of recoveries from the immunized and control cats are included in the Appendix. Overall resistance of cats against challenges is shown in Table 15 and resistance against the different stages is shown in Table 16.

One cat, M89, was autopsied after the 5 "immunizing" doses. Worm recoveries were Lfl - 0.12% and Rfl - 0.09%. The lymphatics of the Lhl was fixed for histological examination. The microfilarial pattern in this cat is shown on Fig.20 (Chapter 6).

TABLE 15. TOTAL WORM RECOVERIES IN CATS INOCUALTED WITH B.PAHANGI AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No.of larvae in inoculation	No.of inoculation	No.of larvae in challenge		% Recovery		% Protection	day from last infection to challenge
			exptl.	control	exptl.	control		
M87	2039	6	981	978	3.60	31.5	88.6	86
M79	2047	6	981	980	16.7	36.6	54.2	96
M85	2029	6	639	641	0	26.8	100	515

TABLE 16. TOTAL ADULT WORM RECOVERIES IN CATS INOCULATED WITH NON.IRRADIATED B.PAHANGI AND CHALLENGED WITH B.PAHANGI.

cat No.	No.of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
M87	299	298	3.80	15.0	74.7
M79	292	292	16.6	22.73	27.5
M85	294	294	0	24.8	100

TOTAL FOURTH STAGE WORM RECOVERIES IN CATS INOCULATED WITH NON-IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No.of larvae in challenge		% Recovery		% Protection
	exptl.	control	exptl.	control	
M87	287	281	2.87	48.0	94.0
M79	296	289	14.9	26.6	44.0
M85	148	147	0	30.2	100

TOTAL THIRD STAGE WORM RECOVERIES IN CATS INOCULATED WITH NON-IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL LARVAE.

cat No.	No.of larvae in challenge		% Recovery		% Protection
	exptl	control	exptl.	control	
M87	395	399	4.0	31.6	87.3
M79	393	399	18.2	54.3	66.4
M85	196	200	0	25.2	100

M79 (female)

This cat was repeatedly infected with a total of 2,047 non-irradiated B. pahangi and challenged 96 days after the final immunizing dose. The animal was challenged following the normal schedule. The animal became patent 64 days after first infection, and the count remained high throughout (see Fig. 17, Chapter 6). At the time of autopsy, the microfilarial count was 543 in 10 [illegible] of blood. The overall resistance to challenges was 54.2%; resistance against the adult stage was 27.5%, against the fourth stage 44.0% and against the third stage 66.4%. Worm recoveries of the repeat infections were Lfl 0.07%, Lfl 0.10% and Rfl 0.09%.

[illegible] (male)

This animal was repeatedly inoculated with 2,039 infective larvae and became patent 78 days after the first infection. The microfilarial pattern is shown in Fig. 19 (see Chapter 6). 193 days after patency, the cat became amicrofilaraemic and was challenged a week later. Overall resistance shown by this animal against challenge was 88.6%; resistance against the adult stage was 74.7%, against fourth stage larvae was 94.0% and against third stage larvae was 87.3%. Worm recoveries of the repeat infections were Lhl 0.03%, Lfl 0.03% and Rfl 0.02%.

[illegible] (female)

This animal was inoculated with a total of 2,029 non-irradiated larvae and challenged 515 days after the last repeat infection. The cat became patent 78 days after the first infection and the

microfilarial pattern is shown in Fig. 18 (see Chapter 6). Three challenges were given with normal larvae, and it resisted 100% of the challenge worms. Both the immunized and the unimmunized legs were fibrosed. Only two gravid females out of the 2,029 worms inoculated were recovered.

Discussion

In these experiments, repeated infections of irradiated larvae conferred on the host a substantial degree of immunity against challenges.

The overall immunity in cats repeatedly infected with B. pahangi larvae irradiated with 25 krads. ranged from 61.3% to 93.0% with a mean of 79.2%. Three animals showed a very high degree of protective immunity against challenges; 82.7%, 90% and 93% respectively. In experiment 6 the results were based on the recovery of parasites at autopsy. Recoveries of B. pahangi from infected cats are usually satisfactory, as developing worms are confined to the afferent lymphatics. If worms of the challenge infection evade the host defence mechanisms and reach maturity, it can be assumed that resistance to challenge was not absolute. One cat, M86, immunized with parasites irradiated with 25 krads. was challenged and the blood examined for the presence of microfilariae after the prepatent period. This procedure was also followed in experiment 5, after the cats inoculated with parasites irradiated with 10 krads. were challenged.

Protective immunity in cats inoculated with parasites irradiated with 10 krads. ranged between 60.3% and 98.5% with a mean of 78.6%. Three cats of the 4 challenged did not become microfilaraemic but during autopsy microfilariae were found in the cardiac blood of two

of these animals. The other 3 animals became patent a few days after the challenge control. In two cases, the protective immunity stimulated by the irradiated inoculations was as high as 90.1% and 98.5%. In the latter case, microfilariae were not detected even during autopsy, indicating total immunity. The lowest degree of immunity was seen in cat A60, where the resistance against challenge infections was 63.5%. Although a mean of 9.81% of adult challenge worms was recovered from this animal, microfilariae were not detected. Microfilariae produced by the challenge worms in the experimental animals looked normal, developed into infective larvae in susceptible mosquitoes and later into adult stages.

Two explanations may be postulated for the failure to detect microfilariae in the immunised cats, despite the presence of gravid, challenge female worms. Firstly, active suppression of microfilariae at various immune centres of the immunised animals is possible but microfilariae were not found in the lungs, kidney or heart of these animals. Duke (1960) has demonstrated that in drills infected with Loa loa, microfilariae were destroyed in the spleen. There is no evidence to suggest that this occurred in cats infected with M. pahangi. Another explanation is that the levels of circulating microfilariae were so low that they were not detected when small samples of blood were examined. 1 ml. of blood was filtered through a Nuclepore filter, on occasions, during the expected period of patency but no microfilariae were found.

Active suppression of establishment of challenge worms by the host immunised with irradiated parasites must occur during the early stages of the life-cycle. This view is supported by the high

degree of resistance shown against third stage larvae. Irradiated parasites do not grow to sexual maturity (see Chapter 3), but they do live for much longer than the duration of the third stage in normal infections. It is to be expected that irradiated larvae stimulate antibodies against the early stages in the life-cycle. Although antibodies had not been positively shown to play a role in protecting the host against *H. pahangi*, the high levels of antibodies detected in the IFA test (see Chapter 7) in cats with irradiated parasites supports this view.

Resistance against challenge worms might manifest itself before the third stage larvae penetrate the lymphatic system. Lymph in the efferent vessels contains few immunologically reactive cells (Hall, 1967) and thus may help the parasite to establish itself in the efferent vessel once it has reached this site. It was thought that adult stages of *D. pahangi* may mask themselves with host antigens as do adult *S. mansoni* (Smithers and Terry, 1969), and thus avoid the host immune mechanisms. Evolutionary factors, intrinsic to the parasites may also enable them to circumvent or block immunological defence mechanisms mounted by the host (Ogilvie and Wilson, 1976). However, McGreevy *et al.* (1975) showed that *H. pahangi* worms do not mask themselves with host antigens.

Denham and McGreevy (1976) suggested that fibrosed tissues and pathological changes in the lymphatic system due to existing infections may act as a barrier to establishment of the challenge worms. Although, this may be the case in cats that have been repeatedly infected with non-irradiated parasites, where the lymphatics are considerably dilated and damaged, there is no reason to suppose that this occurred in cats infected with irradiated parasites. Lymphatics of these cats

were not greatly dilated (see Chapter 5, Figs. 8, 9 and 10.). Thus in cats infected with irradiated worms the lymphatics are not fibrosed, and the lymphatic system acting as a mechanical barrier for the establishment of challenge worms can be ruled out. Support for the above view comes from the failure of the majority of challenge worms to establish in the uninfected leg in the immunised animals. The lymphatics of the "uninfected" Rhl of the repeatedly immunised animals were fine and thread-like, and were not affected by the inoculations of parasites into the other legs.

Varying degrees of resistance were detected in 3 cats repeatedly inoculated with normal B. pahangi. One animal that was challenged when it still had circulating microfilariae did not show very strong resistance (54.2%) when compared with cats inoculated with irradiated parasites. This was in agreement with the findings reported by Denham and McGreavy (1976). The resistance of a cat that was challenged immediately after it became amicrofilaraemic was high (88.6%), and the result is in agreement with the results reported by Denham and McGreavy (1976). However, curious results were obtained when a cat given repeated infections was challenged approximately 11 years after initial infection. This cat, challenged with B. pahangi whilst it still had high circulating microfilariae (150 microfilariae in 20 mm^3 of blood), resisted all of the challenge doses. This absolute immunity was also expressed in the unimmunised Rhl. The lymphatics of this animal were highly fibrosed and may have acted as a barrier to the establishment of some of the challenge worms. Only two gravid females were recovered during autopsy of this animal, despite the high level of microfilariae detected in the peripheral blood.

Absolute immunity against challenge infections seldom occurs in

host-helminth systems. In the present experiment, only one animal prevented the development of microfilaraemia. In this case, host defence mechanisms may have killed the male challenge worms, thus preventing the insemination of the female worms. In other animals, microfilariae were detected either during the expected period of patency, or when cardiac blood was examined during autopsy. Protection against challenges was not absolute in other systems; in D. viviparus (Jarrett et al., 1958a), and in A. caninum (Miller, 1973). Miller (1973) reported that challenge worms in vaccinated dogs produced sterile eggs.

In these experiments, very high numbers of larvae were used as immunizing doses. In experiment 4, the numbers of larvae used ranged between 1,459 and 2,003; and the number of parasites used in experiment 5 ranged from 2,200 to 2,516. This increase in the number of parasites in the second experiment has not greatly changed the resistance mounted by the immunized host. Cats became microfilaraemic normally after many repeated inoculations with 50 infective larvae (Denham and McGreevy, 1976) before becoming "immune". However, Ah et al. (1974a) reported that a protection of 57% was obtained in dogs given a single dose of irradiated larvae, consisting of 500 worms.

A considerable degree of resistance against heterologous challenges with B. patei, i.e. 65.3% and 78.6%, was seen in cats immunized with irradiated B. pahangi. In both these animals, the unimmunized leg was more susceptible to the heterologous challenges. The percentage resistance against challenge worms in the unimmunized legs was low against adult and fourth larval stages, ranging from 16.4% to 48.5%. The higher resistance against third stages (87% - 96%) may have been due to antibodies stimulated by the two previous infections with B. patei.

Heterologous immunity has been demonstrated in other helminth systems. Monkeys infected with an irradiated non-human strain of S. japonicum protected these animals from heterologous challenges with the human strain (Hsu et al., 1962). Miller (1973) vaccinated dogs with irradiated A. caninum and they resisted heterologous challenges with A. brasiliensis and U. stenocephala. Cats vaccinated with A. caninum were also demonstrated to resist infections with the feline hookworms, A. tubaeformis and A. brasiliensis.

Chapter 5

LYMPHATIC CHANGES IN CATS INFECTED WITH B. MALAYI OBSERVED XERORADIOGRAPHICALLY

Introduction

Since Kinmonth (1952, 1954) developed the technique of lymphangiography to visualize the lymphatic system it has been used to study cases of clinical lymphoedema in man (Arora et al., 1958; Cahill and Kaiser, 1964; Da Rocha, 1964; Kanetkar et al., 1966; Caravon et al., 1968; and Cohen et al., 1969) and in experimental filarial infections of animals (Schacher et al., 1969, 1972; Goonaratne et al., 1971; and Ewert et al., 1972).

In preparing lymphangiograms, the blued lymphatic is displayed, and contrast medium injected under pressure directly into the vessel after cannulation. Although the pressure needed to inject the medium into the lymphatic can be carefully monitored, the injection is functionally unnatural for the animal lymphatic system.

Rogers et al. (1975) described a new technique for studying lymphatics and the damage caused by the filarial worms. This technique is based on the method of xeroradiography which was developed for the study of tumours of the human breast (Wolfe, 1968; Hoag, Stacey and Davis, 1971; Gilbe, 1973). This technique has many advantages over conventional lymphangiography. Firstly, because any damage to the integrity of the lymphatics whilst injecting the contrast medium is avoided, as Hypaque is injected subcutaneously and diffuses into the lymphatics. Secondly, lipiodol, the iodine-based contrast medium

used in lymphangiography has at times been toxic to cats studied. During conventional lymphangiography, the stress on the lymphatic system is so great and the retention of lipiodol so long that chronological study of lymphatic pathology at short intervals is impossible. A xeroradiogram is developed in 90 seconds and the whole procedure completed within 5 minutes. No ill effects on the lymphatics or the animals after repeated tests have been recorded (Rogers et al., 1975).

In the xeroradiographic method, the lymphatic system of the test animal transported the contrast medium (Hypaque) from the subcutaneous region of the extremity at the rate of flow which it would use for other material. Thus any damage that may result from outside manipulation is avoided. In their lymphangiographic study of cats infected with Brugia spp., Goonaratne et al. (1971) reported that there was leakage of the medium from the lymphatics. No such malfunction of the vessels was observed in the present experiments nor in unpublished experiments by Rogers (1976) on long term infections with normal larvae.

In this chapter, changes in the lymphatic vessels and nodes of cats infected with normal and irradiated B. pahangi were observed using the xeroradiographic method. In this method, the x-ray intensity transmitted by an object is recorded as a change in density pattern on the surface of a semi-conducting selenium plate. "Edge contrast" patterns are yielded by the powder development (xerox) method which enhances the visibility of fibrous and vascular structures in soft tissues.

The animal to be studied was anaesthetised with Nembutal. 0.5 ml. of [illegible]% Hypaque was injected into each limb. Half the amount was

inoculated ventrally, the rest dorsally into the feet. Full uptake of Hypaque by the lymphatic took place in three minutes. At the end of this period the animal was laid on top of a trolley and secured to prevent movement (see Plate 9). A perspex holder containing the xerox cassette was placed under the limb being studied. A xeroradiogram was taken using a Siemens "Mammomat" x-ray unit with a Molybdenum anode in association with a xerox 125 system (Gillbe, 1973) (Plate 10). The exposure factors were 28kV, 32 mAs at 52 cm. F.F.D. and the xeroradiogram thus processed was developed within 90 seconds. If the result was unsatisfactory, a second xeroradiogram was taken.

Lymphatics of three cats were studied chronologically (A42, A43, A50) during immunization with irradiated (10 krads.) B. pahangi and after a challenge infection with non-irradiated parasites (see Chapter 4). Other cats used in the experiments of Chapter 4 were also studied. Normally the Lhl was inoculated with irradiated or non-irradiated larvae and the Rhl of the same animal served as a control. Plate 11 shows xeroradiograms of the infected (Lhl) and uninfected (Rhl) lymphatics of cat A50.

Observations and conclusions

In all the experimental animals studied the lymphatics remained intact, further suggesting that the xeroradiographic method of observing lymphatic changes was preferable to the lymphangiographic method.

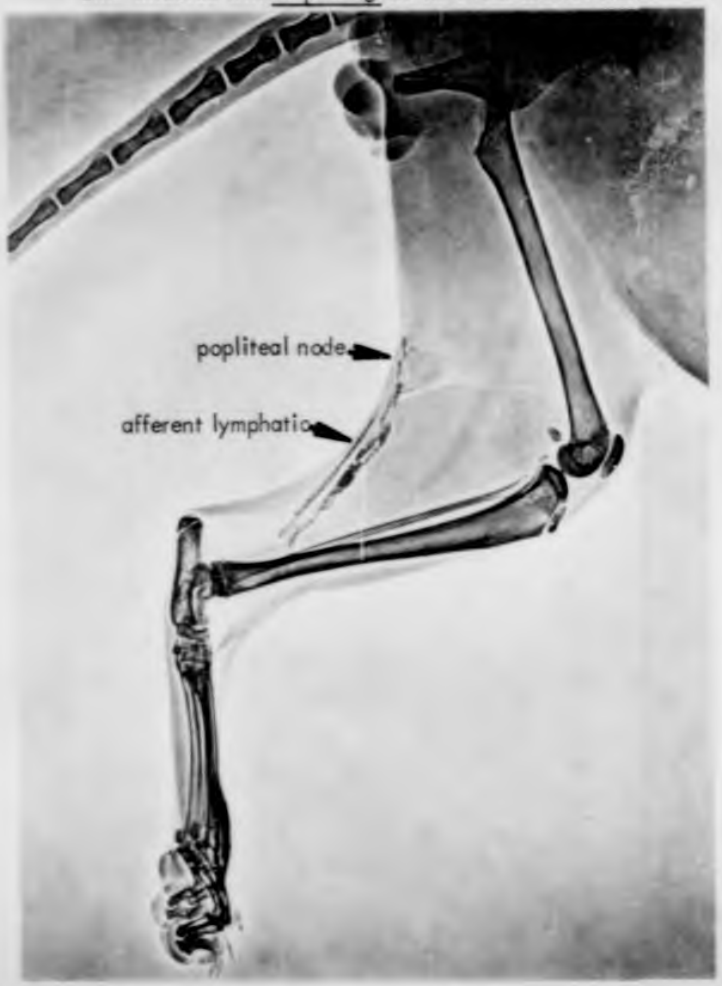
The uninfected Rhl of all cats had fine, thread-like vessels and small nodes. When these legs were challenged with normal larv-

Plate 9. Infected cat secured under a Siemens "Mammomat" X-ray unit

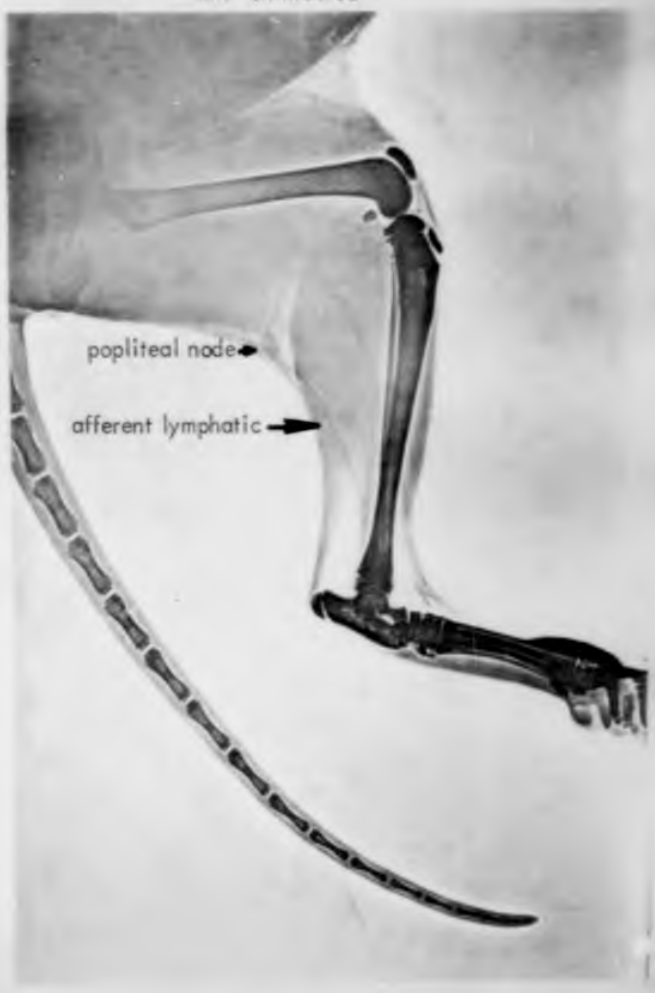
Plate 10. Xerox 125 system

PLATE 11. XERORADIC GRAMS OF CAT A50

Lhl- infected with B. pahangi irradiated with 10 krads.



Rhl- uninfected



both the sizes of popliteal nodes and afferent vessels increased.

Cats M85 and M87 had received totals of 591 and 596 normal infective larvae in the scheduled infection (see Chapter 4).

The initial changes observed after infection were seen in the popliteal lymph nodes into which drained the lymphatics from the site of inoculation of the parasites. When compared with uninfected popliteal nodes, the nodes of the infected legs had enlarged 2 to 3 fold. This increase in the size of the nodes was probably due to the presence of the parasite in the subcortical sinus and the stimulation of intense immunological reactions in the nodes. Goonewardene et al. (1971) and Ewert et al. (1972) have observed similar enlargement of the nodes, and Rogers et al. (1975) reported an increase in the immunological activities of the nodes and the formation of many germinal centres. The lymphatics afferent to the popliteal nodes of these cats were later enlarged in xeroradiograms.

Tracings of the xerograms of these cats are shown in Figs. 8, 9, 10 and 11. Lymphatic dilations also occurred in cats infected with larvae irradiated with 10 krads, (cats A44 and A50, see Figs. 10 and 9). However, dilated lymph vessels did not necessarily indicate the presence of live worms. During autopsies of the two cats, very few worms were retrieved and these were worms of the challenge dose. It is probable that the worms which had caused the dilation had died before the challenge with normal larvae. In this case the lymphatic damage would most likely have resolved (Denham and Rogers, 1976).

Xeroradiograms of lymphatics of cats infected with irradiated parasites are shown in Fig. 8, Fig. 9 and Fig. 10. Cats A42, A43,

Fig. 8. LYMPHATIC CHANGES IN CATS INFECTED WITH B. PAHANGI irradiated with 10 krads.)
(XERORADIOGRAPHY TRACINGS)

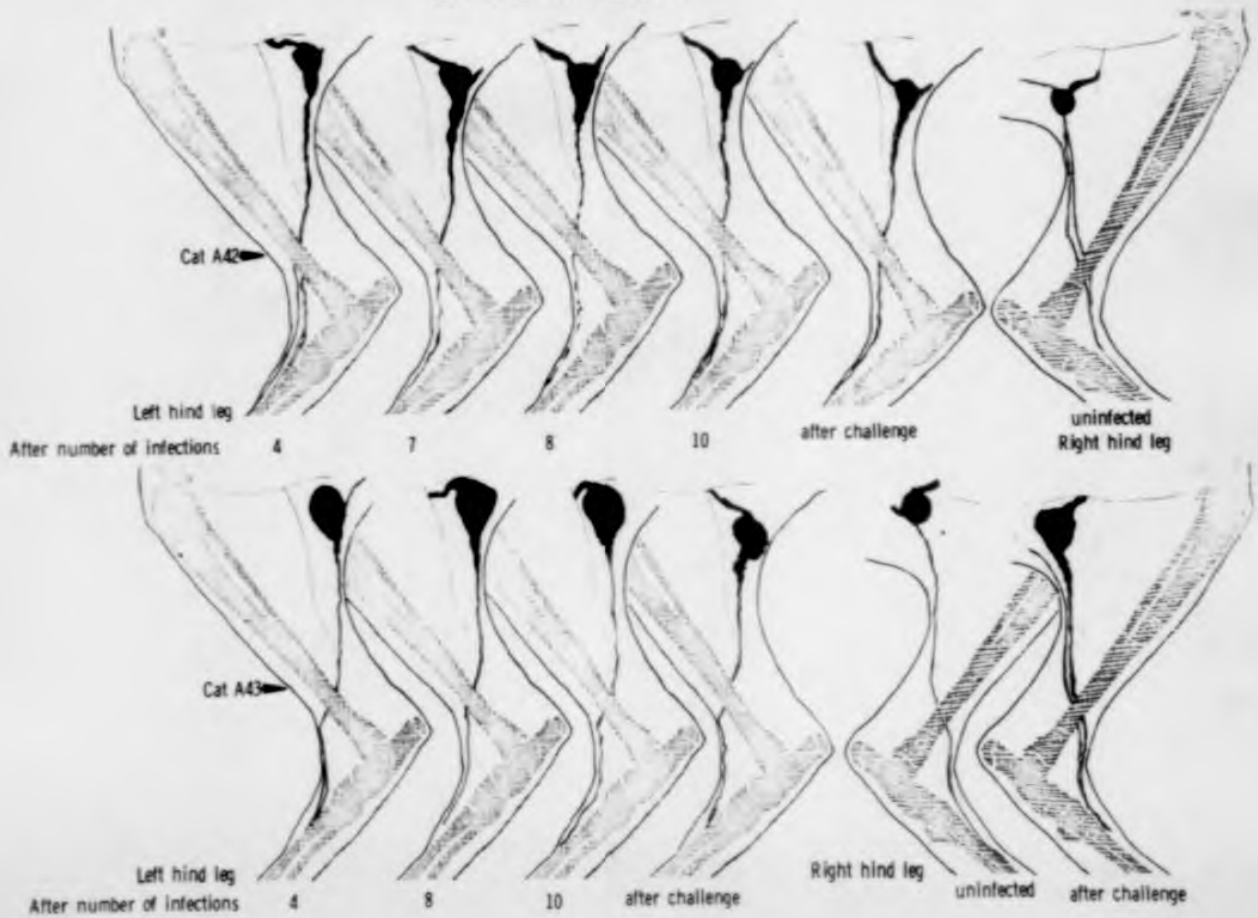


Fig. 9. LYMPHATIC CHANGES IN CATS INFECTED WITH B. PAHANGI (irradiated with 10 krads.)
(XERORADIOGRAPHY TRACINGS)

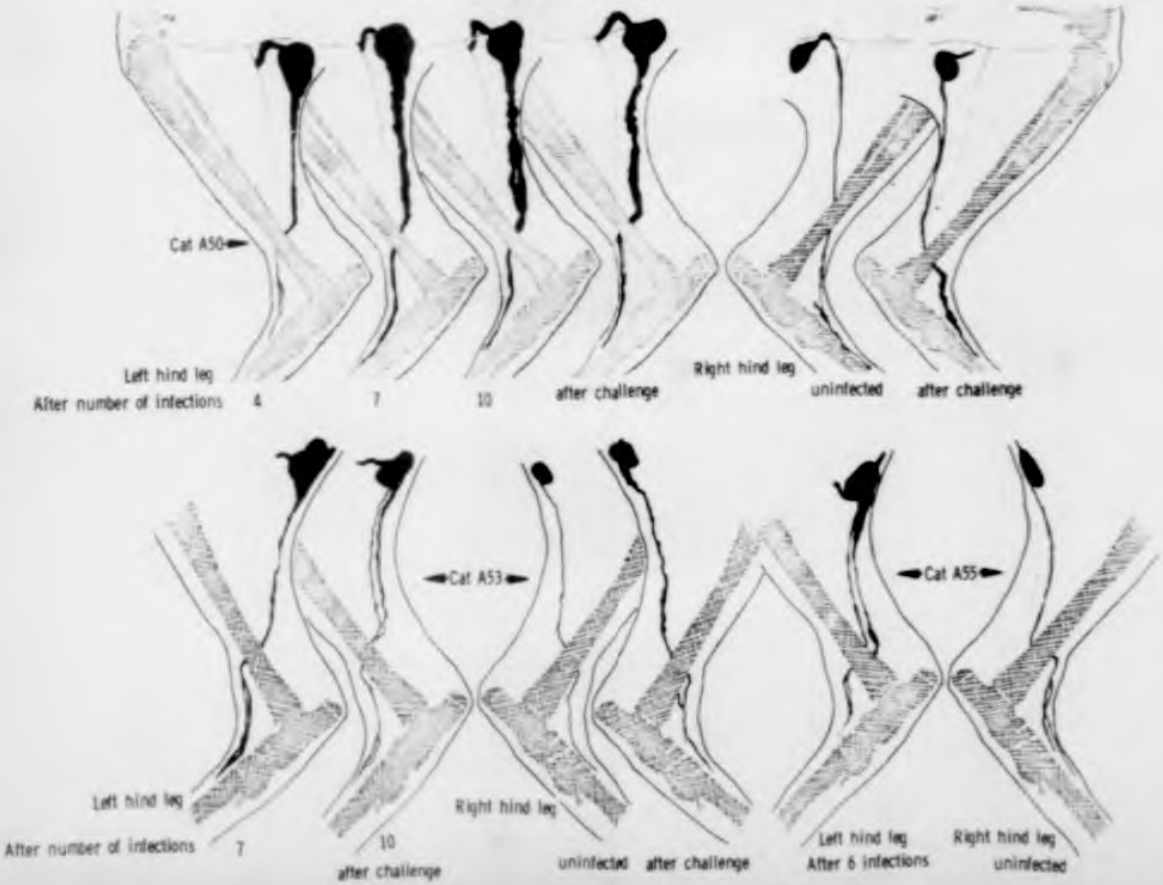
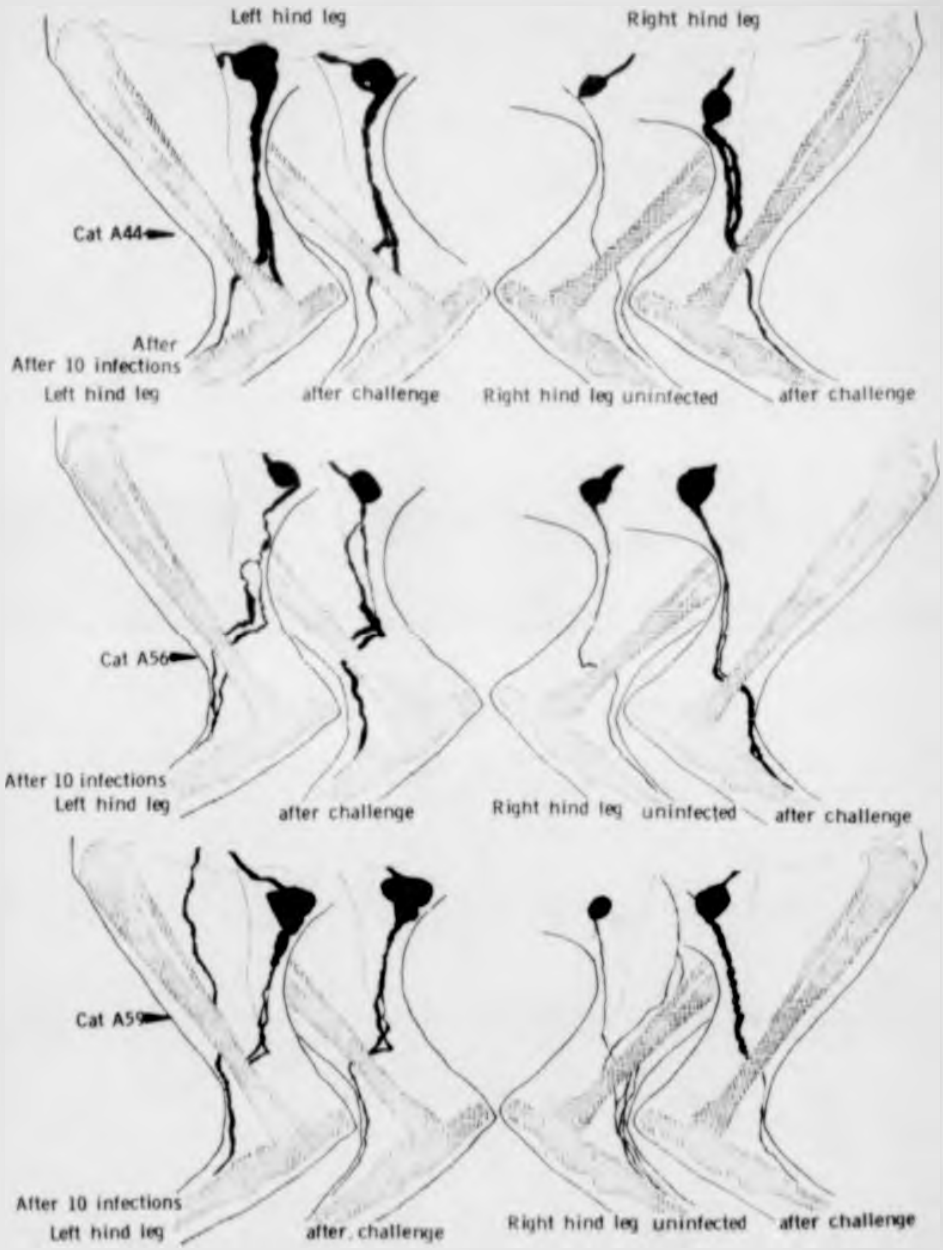


Fig. 10. LYMPHATIC CHANGES IN CATS INFECTED WITH B. PAHANGI (Irradiated with 10 krads.)
(XERORADIOGRAPHY TRACINGS)

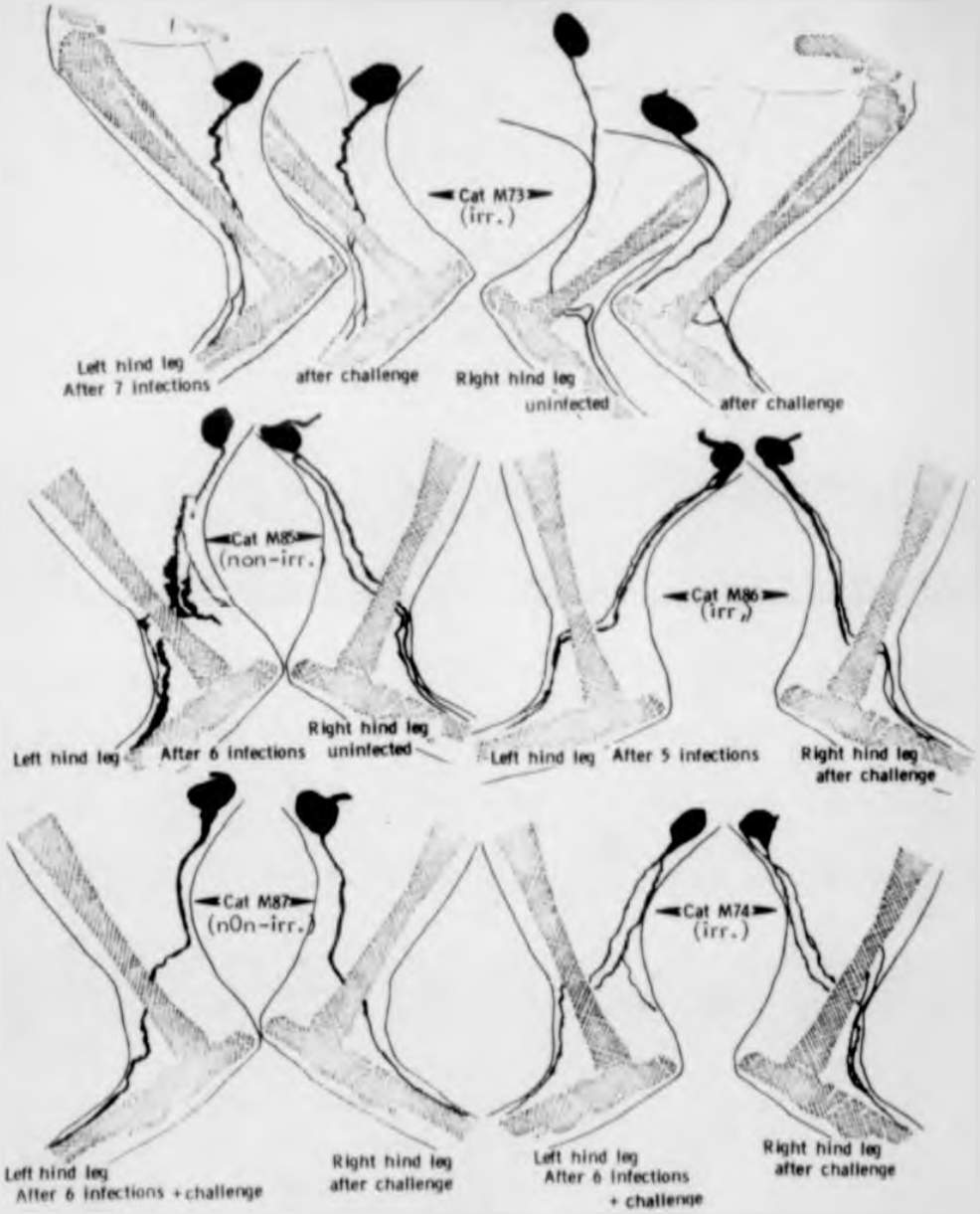


A44, A50, A53, A56 and A59 were infected in the Lhl with 900-1,000 B. pahangi irradiated with 10 krads. In all these cats, except A44 and A50, the lymphatic vessels were not distended or tortuous. This was due to three reasons. Firstly, the parasites did not grow to their natural sizes as they had been exposed to irradiation. Secondly, very few irradiated parasites migrated back to the afferent vessels. Thirdly, the irradiated parasites have a shorter life span (see Chapter 3).

In sequential studies of lymphatics of cats infected with irradiated parasites (A42, A43 and A50, see Figs. 8 and 9), the nodes were enlarged after infections as compared with their uninfected Rhls. The nodes were largest after 7 to 8 immunizing infections, after which there was a slight decrease in the sizes of the popliteal nodes. The sections of afferent vessels immediately next to the nodes were dilated in most cases, but in no case were the afferent vessels near the ankle dilated, as observed in long term infections with non-irradiated B. pahangi (see Fig. 11, M85 and M87).

It is interesting to note that the lymphatics of Lhl which had been previously inoculated with about 1,000 worms irradiated with 10 krads. of Co.60 did not enlarge greatly except in two cases. This did not change 2 months after the challenge dose with non-irradiated parasites. The lymphatics of the Rhl on the other hand were distended and had a beaded and dilated appearance. This indicated that most of the challenge worms in the Lhl were either killed by the host or never returned to the afferent vessels after being encapsulated in the nodes. The beaded appearance of the lymphatics in the Rhl was due to the parasites positioning themselves between

Fig. 11. LYMPHATIC CHANGES IN CATS INFECTED WITH B. PAHANGI
(PERORADIOGRAPHY TRACINGS)



irr. = infections with larvae irradiated with 25 krads.

non-irr. = non-irradiated larval infections

the valves in the lymphatics. The functioning of these lymphatics were seldom hindered, as the vessels and the nodes blued readily during autopsies. In some cases, a slight malfunction could be surmised, as the popliteal nodes did not colour as deeply as the afferent vessels. This happened more frequently if dead parasites were present. This view is supported by Rogers and Denham (1975) who say that the rate of lymph flow was unaltered in lymphatics that had been repeatedly infected with B. pahangi.

Discontinuities of the lymphatics occur in lymphangiograms due to inadequate filling of the vessels by the contrast medium (Cahil et al., 1964; Schacher, 1973; and Burns, 1975). This often leads to a condition known as dermal backflow, where retrograde filling of the dermal vessels occurs (Goonewatne et al., 1971; and Schacher et al., 1973). When the main lymphatic of the infected limb is blocked, compensatory structures appear in the form of collateral vessels (Schacher, 1973; Burns, 1975; Ewert et al., 1972). Dermal backflow was observed in cat A59 (see Fig. 10) and collateral vessels in cats M85, M86, M74 (Fig. 11) A44, A50, A36 and A53 (Fig. 10).

All the changes in the lymphatics due to infection, such as enlargement of nodes, dilated, tortuous vessels, obstructive vessels, and the resultant changes had been observed in humans roentgenographically (Arora et al., 1958; Cahil et al., 1964; Da Rocha, 1964; Carevons et al., 1968; Cohen et al., 1961; and Kamatkar et al., 1966).

In the present experiments there was no change in the size of the afferent vessels of animals infected with B. pahangi. This was because parasites very rarely passed through the filter mechanisms of the nodes. Ewert et al. (1972) reported that the afferent vessels were only outlined if the limb was uninfected.

If the xeroradiography method became readily available, it would be a very quick method of diagnosing lymphatic infection in early cases of lymphostatic verrucosis and filarial lymphoedema.

Chapter 6

HAEMATOLOGICAL CHANGES IN CATS INFECTED WITH B. PAHANGI WITH SPECIAL EMPHASIS ON THE EOSINOPHIL RESPONSE

Introduction

A short review on eosinopoiesis and its function

Although eosinophils have been known to exist since 1879, it is only recently that the actual mechanisms involved in eosinophil production and function have been investigated. The function of the eosinophil is by no means clear and no specialised function has yet been ascribed to them. The subject has been reviewed by Archer (1961, 1970), Hirsch (1965), Editorial of Lancet (1971) and Zucker (1974).

Increased numbers of eosinophils in the circulation are associated with hypersensitivity states, drug reactions, parasitic infections, dermatoses and certain neoplastic diseases. Eosinophils are produced in the bone marrow from precursor cells, undergo maturation within 2 days, and appear in the circulatory system. It has been estimated that for every eosinophil in the peripheral blood of guinea pigs there are 400 in the bone marrow (Hudson, 1960). Eosinophils in the circulatory system are merely 'en route' before they infiltrate various tissues (Archer, 1970).

The mechanism of eosinophilia has been mainly elucidated by experiments on T. spiralis infections in rats and mice. The great difficulty has been in ascribing to eosinophils functions that are not shared by neutrophils. Both cells show amoeboid movement,

respond to chemotaxis, phagocytose and degranulate.

Working with T. spiralis in rats, Basten et al. (1970a) showed that whole worms were needed to induce eosinophilic response. It did not matter if these were alive or dead but the eosinophilia did not occur when they used homogenates of T. spiralis. Rats that were T cell deprived by thymectomy, administration of ALS or irradiation, did not produce circulating eosinophilia (Basten et al., 1970b). Reconstitution with sensitized T cells resulted in the release of eosinophils into the peripheral blood. Thus sensitized lymphocytes play an intermediary role. It appears that eosinophils share with lymphocytes, plasma cells and macrophages the property of proliferation after antigenic challenge. Litt (1961) demonstrated conclusively that immune complexes attract eosinophils, but on occasions they responded to antigens at first exposure (Archer, 1970). Preliminary findings of Butterworth et al., (1974) show that an eosinophil-rich polymorphonuclear leukocyte fraction damaged schistosomulae of Schistosoma mansoni in vitro, in the presence of sera from infected patients.

Kay et al. (1971) found that lung tissue from a sensitized guinea pig released a chemotactic factor, which was accompanied by the elaboration of histamine and SRS-A factor. This in turn caused the production of eosinophilia. Cohen and Ward (1971) found that antigenically stimulated sensitized lymphocytes release a substance which combines with immune complexes in vitro to produce a factor chemotactic for eosinophil production. The phagocytic function of eosinophils has been demonstrated (Archer, 1963; Sabesin, 1963) and during this process these cells degranulate.

In this chapter a study of the cellular component of the blood

of cats infected with irradiated and non-irradiated B. pahangi is reported.

Materials and Methods

Total red blood cells (RBC), total white blood cells (WBC), packed cell volume (PCV) and eosinophil counts of animals used in the experiments of Chapter 4 were carried out at weekly intervals. This included cats infected with non-irradiated B. pahangi and larvae irradiated with 25 krads. However, only PCV and eosinophil levels of cats infected with larvae irradiated with 10 krads. were monitored.

Blood cell counts

When counts of erythrocytes, total leukocytes and eosinophils were to be done, blood was collected into an EDTA tube (Stayne Lab.) and mixed well by gently rotating it. The counts were either done on the same or the following day in which case they were kept at $4^{o}C$.

Total red blood cell and white blood cell counts

A coulter counter (Coulter Counter Model-E, Coulter Electronics Ltd., Bedfordshire) was used to count red and white blood cells. Dilutions of erythrocytes (1:50,000) and leukocytes (1:500) of blood samples were prepared in isoton, in glass universal tubes. When counting WBC, a drop of zaponin (Coulter Electronics Ltd.) a strong stromatolysing agent, was added to lyse the erythrocytes.

At the outset, the coulter counter was calibrated to count cat blood cells. This was accomplished by counting the same blood samples at different values of aperture current and amplification. As only the lower threshold values (LTV) needed to be calibrated, the upper threshold value dial was switched off. A graph was constructed by plotting the counts obtained for the blood sample at various levels of LTV, starting from 0 to 60. A plateau appeared on the graph and a LTV corresponding to the mid-point of the plateau was chosen. A similar graph was constructed for WBC counts, and the value corresponding to the further end of the plateau was chosen. A total of 5 consecutive counts was made for each sample of blood and the mean calculated. Total counts of RBC also included WBC but their numbers were insignificant in comparison. The aperture was rinsed with isoton whenever a new sample of blood was counted. When counting cat blood cells the aperture was set at [illegible] and the amplitude at $\frac{1}{4}$. The LTV for RBC was 13 and for WBC was 38.

Eosinophil counts in the peripheral blood

The following diluting fluid was prepared:

1% eosin (orange, water and alcohol soluble) 5%

Acetone 5%

Distilled water .....[illegible]

The blood sample was diluted 1:200 in the mixture with a white blood counting pipette. The pipette was rotated in the hand until a clear pink solution formed. Part of the solution was drawn out and a sample introduced immediately under a cover slip on an

Improved Neubauer Counting Chamber. The eosinophils were counted after 2-3 minutes. The granules in these cells stained pink and were prominent when observed under a x 100 magnification of a microscope. The total number of cells in all the 8 corner squares in the chamber was counted, and the eosinophil count calculated using the formula:-

Total number of eosinophils counted x 25 = number of eosinophils in 1 cu.mm. of blood

Packed cell volume (PCV)

Blood from animals was collected into heparinised capillary tubes (Gelman-Hawksley Ltd.) and one end sealed using plasticine. The capillary tubes with the blood samples were spun in a micro-haematocrit centrifuge at 10,000 r.p.m. for 5 minutes, and a Hawksley Micro Haematocrit Reader used to read the percentage of cells by volume in the blood.

Results

PCV of all cats ranged from 26% to 35% and were normal.

Total RBC counts of cats did not change significantly after infection with *B. pahangi* (see Fig. 12).

Total WBC counts

There was no significant change in the total WBC counts in cats infected with parasites irradiated with 25 krads. (see Fig. 13

Fig. 12 TOTAL RBC COUNTS IN CATS INFECTED WITH B. PAHANGI IRRADIATED WITH 25 krads.

↓ immunization

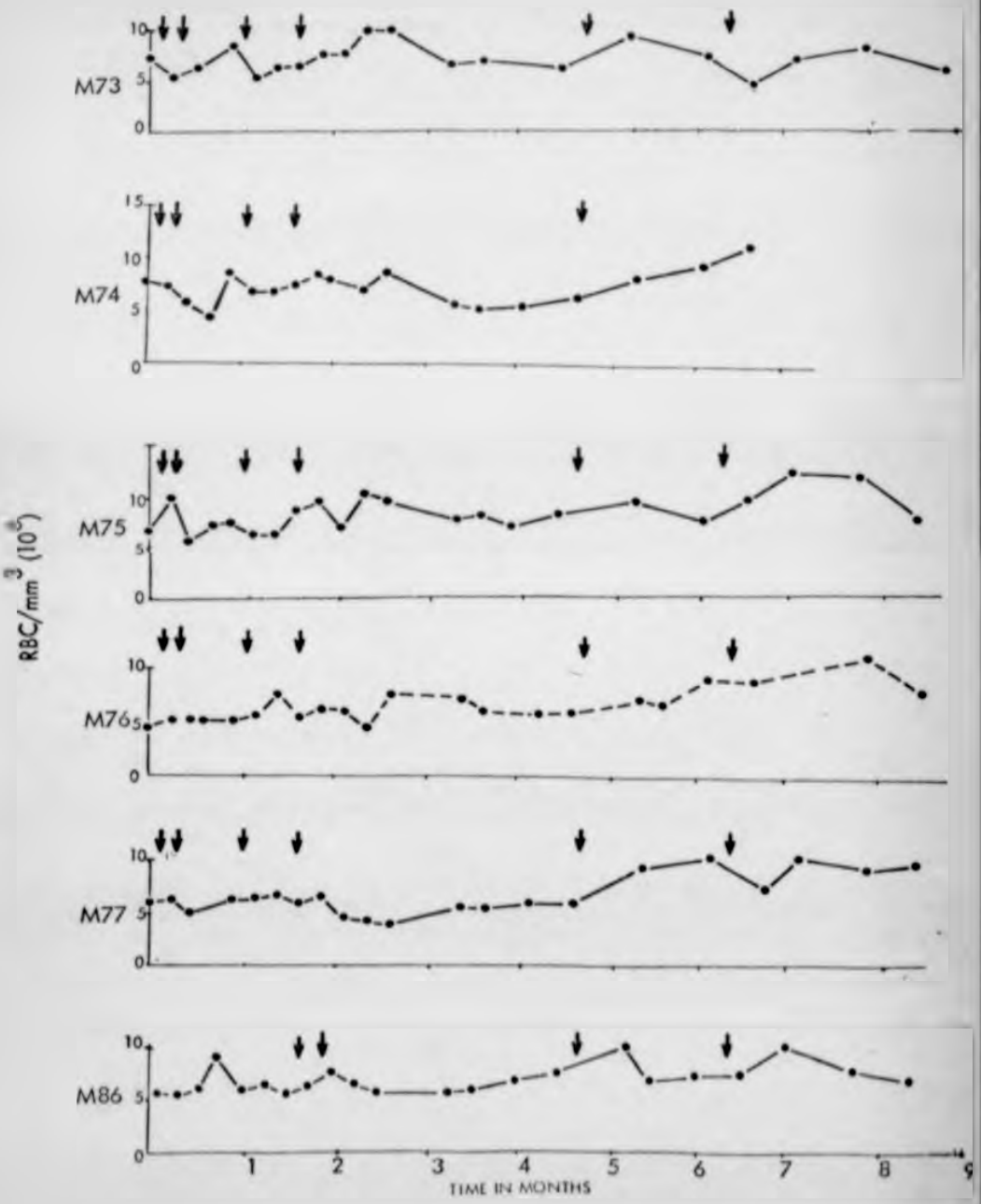
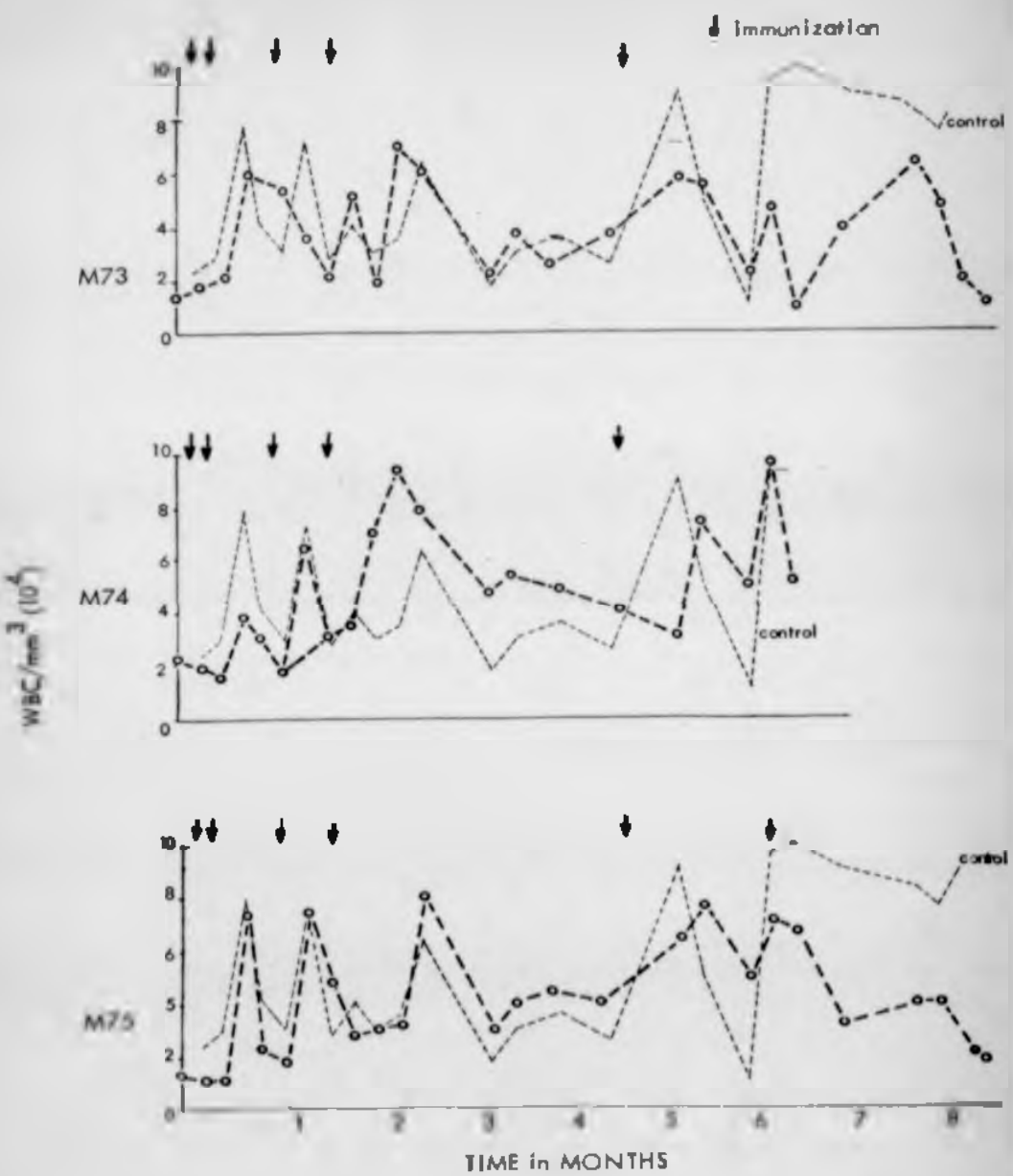


Fig. 13. TOTAL WBC COUNTS IN CATS INFECTED WITH B. PAHANGI IRRADIATED WITH 25 krads.



and 14) and in cats infected with non-irradiated B. pahangi (see Fig. 15 and 16). Although the total WBC counts fluctuated, with occasional high counts, in no case did the counts remain uniformly high. The general increase in the WBC counts in these animals was probably due to the animals growing older.

Eosinophil counts

1. Infections with non-irradiated parasites

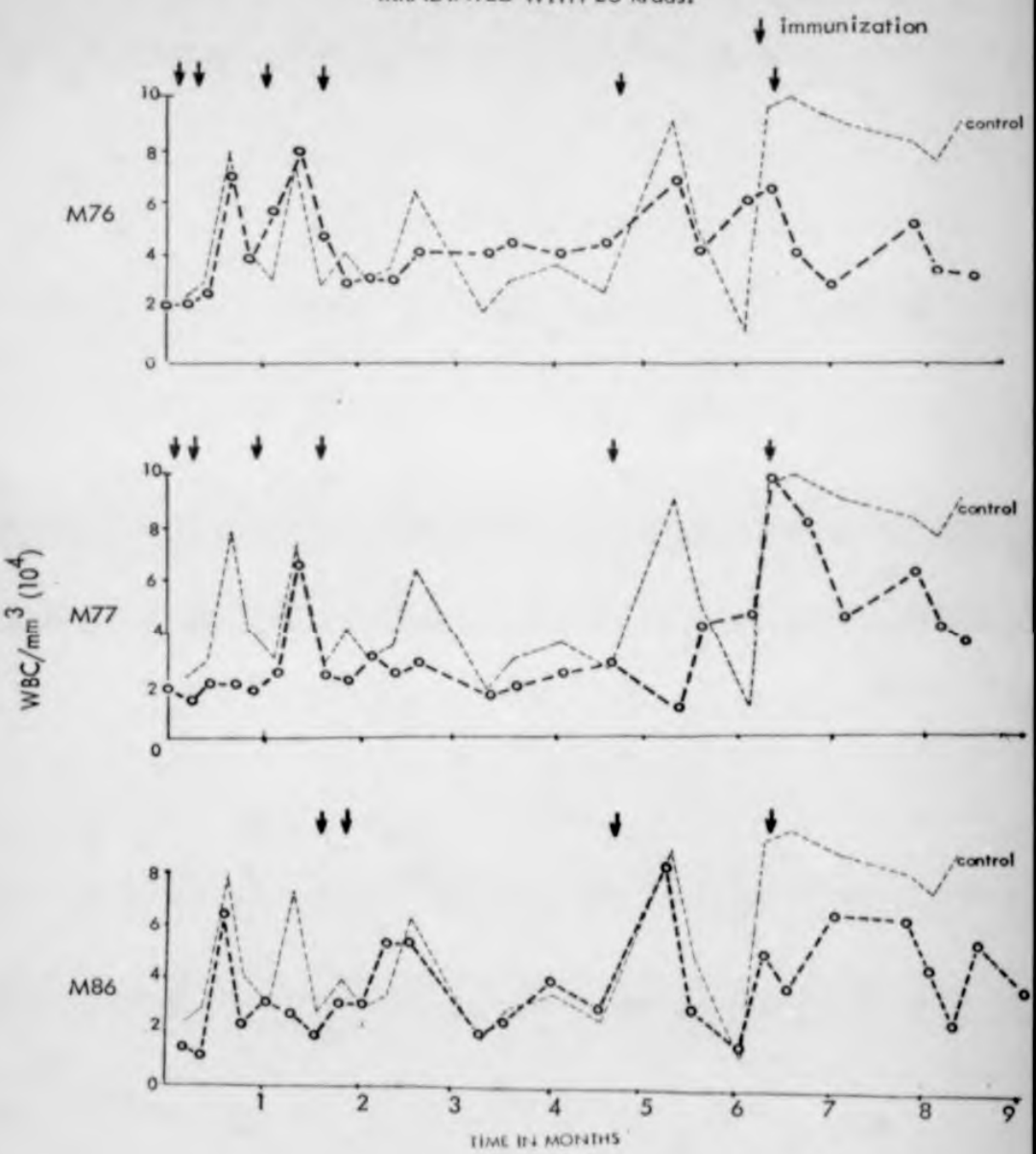
The changes in the eosinophil counts of cats infected with non-irradiated B. pahangi are shown on Figs. 17, 18, 19 and 20. The mean eosinophil counts of two uninfected cats are also included.

These figures indicated that eosinophil levels in cats increased after infection with B. pahangi. The high levels of eosinophils noticed in these cats could not be correlated with the time of moulting of the parasites (3rd. to the 4th. stage and 4th. to adult stage). This was because these animals were repeatedly infected and injections of parasites may produce eosinophilia in themselves in these animals. Except in one case (M89), the animals (M79, M85 and M87) had received 4 immunizing doses before the maximum level of eosinophils were recorded.

Eosinophilia and microfilaraemia

A definite correlation between the onset of patency of the parasite in cats and increased eosinophil responses can be seen in Figs. 17, 18, 19 and 20 (M79, M85, M87 and M89). In cat M89, the

Fig. 14. TOTAL WBC COUNTS IN CATS INFECTED WITH B. PAHANGI IRRADIATED WITH 25 krads.



TOTAL WBC COUNTS IN CATS INFECTED WITH BRUGIA PAHANGI

Fig. 15.

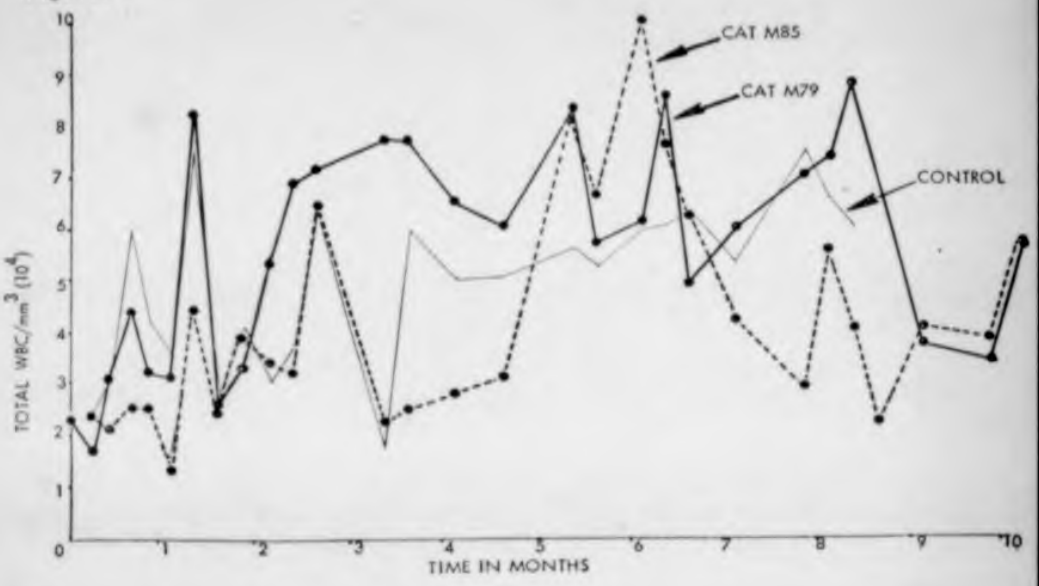
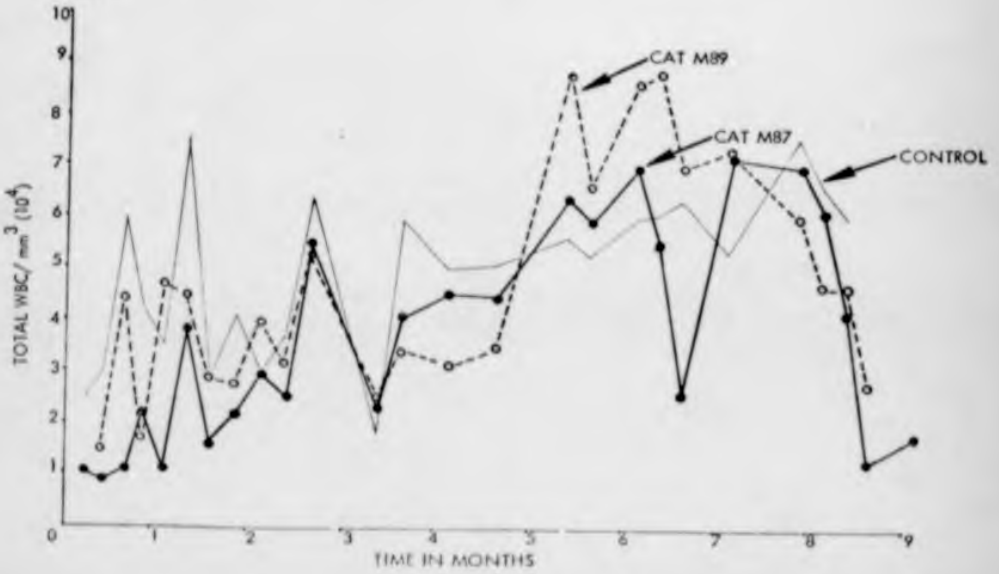


Fig. 16.



time of the onset of microfilaraemia, corresponded with the second highest eosinophil level; in cat M79, the highest level of eosinophils observed corresponded with the time when the cat became microfilaraemic. This feature can also be observed in cats M85 and M87. The precise time of the onset of microfilaraemia did not correspond with raised eosinophilia because microfilariae could only be detected if sufficient numbers of them appeared in circulation (100 mm^3 of blood was examined from the expected date of patency). After this initial raised eosinophilia in response to microfilariae being released into the circulation, the eosinophil levels dropped in all the animals studied. However, the eosinophil levels in these animals remained higher than those of control animals.

In cat M87 (Fig. 19), which became amicrofilaraemic 11 months after first infection, the eosinophil counts did not change. In another animal, M85 (Fig. 18), which remained microfilaraemic for over 2 years, the eosinophil levels remained moderate; and the raised levels of eosinophils during the first 12 months after initial infection did not appear. Throughout the second year of observation (not included in diagram) the level of eosinophils/mm^3 remained between 1,350 - 2,500. This was within the normal eosinophil levels of cats.

It is interesting to note that after the highest level of eosinophils was recorded (at the onset of microfilaraemia) further inoculation with infective larvae did not produce an increase in the eosinophilia (except in M89, see Fig. 20).

Fig. 17 CIRCULATING EOSINOPHILS AND MICROFILARAEMIA IN CATS INFECTED WITH NON-IRRADIATED B. PAHANGI.

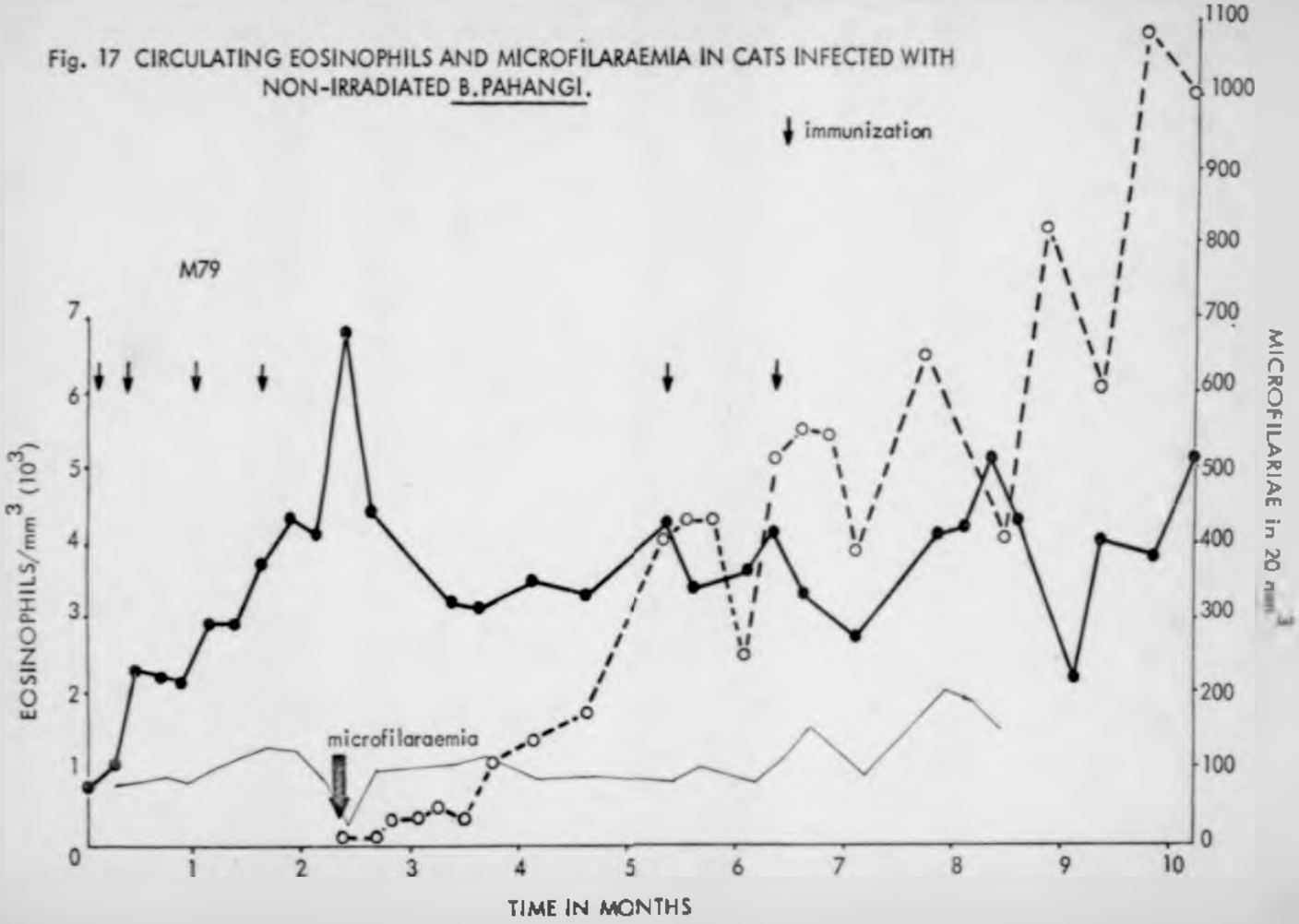


Fig. 18 CIRCULATING EOSINOPHILS AND MICROFILARAEMIA IN CATS INFECTED WITH BRUGIA PAHANGI (n0n-irradiated)

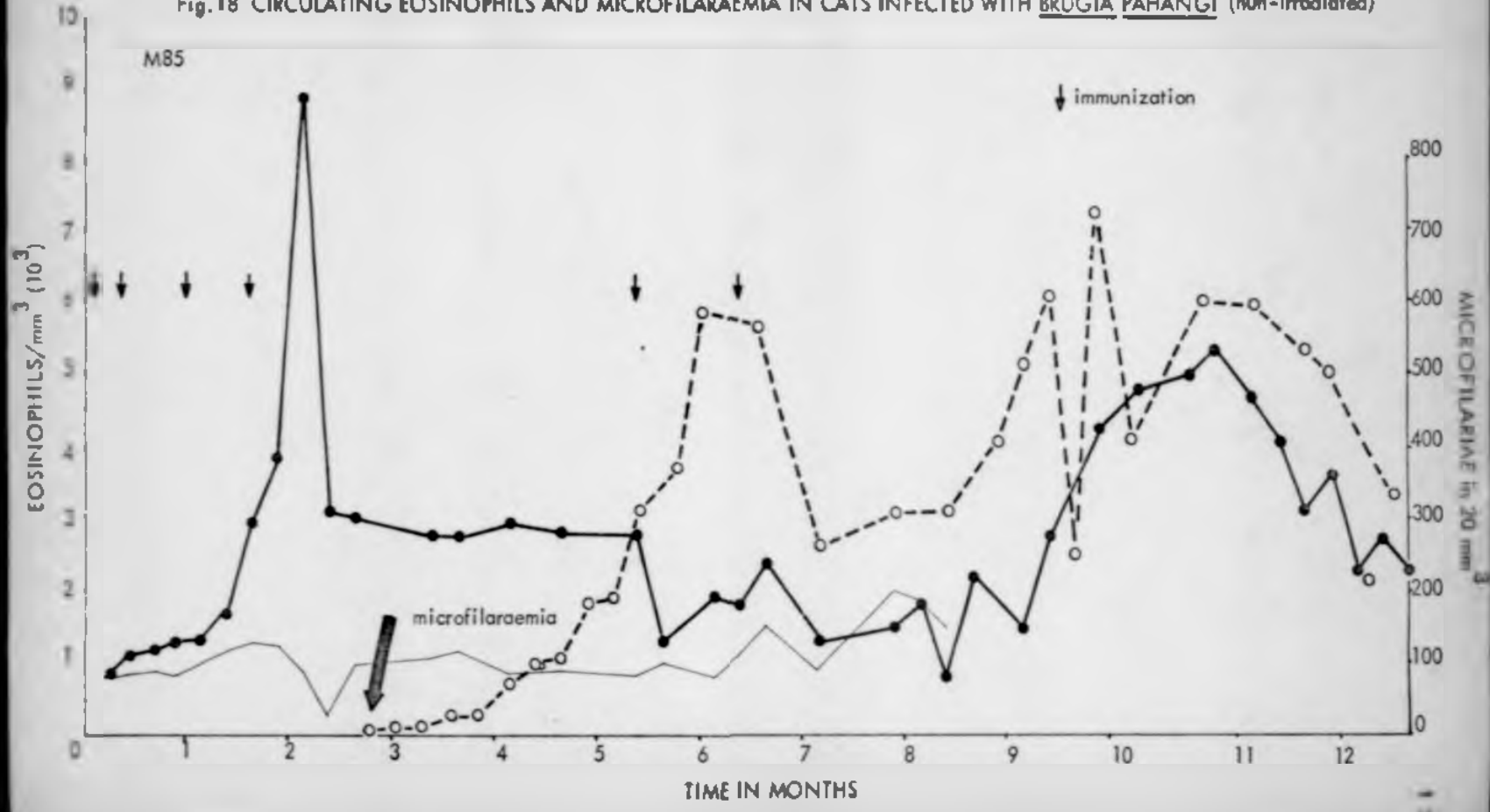


Fig. 19 CIRCULATING EOSINOPHILS AND MICROFILARAEMIA IN CATS INFECTED WITH BRUGIA PAHANGI (non-irradiated)

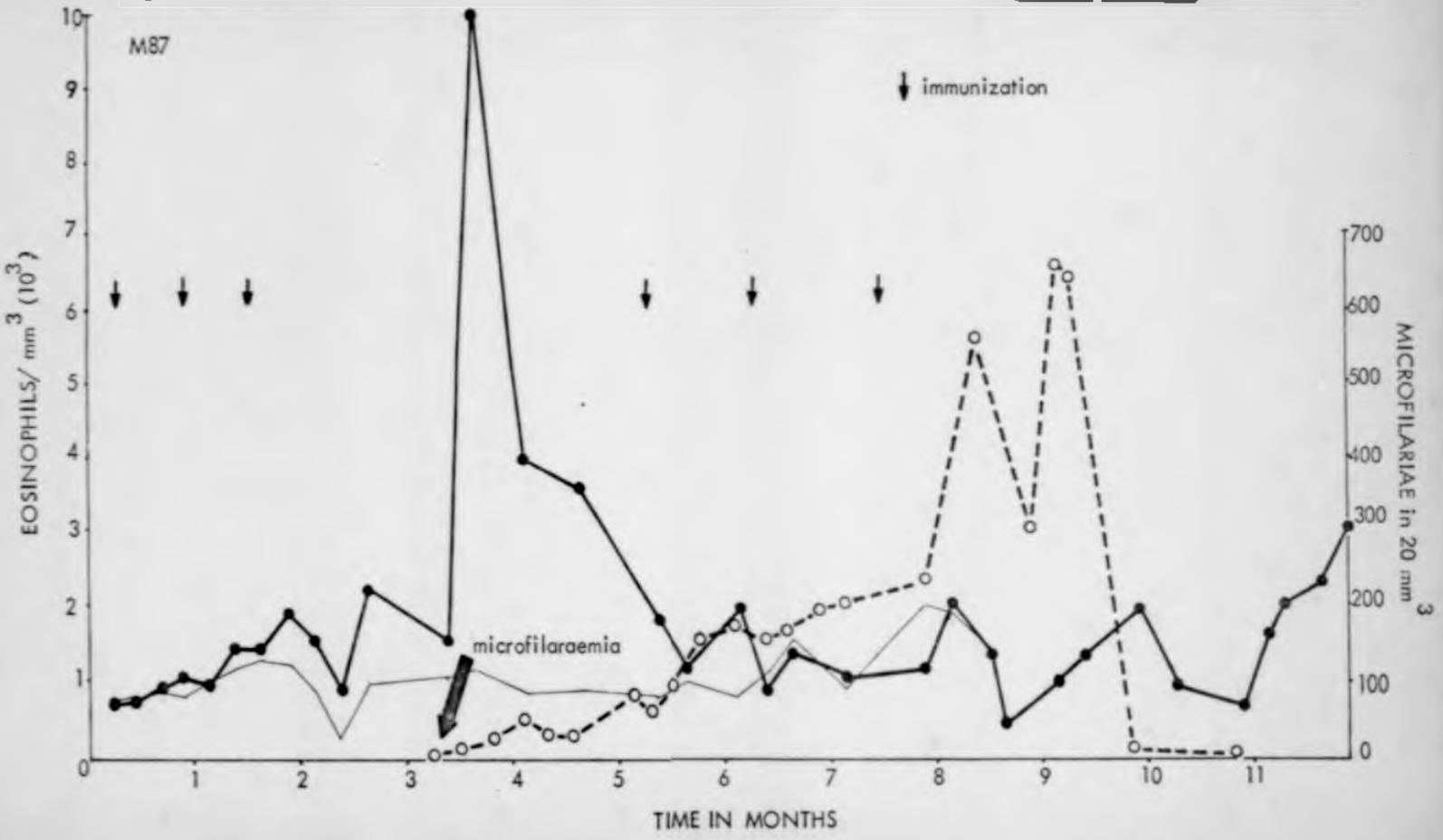
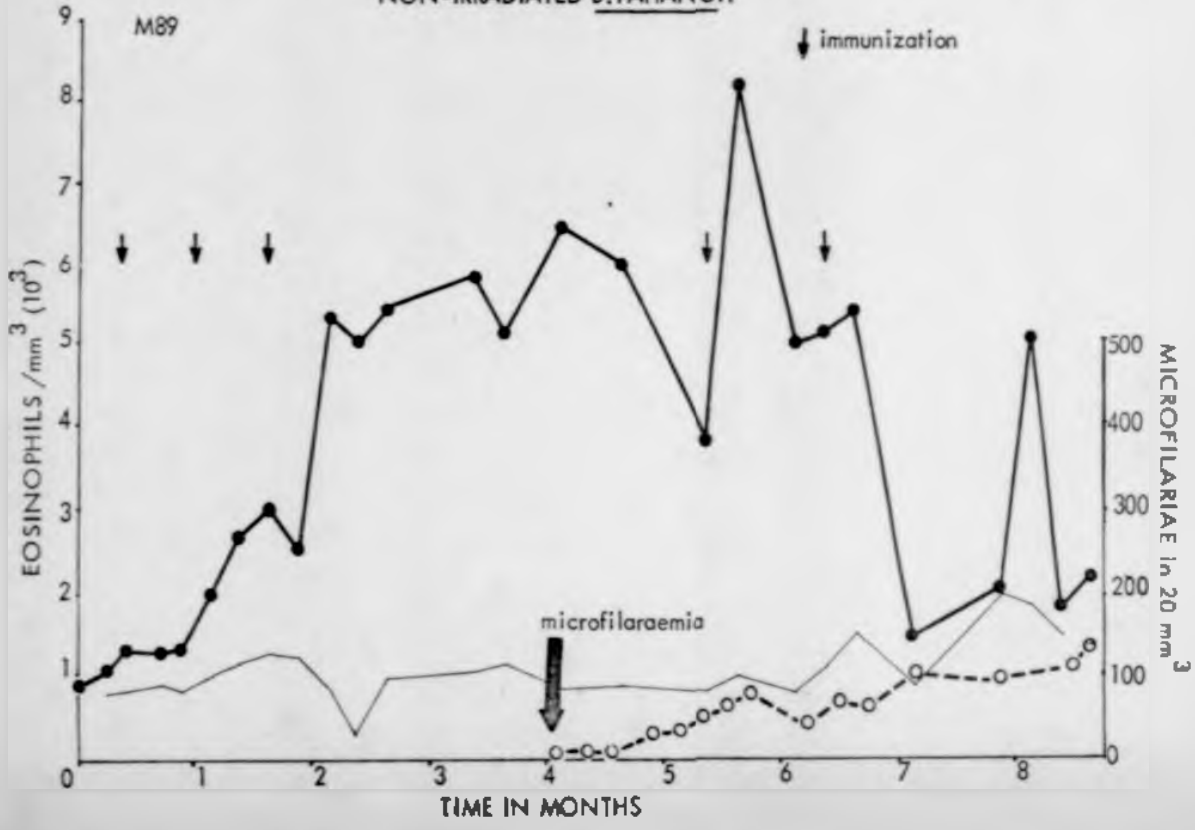


Fig. 20 CIRCULATING EOSINOPHILS AND MICROFILARAEMIA IN CATS INFECTED WITH NON-IRRADIATED B. PAHANGI.



2. Infection with irradiated B. pahangi

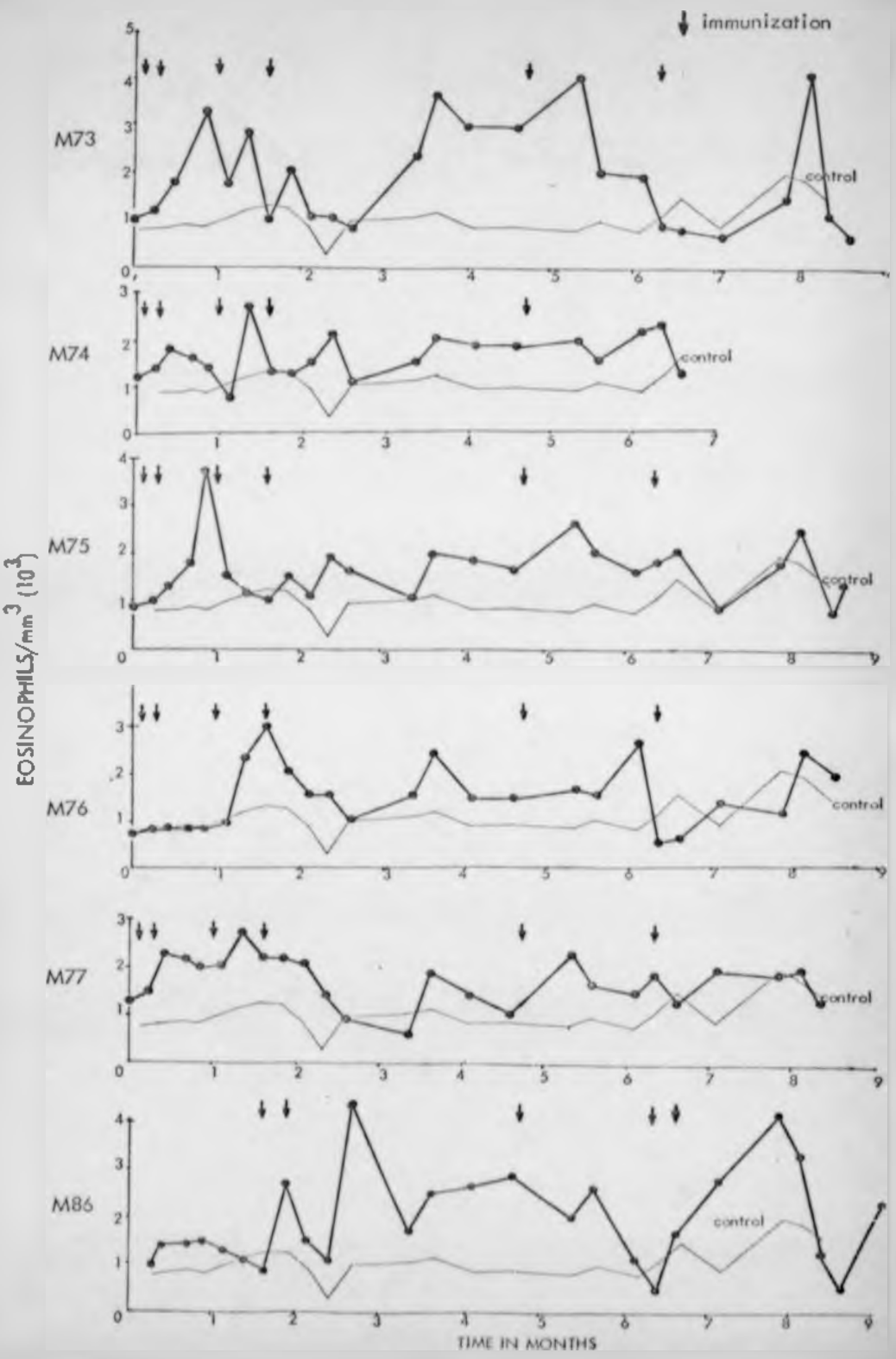
A. Eosinophil levels in the peripheral blood of cats infected with infective larvae irradiated with 25 krads. are shown in Fig. 21.

The eosinophil counts in the peripheral blood of cats inoculated with larvae irradiated with 25 krads. was generally lower throughout the period of observation than in cats infected with non-irradiated larvae. The highest levels recorded were 4,200 and 4,150 eosinophils/mm^3 (M86 and M75 respectively). Infective larvae irradiated with 25 krads. of Co.60 do not develop to sexual maturity to produce microfilariae (see Chapter 3). However, infection with irradiated parasites did produce an increase in the production of eosinophils when compared with the control animals.

It was difficult to correlate the eosinophil levels with the time of moulting of the parasite, as the parasites were inhibited in their development and did not develop beyond the fourth stage. The lower levels of eosinophilia recorded in these cats may be due to the lack of moulting fluid and secretory and excretory fluid from ecdysing worms. In many instances, immediately after inoculation with parasites, there was an increase in the eosinophil levels but this level returned to the plateau level subsequently.

After initial inoculation with the irradiated parasite, the time of the first peak of eosinophilia occurred between days 14 and 49. Again, no correlation between eosinophil response and the developmental stages of the parasite was apparent.

Fig. 21. EOSINOPHIL COUNTS IN CATS INFECTED WITH B. PAHANGI IRRADIATED WITH 25 krads.



B. Eosinophil counts of animals infected with larvae irradiated with 10 krads. are illustrated in Fig. 23, 23 and 24 (Cats A42, A43, A44, A50, A53, A55, A56, A58, A59 and A60). Eosinophil responses in these cats could be broadly divided into 2 groups. In the first group of animals, comprising cats A44, A55 and A56, the level of circulating eosinophilia was moderate. The other animals had raised eosinophilia in the peripheral blood, which remained high throughout the period of the experiment.

The eosinophil responses in cats infected with parasites exposed to 10 krads. were generally higher than in infections with parasites exposed to 25 krads. This was probably due to three factors. Firstly, the former group of cats were immunized on 10 different occasions (the latter group received 6 immunizing doses). Secondly, parasites irradiated with 10 krads. grew to become sexually sterile adult worms whilst those irradiated with 25 krads. reached only the fourth stage of the life-cycle. This indicated that adult stages induced greater eosinophilia. Lastly, worms irradiated with 10 krads. survived longer than did those irradiated with 25 krads.

Cats A44 and A56 (Fig. 22 and 23) showed only moderate numbers of circulating eosinophils. There were only two raised peaks of eosinophil response, one recorded during immunization and the other when the animals were challenged with non-irradiated worms. The other animals in this group (A42, A43, A50, A53, A58, A59 and A60) all had higher numbers of circulating eosinophils.

Fig. 22 EOSINOPHIL LEVELS IN CATS INFECTED WITH B. MALAYI IRRADIATED WITH 10 KRADS.

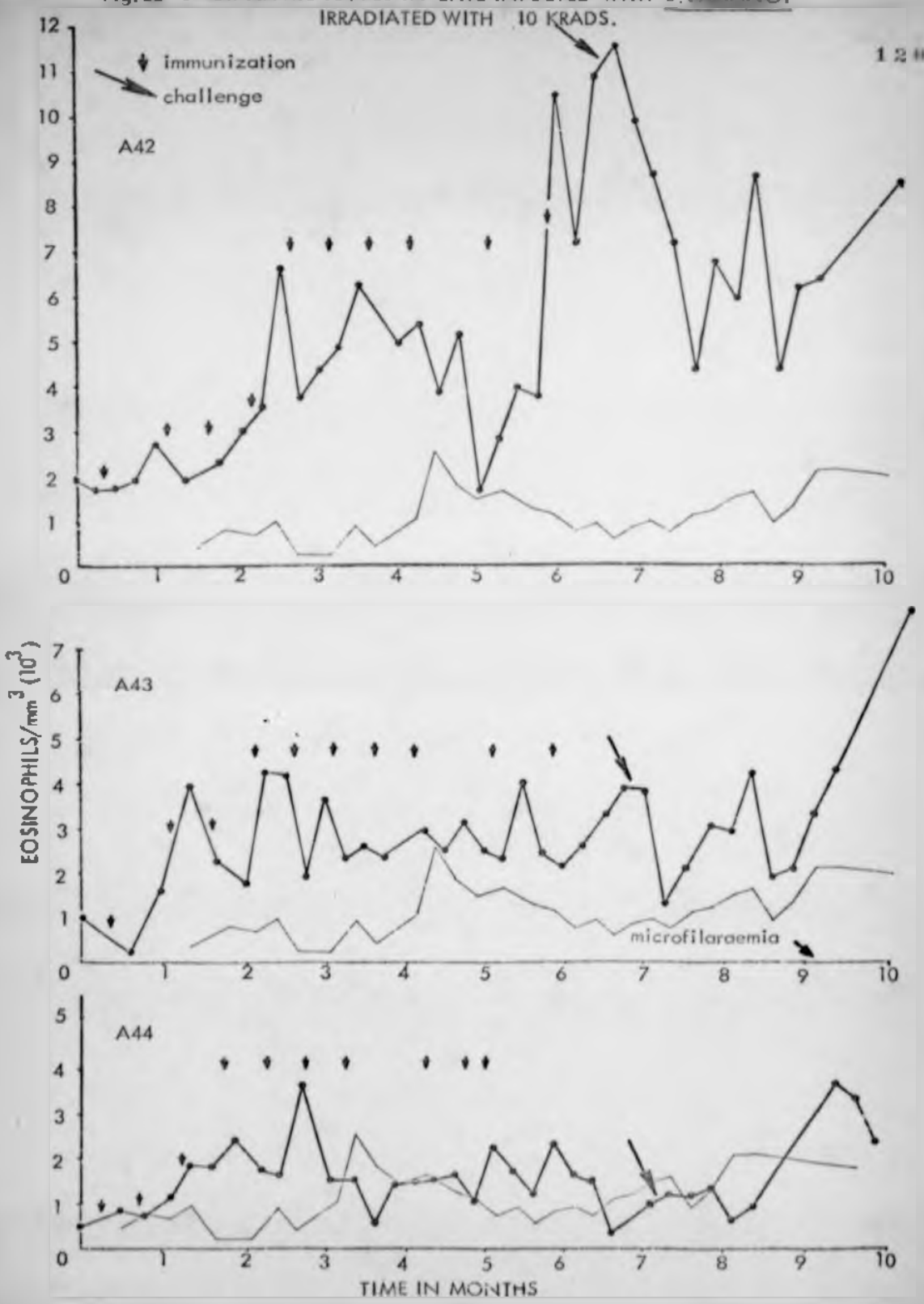


Fig. 23 EOSINOPHIL LEVELS IN CATS INFECTED WITH B. PAHANGI

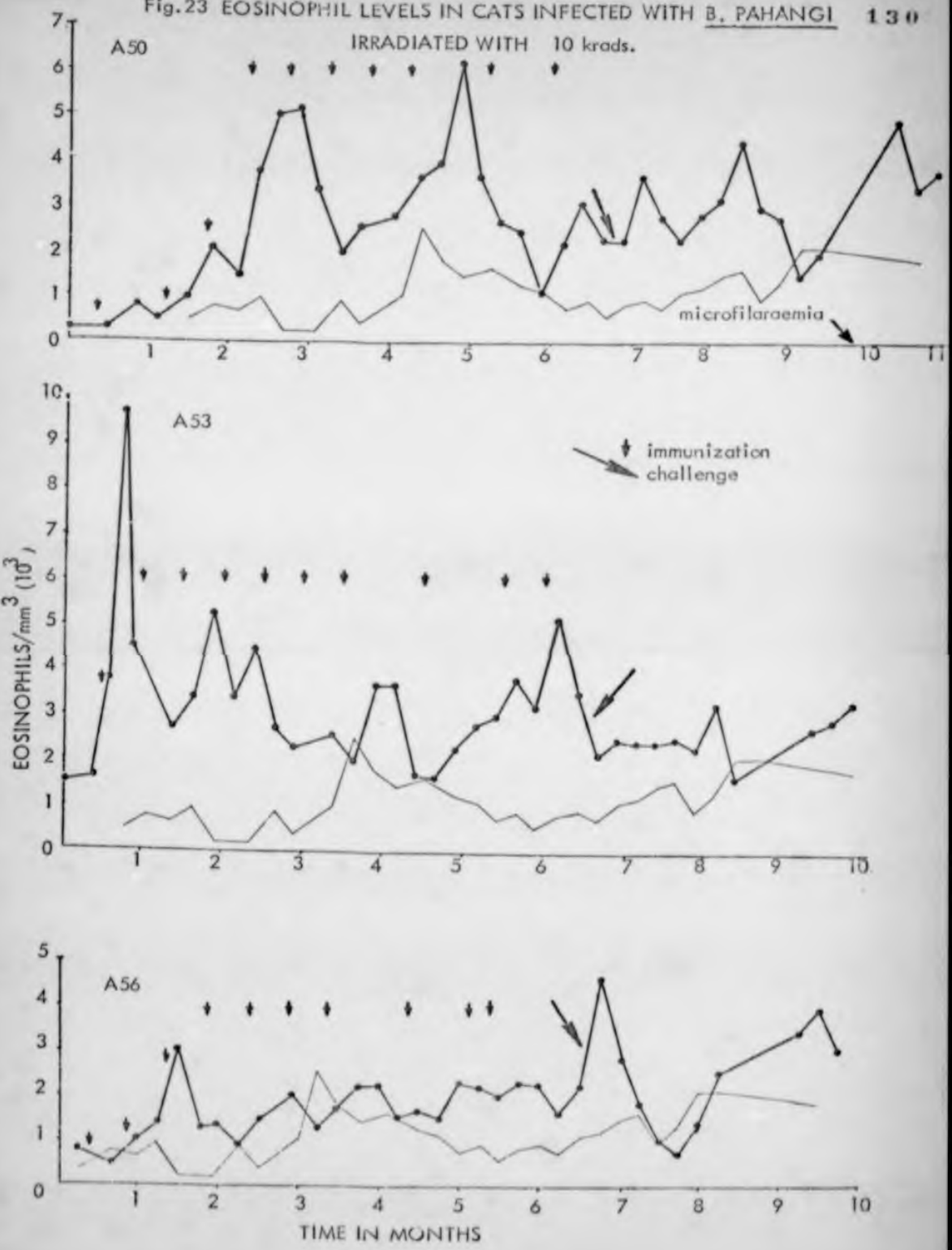
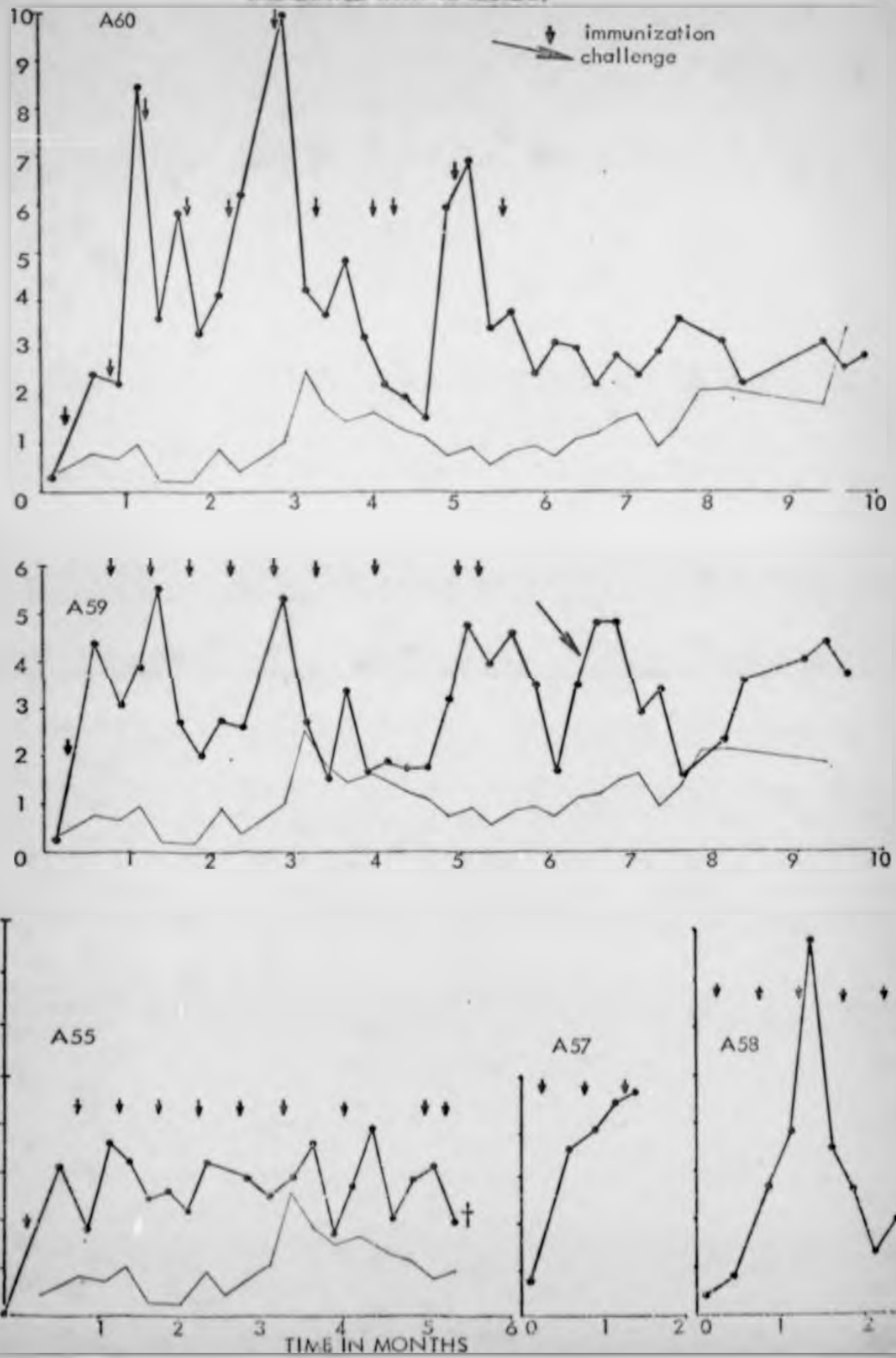


Fig. 24 EOSINOPHIL LEVELS IN CATS INFECTED WITH B. PAHANGI

IRRADIATED WITH 10 KRADS.



Eosinophil response of cats after challenge

Some of the immunised animals showed a sudden increase in the numbers of circulating eosinophils after challenge with non-irradiated *B. pahangi*. Cat A42 generally had high numbers of peripheral eosinophils. When this animal was challenged with non-irradiated *B. pahangi*, the highest level of eosinophilia in all these experiments was recorded (10,450 eosinophils/mm^3). Subsequently the number of eosinophils dropped to a lower level but remained much higher than in the uninfected control animals. In cat A43, the level of eosinophils after challenge with *B. pahangi* remained elevated for 4 days, but this level dropped subsequently. Cat A50 showed a similar pattern.

Cats A36 and A39 were given heterologous challenges with *Brugia patei*. Immediately following these inoculations there was an increase in the level of eosinophils in the circulation of these animals.

Cats A43 and A50 (Fig. 23 and 24) became patent as a result of some of the challenge worms maturing. In these two animals the onset of microfilaraemia was followed by an increase in the eosinophils.

Discussion

Leukocytosis and eosinophilia have been reported in human and experimental filariasis (Goodman *et al.*, 1945; Hodge *et al.*, 1945; Buckley, 1958 and Wong, 1974, 1975). The major change in the blood in filariasis and many other helminthic infections is the high level of eosinophils in the circulation. The studies in this chapter

indicated that cats that were repeatedly infected with non-irradiated and irradiated D. pahangi produced varying degrees of eosinophilia in the peripheral blood. However, this study gives no correlation between the numbers of circulating eosinophils in these infections and those in various tissues.

In cats infected with normal larvae, there were no distinctive peaks of eosinophils within the first 2 months that could be correlated with the moulting phases of the parasite. In experiments with repeat infestations, however, such correlations would be inaccurate. To elucidate, if moulting larvae released antigenic material that causes an increase in the production of eosinophils, animals given single infections with the parasite would have to be studied. When cats were infected with D. pahangi irradiated with 10 krads., these parasites underwent moulting from one stage to another at a slower pace.

The invasive stage of parasites causes appreciable tissue damage that normally resulted in raised eosinophilia (Archer, 1963). This statement is generally accepted to be true, as peripheral eosinophils are thought to be 'en route' to tissue sites. However, Weber (1958) could not correlate raised eosinophil response to the time when D. viviparus penetrate the host lung.

Eosinophil response during a parasitic infection can be divided into 3 phases (Lavier, 1944-45). After infection, a period of induction is followed by rapid increase, ending with high eosinophil counts in the form of a plateau value. These features were observed in experimental infections of cats with D. pahangi.

The onset of microfilaraemia induced a high level of eosinophilia in cats, and often this level was found to be the highest count

recorded in these animals. Animals immunized with irradiated parasites that failed to show complete protection against challenge, also showed an increase in the eosinophil response at the time of microfilaraemia after challenge. Such high levels of circulating eosinophilia, however, did not persist for long periods, even though increased numbers of microfilariae were being released into the peripheral blood. It can be conclusively said that microfilariae and/or uterine products released during birth of microfilariae were eosinotactic. Antigen-antibody complexes play a role in the induction of eosinophil response (Sabesin, 1963). Immune complexes may be formed in the blood of cats infected with B. pahangi and induce eosinophilia, but eosinophil levels declined despite increased numbers of microfilariae appearing in the peripheral blood. This may be due to the host becoming tolerant to microfilarial antigen. One animal observed over 2 years had normal levels of eosinophils although the animal had high numbers of circulating microfilariae.

Various authors have demonstrated, using *in vitro* techniques, that microfilariae are particularly attractive to eosinophils. Only the exsheathed microfilariae of W. bancrofti, incubated in eosinophil preparations from patients with high eosinophilia (Fros et al., 1952; Bergman, 1932), attracted eosinophils. Bergman (1932) hypothesised that exsheathed microfilariae may release metabolic products that cause such adherence. However, this adherence could not be demonstrated when microfilariae of L. carinii were incubated with eosinophil preparations obtained from pleural exudates of albino rats (Mahan, 1974). Higashi and Chowdhury (1970) showed that eosinophils from sensitised persons adhered to infective larvae of W. bancrofti in the presence of [illegible].

In this experiment it was clear that live parasites caused an increase in eosinophilia. However, Wong et al. (1974) found that higher eosinophilia was recorded when adult D. immitis died in the infected monkeys.

Less eosinophilia resulted when cats were infected with irradiated parasites, and the response decreased when the dosage used to irradiate the parasite was increased. Thus, it is evident that parasites allowed to mature, without interference, induced higher eosinophil responses. This was due to the antigenic materials released by developing parasites in the form of secretory and excretory substances. If the parasite was severely inhibited in its development, as they were when irradiated with 25 krads., the eosinophil response was moderate. A less severe irradiation (when irradiated with 10 krads.) caused a higher response in the host. T. spiralis irradiated to sexual sterility did not produce an eosinophilic response as did the untreated worms (Scardino and Zaiman, 1962).

Tropical eosinophilia or 'eosinophilic lung' has long been associated with filarial infections (reviewed by Donoghue, 1963). Wong (1974) found microfilariae of D. immitis trapped in the lungs of dogs, with granulomatous lesions surrounding these microfilariae, and postulated that this may correspond to the eosinophilic lung described by Damaraj et al. (1966).

Chapter 7

ANTIBODY RESPONSES OF CATS INFECTED WITH B. PAHANGI DETECTED BY INDIRECT FLUORESCENT ANTIBODY TECHNIQUE (IFAT)

Introduction

Many serological tests have been used to detect filarial infections and Kagan (1974) has reviewed the subject. The most extensively used standardised methods of diagnosing filarial infections are the skin test, using the Sawada antigen (prepared from adult D. immitis) (Sawada et al., 1965) and complement fixation test (Damaraj, 1957). Smith et al. (1971) evaluated the intradermal test by asking persons from different countries to use this antigen and concluded that clear diagnosis of filariasis was difficult when Sawada antigen was used. Dondero and Ramachandran (1972) also met with lack of specificity when they used this test. Ambroise-Thomas and Kien Truong (1974) tested the specificity of D. viteae antigen to diagnose many other parasites and found that the antigen can be used in the same way as the Sawada antigen.

Coons et al. (1942) first described the fluorescent antibody test and it has been used to detect antibodies against various helminth infections; T. spiralis (Jackson, 1959), S. mansoni (Sadun et al., 1960), Ascaris suum (Taffs and Voller, 1962), D. filaria (Movsesijan and Lalic, 1971) and N. brasiliensis (Sousse, Wescot and Gorham, 1976). Lucasse (1962) and Lucasse and Hoeppli (1963) adapted this technique for detection of onchocerciasis. The fluorescent antibody test has been used to detect antibodies against other filarial infections; Chowdhury and Schiller (1962) for W. bancrofti

and B. malayi; Duxbury and Sadun (1967) for W. bancrofti and Onchocerca volvulus; Ishii and Tanaka (1968) for L. carinii; Jayawardane and Wijayaratnam (1968) for W. bancrofti, B. ceylonensis, and Dirofilaria repens; Wong and Guest (1969) for B. malayi; Muller (1970) for Dracunculus medinensis; Wong (1974) for D. immitis; and Ponnudurai et al. (1974) for B. pahangi.

The FA test is now routinely used for the diagnosis of several protozoan diseases (African trypanosomiasis, amoebiasis, leishmaniasis, Chagas disease, malaria and toxoplasmosis) and a few helminth infections (trichinosis, schistosomiasis and echinococcosis) (Kagan, 1974). The test is easily performed, and requires only small quantities of reactants.

In this chapter the FA test was used to study the antibody responses of cats given either single infections or repeated infections with irradiated and non-irradiated infective larvae of B. pahangi. Infective larvae, frozen sections of fourth stage larvae and adults, and microfilariae were used as antigens.

Materials and Methods

Collection of blood samples

In cats, the marginal vein was found to be very convenient for collecting blood samples, as much as 5 ml. of blood being easily obtained from cats anaesthetised with Saffan (Glaxo Lab. Veterinary, Middlesex, England), a specific anaesthetic for cats. The blood samples were kept at 37°C for 20 minutes, and centrifuged at 2,000 r.p.m. for 15 minutes. Serum was stored in the deep freeze at -20°C.

Whole worm antigen

Indirect FAT was carried out using whole infective larvae and microfilariae of B. pahangi. Microfilariae were obtained by collecting citrated blood from infected cats with high microfilarial counts. This blood was mixed with 10 times its volume of cold distilled water and the mixture poured through a stainless steel sieve (500 meshes per inch). A jet of cold distilled water was directed onto the surface of the sieve in order to lyse any remaining red blood cells. The microfilariae trapped on the sieve were transferred to centrifuge tubes and washed with PBS. The washed microfilariae were stored at -20°C.

Third stage larvae of B. pahangi were obtained by the method described in Chapter 2 and washed several times in PBS before being stored at -20°C.

Cryostat sections of fourth stage and adult worms

PTFE coated multispot microscopic slides were cleaned in a solution containing a mixture of alcohol and acetone to remove any trace of grease. These slides were subsequently used to mount frozen sections of fourth and fifth stage worms. A small knot of worms was embedded in Tissue Tek (Ames Company) medium contained in a capsule, frozen with solid CO_2 and placed in the cryostat at -30°C for at least one hour before use. 5µ sections were cut and transferred onto the multispot slides. These were wrapped in aluminium foil and stored at -20°C.

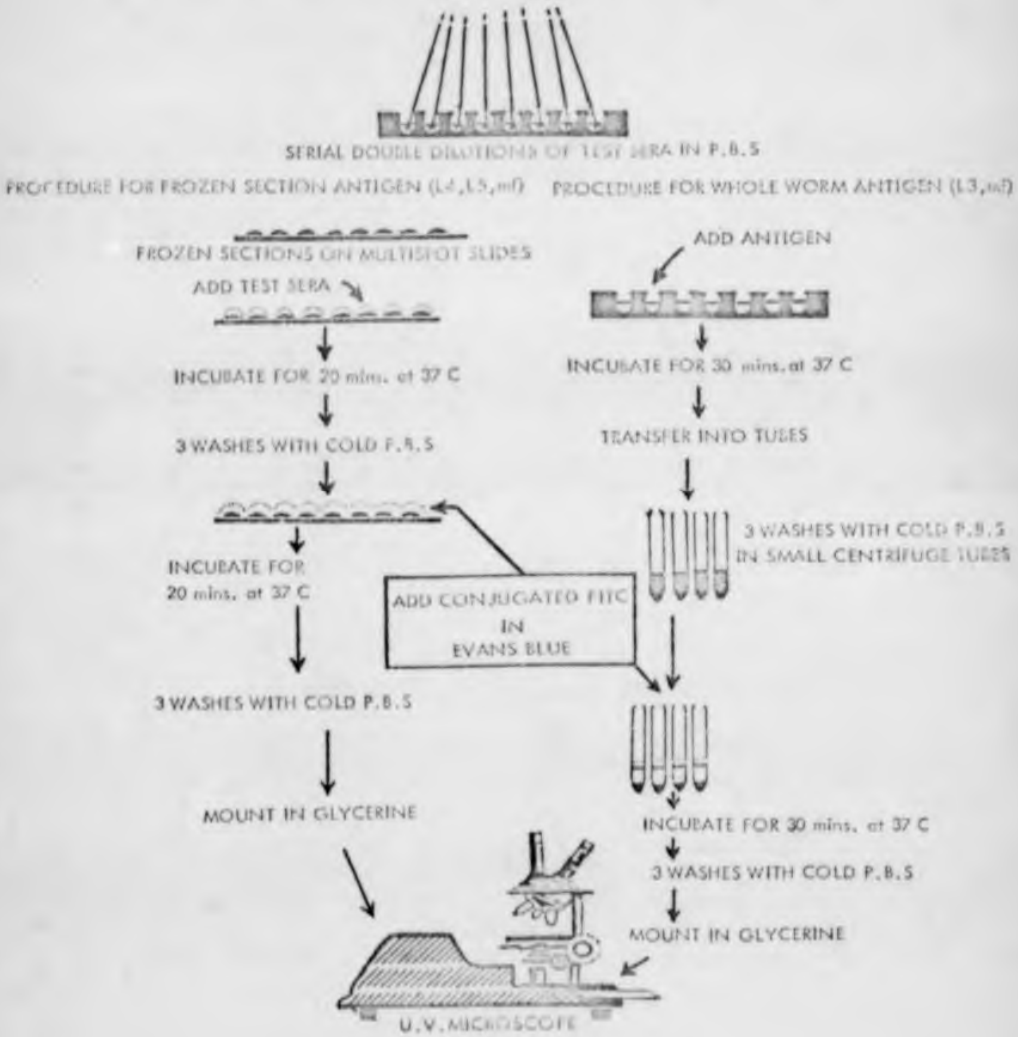
Techniques using whole worm antigen in the FA test

Small centrifuge tubes were prepared, by cutting the thinner end off Pasteur pipettes and sealing them with a flame. These smaller tubes enabled more tests to be done at any one time and also reduced antigen loss during washing. (A summary of the technique is illustrated in Fig. 25a).

Complement fixation trays (Microtiter) were employed in making serial double dilutions of serum. 0.025 ml. of cold PBS was placed in each well. 0.025 ml. of test serum was mixed in the first well and serially diluted with the aid of microtiter diluters. The antigen to be used was made in PBS such that each drop of 0.025 ml. contained approximately 6 inactive larvae or about 50 microfilariae. One drop of this preparation of antigen was added to the dilutions being investigated. (It is to be remembered that due to adding antigen suspended in 0.025 ml. of PBS, the sera were diluted 1 in 2.) The plate was sealed with a microtiter plate sealer and kept at 37°C for 30 minutes. The contents were transferred into small centrifuge tubes and spun at 1,000 r.p.m. for 5 minutes. Most of the fluid was removed, fresh cold PBS was added and the tubes recentrifuged. The larvae were washed three times in this way.

Most of the PBS used for the final wash was discarded, leaving a small quantity suspending the antigen. To this 0.025 ml. of fluorescein labelled rabbit anti-cat serum diluted in 0.25% of Evans Blue (EB) was added. The tubes were agitated and incubated at 37°C for 30 minutes. Excess unbound conjugate was removed by repeated washing with PBS as described above. During the final wash, PBS was replaced by buffered glycerine. The worms were transferred

Fig. 25a. INDIRECT FLUORESCENT ANTIBODY TECHNIQUE



te microscope slides and cover slips placed over them. Results were read under a Nikon fluorescence microscope with a 200 watt high pressure mercury lamp.

Technique using frozen sections for the FA test

Multispot slides with sections of antigen were removed from the -20°C deep freeze and kept in a desiccator at room temperature for 1½ hours. The sections were fixed with acetone for 30 seconds and placed inside a black perspex humidity chamber. Care was taken not to allow the frozen sections to dry at any stage of the test.

The sera under test were serially diluted, as described earlier, and the required dilutions of serum transferred onto the frozen sections on the multispot slides. The humidity chamber was incubated at 37°C for 20 minutes. At the end of the incubation period, slides were washed in a trough of cold PBS and agitated for 15 minutes. The slides were then dried and returned to the humidity chamber. Aliquots of 0.025 ml. of diluted fluorescein labelled rabbit anti-cat serum in EB were added to cover the sections of antigen, and later incubated for 20 minutes at 37 C. The slides were washed in cold PBS for 15 minutes, dipped into acetone to differentiate the sections, then mounted in buffered glycerine. The slides were examined under a Nikon fluorescent microscope.

In all tests done, three controls were included, a positive serum for the antigen, a control serum from an uninfected cat, and finally a PBS control.

A positive reading for the serum was recorded when the cuticle of the cryostat section of the worm fluoresced (see Plates 12 and 13),

Plate 12. Positive IFAT reaction with adult frozen sections (x200)

Plate 13. Negative IFAT reaction with adult frozen sections (x200)

Plate 14. Positive IFAT reaction with microfilaria (x350)

Plate 15. Negative IFAT reaction with microfilaria (x350)

Plate 16. Positive IFAT reaction with third stage larva (×200)

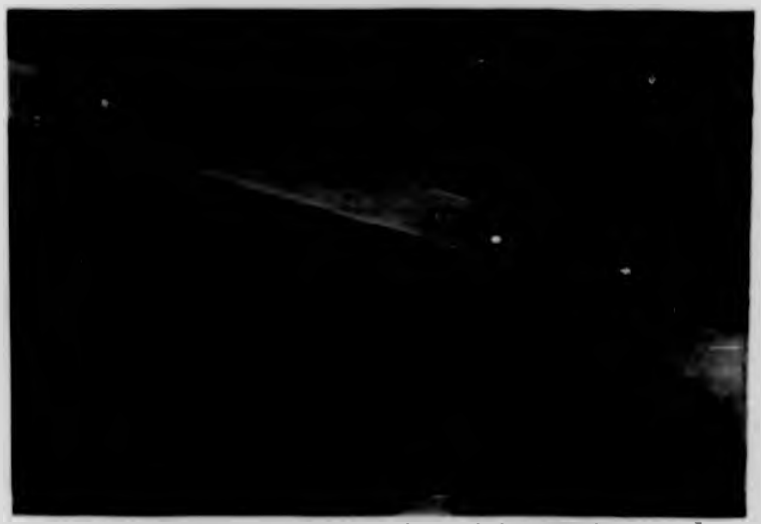


Plate 17. Negative IFAT reaction with third stage larva (×350)

or in the case of whole worm antigen, when the cuticle of the worm fluoresced in its mid-region. Plates 14 and 15 show positive and negative readings for microfilariae; and Plates 16 and 17 show the positive and negative readings for third stage larvae by the IFAT. The last dilution of the serum in which fluorescence was observed was considered as the end point of the titration.

Serum samples

Serum samples were collected from cats before infection and on days 4, 8 and every week after inoculating infective larvae of B. pahangi. The samples were stored at -20°C.

Results

Single infections

Four cats were given one infection with 100 infective larvae of B. pahangi in the Lhl, and antibody responses of these cats to different antigens studied over a period of 200 days.

Antibody responses against the 4 different antigens are shown in Fig. 23b. Table 17 shows the mean geometric titres.

Antibody responses against infective larvae were first detected between days 56 and 60 after initial infection. The antibody titres of the sera of these cats against infective larvae increased from 8 (geometric) to 32, 32 and 16 in cats P1, M50 and M53 respectively. The titres remained at this level until the end of the experiment. One animal, P2, did not become infected, and at no stage of the

Fig. 25b ANTIBODY RESPONSE OF CATS INFECTED WITH B. PAHANGI (non-irradiated)
(SINGLE INFECTIONS)

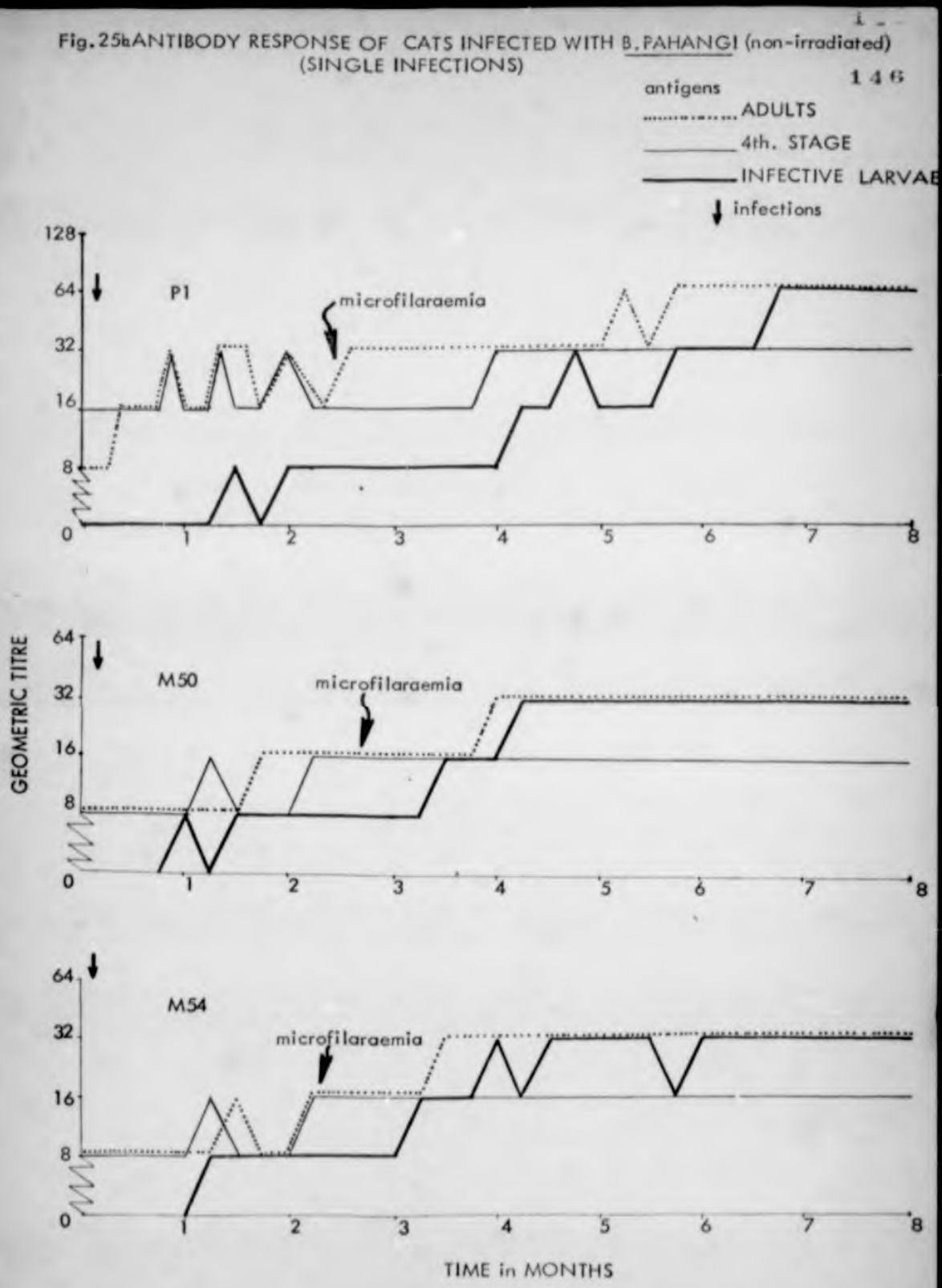


TABLE 17. ANTIBODY TITRES OF CATS GIVEN A SINGLE INFECTION OF B.PAHANGI AGAINST DIFFERENT ANTIGENS. (HIGHEST TITRES)

cat No. / antigen	P1	P2	MSD	MS4	mean titre
3rd. stage larvae	64	0	32	32	32
4th. stage larvae	32	0	16	16	16
adults	32	0	32	32	24

experiment did the serum from this cat react positively against the various antigens used.

Antibodies against fourth stage larvae (frozen sections) were first observed from days 25 and 32 after initial infection. There was non-specific fluorescence when this antigen was used at geometric titres of 8 and 16. Antibodies against this stage did not increase above a titre of 32.

When adult frozen sections were used as antigens, the antibody was first observed from day 9 to day 38. There was non-specific fluorescence at titres of 8. The titres increased to 32 and remained at this level.

In none of these cats were antibodies against microfilarial antigen found.

Repeat infections

Five cats were infected initially with 100 infective larvae of B. pahangi in the Lhl, and when these animals became patent they were inoculated in the Lhl with 50 infective larvae on 10 occasions. The antibody responses to different antigens were studied throughout this period.

Antibody response of cats given repeat infections of B. pahangi are shown on Fig.26a and 26b. The mean geometric titre against the three different antigens is shown on Table 18.

Titres against infective stages of B. pahangi were first observed from days 25 and 62 after initial infection. Antibody titres when these animals became microfilaraemic were 16, and this level increased with repeated infections, to a geometric mean titre

Fig. 26 ANTIBODY RESPONSE OF CATS INFECTED WITH B. PAHANGI (non-Irradiated)
(REPEAT INFECTIONS)

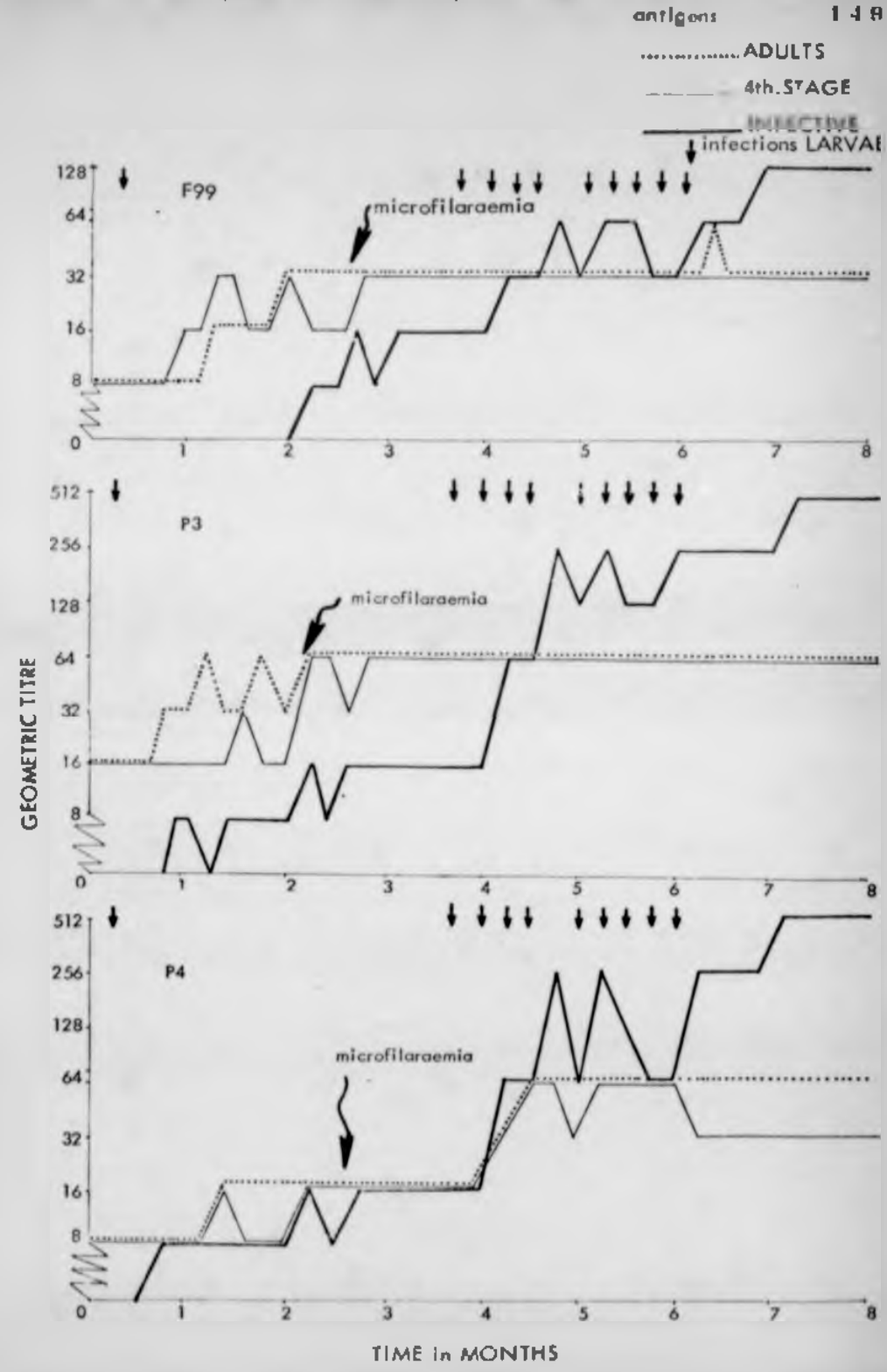


Fig. 26b ANTIBODY RESPONSE OF CATS INFECTED WITH B. PAHANGI (non-irradiated)
(REPEAT INFECTIONS)

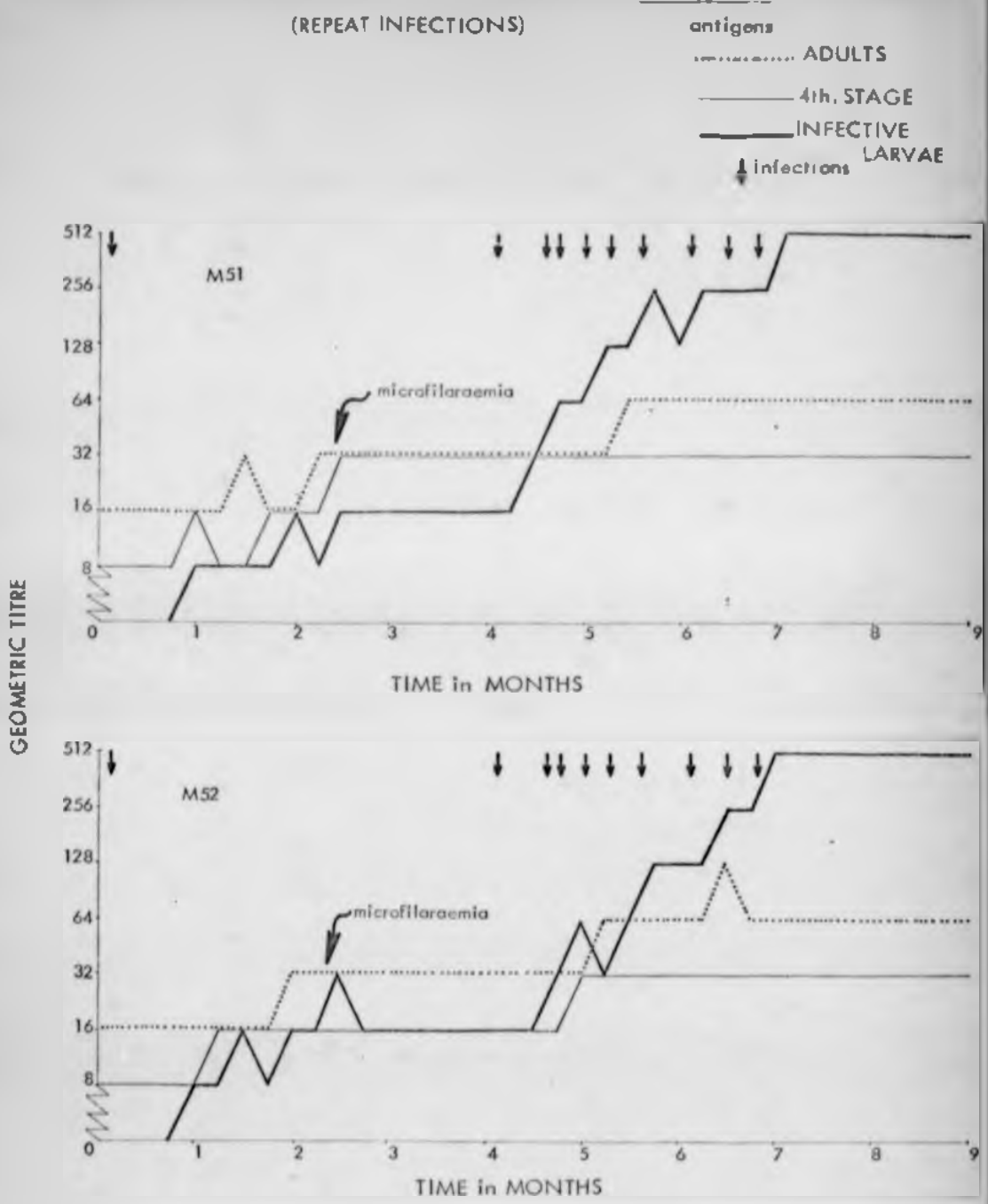


TABLE 18. ANTIBODY TITRES OF CATS GIVEN REPEAT INFECTIONS OF B.PAHANGI AGAINST DIFFERENT ANTIGENS. (HIGHEST TITRES)

cat No. / antigen	P3	P4	F99	M51	M52	titre
3rd. stage larvae	512	512	128	512	512	444.0
4th.stage larvae	64	64	32	32	32	44.8
adults	64	64	64	64	128	76.8

of 444.0.

Antibody titres against fourth stage larvae of B. pahangi were first observed from day 35 and day 44 after initial infections. Titres against this stage did not increase above 64.

When frozen adult worm sections were used as antigens, titres were first seen from day 21 and 33. Despite repeated infections with infective larvae amounting to a total of 500 worms, the titres against adult stages did not increase significantly. The highest titre value recorded against this stage was 128.

One of these cats (M52) became amicrofilaraemic; from this time onwards the sera reacted positively against microfilariae at serum dilutions of 1 in 128 but in none of the other cats was antibody against microfilariae found.

Antibody studies were also made on the cats repeatedly infected with normal larvae, reported in Chapter 4. In view of the results obtained with the detailed study of the repeatedly infected cats, described above, only infective larvae were used as antigen for these cats. The antibody titres are shown in Fig. 27-30.

Cat M87 which was infected 6 times with 300 infective larvae each inoculation, became amicrofilaraemic 124 days after initial infection. From this time, the sera of this animal reacted positively against microfilariae.

Infections with irradiated [redacted]

Antibody studies were also made on some of the cats infected with irradiated larvae as reported in Chapter 4.

Antibody response of a cat infected with B. pahangi irradiated with 25 krads. of Co.60 is shown in Fig. 29.

Fig. 27 ANTIBODY RESPONSE OF CATS INFECTED WITH IRRADIATED B. PAHANGI (10krads.) (Ag: infective larvae)

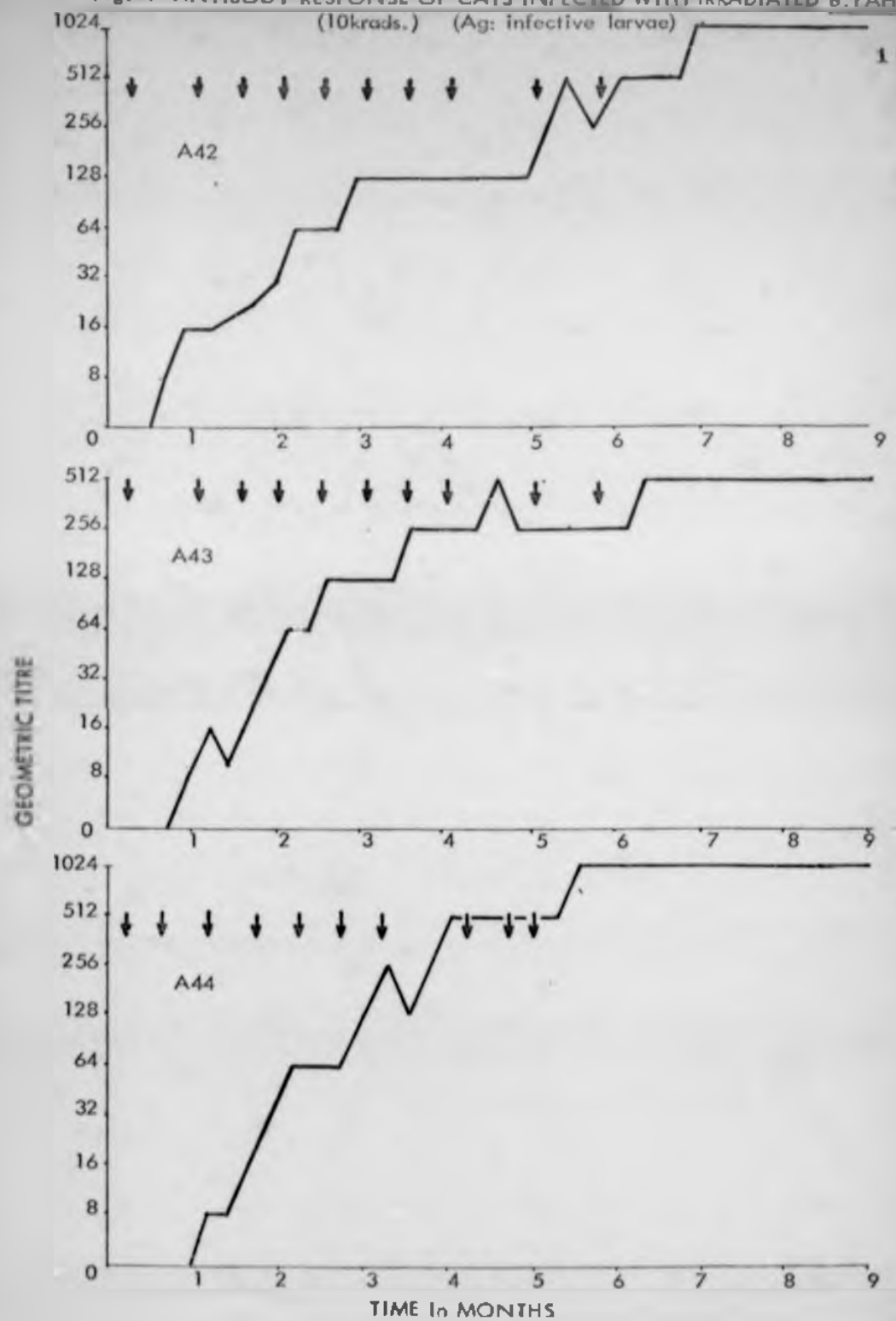


Fig. 28 ANTIBODY RESPONSE OF CATS INFECTED WITH IRRADIATED B. PAHANGI (10 krads.) (Ag. infective larvae)

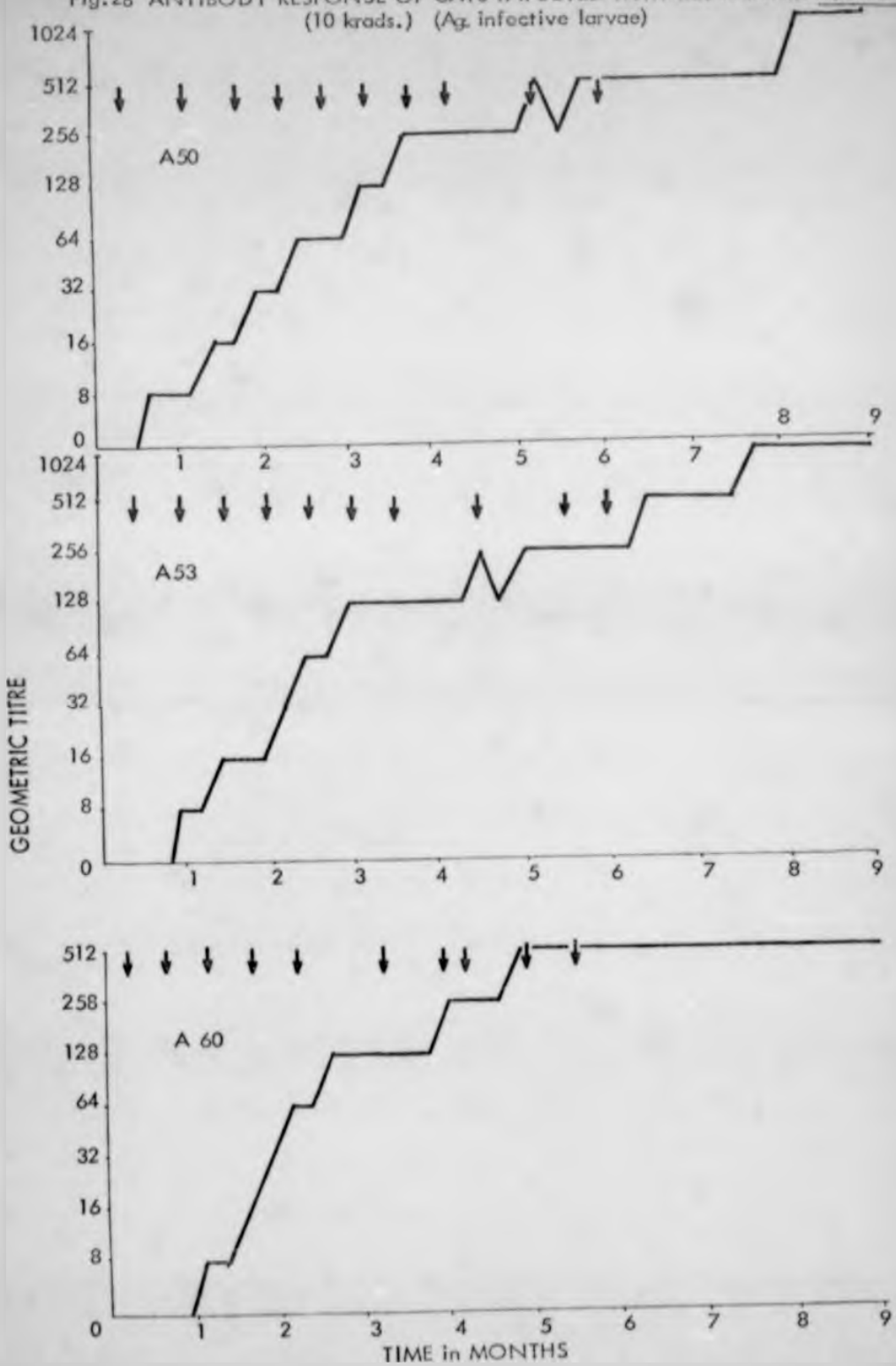


Fig. 29 ANTIBODY RESPONSE OF CATS INFECTED WITH IRRADIATED B. PAHANGI (10 krads.) (Ag: infective larvae)

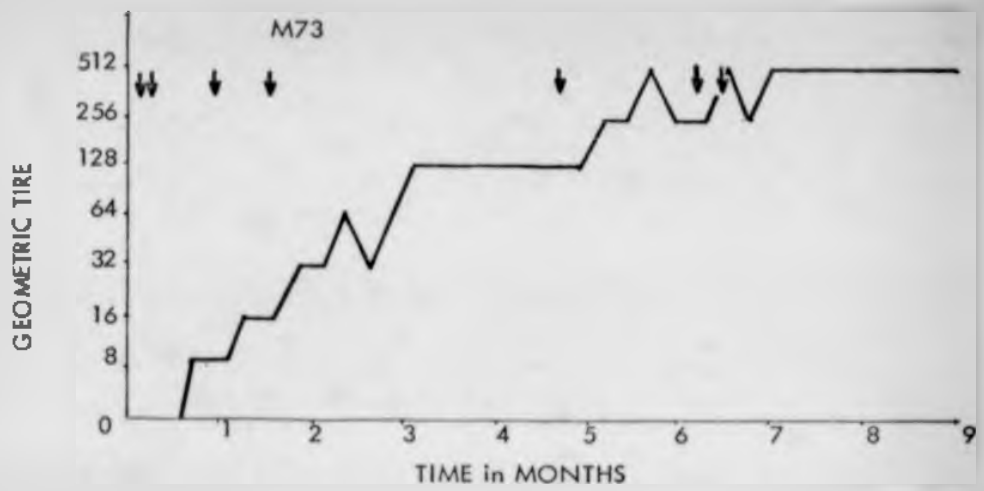
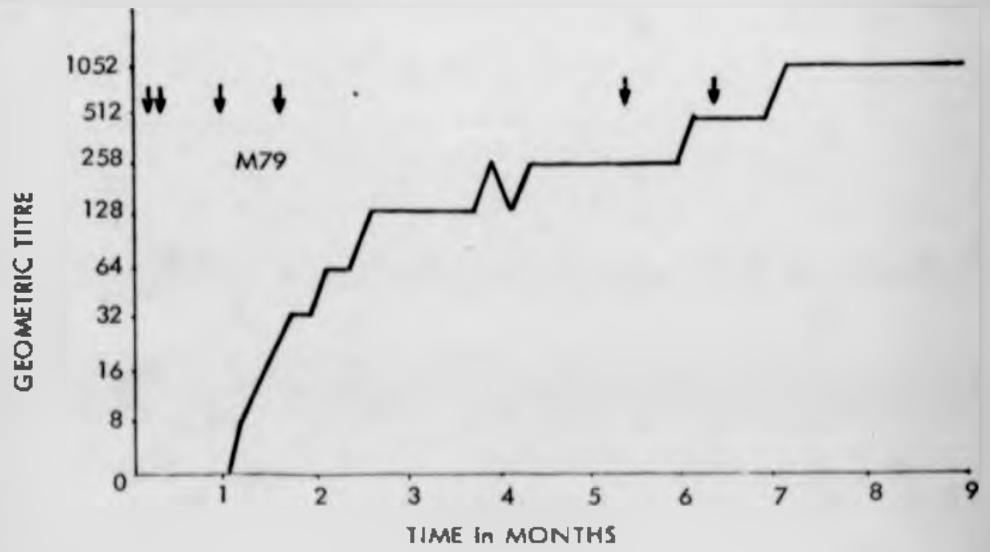


Fig. 30 ANTIBODY RESPONSE OF CATS INFECTED WITH NON-IRRADIATED B. PAHANGI



Antibody responses against infective larvae was first detected from day 21 to 35 in these animals. The titres increased steadily, with repeat infections. The mean of highest geometric titres recorded in the two groups of animals are shown in Table 19.

Lower mean titres were found after the use of irradiated larvae than were found in cats similarly infected with normal (non-irradiated) larvae. As reported above, sera collected from cats infected with non-irradiated B. pahangi reacted positively against fourth and adult stages of the parasite. However, sera obtained from cats immunized with irradiated larvae did not react positively against these antigens.

Antibody responses of cats immunized with B. pahangi irradiated with 10 krads. of Co.60 against infective larvae are shown on Figs. 27 and 28, and titres were first observed from day 21 to 31. The highest titres recorded varied, and the mean of the geometric titres in these cats was 847.4 (see Table 20). Sera from these cats did not react positively against fourth, adult stages or microfilariae.

Studies with heterologous antigens

Sera from cats infected with B. pahangi were used to determine if fluorescent antibodies against B. pahangi antigens cross reacted with other filarial antigens (B. malayi, B. patei, W. bancrofti and D. witeae).

Tables 21, 22 and 23 show the results of heterologous antibodies detected by FAT in cats infected with B. pahangi against third stage larvae of B. patei, D. witeae; against adults of B. patei and D. witeae; and against microfilariae of W. bancrofti, B. patei and B. malayi.

TABLE 19. ANTIBODY RESPONSES AS DETERMINED BY IFAT OF CATS IRRADIATED AND NON-IRRADIATED B.PAHANGI.(antigen: 3rd.stage larvae)

IRRADIATED WITH 25 KRADS.

cat No.	M73	M74	M75	M76	M77	M86	mean titre
highest antibody titre	512	512	256	1024	1024	512	565.1
day first titre recorded	21	28	28	21	21	35	25.7

NON-IRRADIATED INFECTIONS

cat No.	M79	M85	M87	M89	mean titre
highest antibody titre	1024	1024	512	1024	896
day first titre recorded	21	28	28	21	24.5

TABLE 20 . ANTIBODY TITRES OF CATS INFECTED WITH B.PAHANGI IRRADIATED WITH 10 KRADS.

cat No.	A42	A43	A44	A50	A53	A56	A60	mean titre
highest antibody titre	1024	512	1024	1024	1024	512	512	847.4
day first titre recorded	21	28	35	21	28	21	35	27.0

FA TITRES OF CATS IMMUNIZED WITH B.PAHANGI, USING OTHER FILARIAL PARASITES AS ANTIGENS

TABLE 21. antigen: third stage larvae
sera dilution: 1/32

filaria spp. / cat sera	B.pahangi	B.patei	D.witeae
positive	+	+	-
control	-	-	-
PBS	-	-	-

TABLE 22. antigen: adult worm sections
sera dilution: 1/32

filaria spp. / cat sera	B.pahangi	B.patei	D.witeae
positive	+	+	-
control	-	-	-
PBS	-	-	-

TABLE 23. antigen: microfilariae
sera dilution: 1/32

filaria spp. / cat sera	B.pahangi	B.malayi	B.patei	W.bancrofti
positive	+	+	-	+
control	-	-	-	-
PBS	-	-	-	-

Heterologous fluorescent antibodies were detected against infective larvae and adults of B. patei but not against those of D. witesa. Cats which had lost their circulating microfilariae, had antibodies that reacted positively with microfilariae of W. bancrofti and B. malayi but not against microfilariae of D. patei.

Discussion

In the present investigations, the sequential antibody responses of cats infected with B. pahangi to various stages of the same parasite were studied. Antibodies against infective larvae were detected only after 38 days in single infections. It may be that antibodies were present earlier than this but their presence was not demonstrated with the FA test. After antibody against third stage larvae had been detected, the titres increased gradually even after the worms had developed to adults. It is possible that infective larvae are more immunogenic than the other life cycle stages, but it is equally possible that the whole worm test using third stage larvae is more sensitive than the test using sections of adult worms even for detecting anti-adult antibodies.

Infections with infective larvae irradiated with 25 krads. did not evoke as high antibody titres as were seen in cats infected with non-irradiated B. pahangi. This may be explained by the altered metabolic activities of irradiated parasites. Infective larvae irradiated with 25 krads. did not grow beyond the fourth stage (see Chapter 3) and cats infected with such irradiated parasites had antibody against infective larvae, but none against the fourth and adult stages.

After irradiation with 10 krads. larvae developed to the young juvenile stage but even with this type of infection no antibodies were found against fourth larval stage and adult antigens. Higher antibody titres against third stage larvae were found than after infection with larvae irradiated at 25 krads.

The fourth stage larvae of D. pahangi appear to be the least active antigen in the FA test.

Antibodies against adult worms were first observed from day 21 to 33 after initial infection. Titres against this stage did not increase significantly in repeat infections compared with single infections. Titres recorded immediately before the infected animals became microfilaraemic did not change significantly despite repeat infections with 500 infective larvae. Ponnudurai et al. (1974) recorded that the highest antibody titres against adults were detected when the animals became microfilaraemic, and the level did not increase even if the animals received further infections.

In this experiment, two cats showed antibody against microfilariae during their post-microfilaraemic phase. This is in agreement with the observations of Ponnudurai et al. (1974). In none of the cats in which microfilariae were still circulating were antibodies against microfilariae detected. Mantovani and Sulmer (1967) used microfilariae of D. immitis, Dirofilaria repens and D.viteae, disintegrated by ultrasonic vibrations, to test the sera from infected dogs with circulating microfilariae and demonstrated fluorescence at the broken ends of the microfilariae. Muller (personal communications) used the same technique as Mantovani and Sulmer for the diagnosis of onchocerciasis but obtained a high degree of non-specific fluorescence. Mantovani and Sulmer suggested that intact microfilariae of D. immitis

were not immunogenic. In the present investigations, both the sheath and the cuticle showed fluorescence when tested against sera obtained from amicrofilaraemic cats.

Lucasse (1962) and Lucasse and Hoeppli (1963), however, were able to detect antibodies against microfilariae of O. volvulus. This discrepancy in the reactivity of microfilariae of O. volvulus and other filarial worms (i.e. non-reactivity in subjects with circulating microfilariae) may be due to the fact that whilst other microfilariae are in the blood, microfilariae of O. volvulus are mainly found in the skin. Chowdhury and Schiller (1962) and Smithers (1968) suggested that microfilariae may be immunologically inactive. A more probable view is that the great abundance of microfilariae circulating in blood may be actively absorbing antibodies (Capron et al., 1968). With the removal of microfilariae from the circulation in cats, antibody against this stage appears in circulation. In onchocerciasis, microfilariae may not absorb antibodies from the blood.

The role of antibodies reacting in the FA test in protective immunity has not been demonstrated. Denham and Fakeer (unpublished) have found that such antibody does not confer passive immunity against challenge with infective larvae although kittens born to immunized mothers frequently showed very strong resistance to challenge.

It may be coincidental that after repeated infection with normal larvae, strong resistance to challenge was only shown by amicrofilaraemic cats and that these all showed an antimicrofilarial antibody. Though the appearance of free anti-microfilarial antibodies and protective immunity may not be related at all, it is significant to

note their simultaneous occurrence. There was little correlation between antibody response detected by FAT and resistance to challenges in other cases. There was no significant difference between the antibody titres and the degree of resistance of cats immunized with irradiated B. pahangi (as in Chapter 4). Cats repeatedly infected with B. pahangi (showing high antibody titres) challenged when they still had circulating microfilariae, were not protected.

There is an interesting similarity between the antibody responses of cats infected with B. pahangi and human patients infected with B. malayi. For example, cats after repeated infection with normal larvae show high anti-third stage larva titres but are negative in the anti-microfilaria test unless they have become microfilaria negative.

Both symptomatic and asymptomatic filarial patients had antibodies against infective larvae of B. malayi. (Wong and Guest, 1969) but antibodies against microfilarial stage was demonstrable only in patients who had no circulating microfilariae but had clinical filariasis.

Demonstration of antibodies against microfilariae was not possible in infected subjects with circulating microfilariae (Sadun, 1963; Jayawardene and Wijayaratnam, 1968; Woodruff and Wiseman, 1968; Wong and Guest, 1969; Muller, 1970; Ponnudurai et al., 1974).

Jayawardene and Wijayaratnam (1968) attempted to differentiate patients with filarial infections from patients suffering from tropical pulmonary eosinophilia by using the FA test, with microfilarial antigens from human and animal filarial parasites. However, there was lack of specificity, and sera from both groups reacted positively with microfilariae of W. bancrofti more than with the animal parasites. Others (Garcia, Cabrera and Lara, 1968; Fujita,

Tanaka and Sasa, 1970) also found that filarial worms showed much cross reactivity.

A sensitive serological test that can be easily performed in endemic areas will be invaluable as an aid to diagnosing early stages of filarial infections. It would be especially useful when parasitological demonstration of microfilariae in persons with low levels of circulating microfilariae is impossible, and in infections with parasites whose microfilariae show [illegible] periodicity. The intradermal test shows much cross reactivity with other helminth infections. A potentially more sensitive method of diagnosing infections, the enzyme-linked immunosabsorbent assay (ELISA) (Engvall and Perlmann, 1972) has been tested for diagnosis of various parasitic infections, including onchocerciasis (Bartlett, Bidwell and Voller, 1975).

Standardization of the FA test is greatly needed so that tests done in different laboratories could be compared. An attempt was made by Manawadu and Voller (1971) to rule out subjective estimation of end-point in the FA test by incorporating a fibre optic probe to measure the intensity of fluorescence.

Cross reactivity in filarial antigen-antibody reactions is a common feature. Sera from cats infected with B. pahangi which reacted positively against infective stages of B. pahangi, also showed similar high titres against infective larvae of B. patei, but not against those of D. witenae. Adults of D. witenae tested similarly did not react positively whilst there was positive reaction against adult worms of B. patei. Microfilariae (W. bancrofti, B. malayi, B. pahangi) reacted positively in FA test against sera from amicrofilaraemic cats, but microfilariae of B. patei were inactive. The

negative reaction obtained against microfilariae of B. patei is a curious feature. This may indicate that phylogenically, B. patei is distant from other human filarial parasites. A more detailed study, using different stages, may help in establishing the order of phylogeny of filarial parasites.

Chapter 8

HISTOLOGICAL CHANGES IN TISSUES OF CATS INFECTED WITH B. PAHANGI

Materials and Methods

During the autopsies of cats immunized with irradiated and non-irradiated B. pahangi, lung, heart and kidney tissue of these animals was fixed in Bouins fluid. The cervical lymph node and vessels of cat M89 repeatedly infected with B. pahangi, and M76, repeatedly infected with B. pahangi irradiated with [illegible] were also fixed in Bouins fluid. Paraffin wax sections were examined to determine the locations of irradiated and non-irradiated worms. During the autopsy of cat M76, a small nodule with degenerating worms was found. This nodule was fixed to study the degree of degeneration and calcification of the worms. Serial sections of this nodule were prepared. Lymphatics of cats A55, A57 and A58, infected with parasites irradiated with 10 krads., were also fixed in Bouins fluid. Sections were examined microscopically.

Observations

Lung

There was a slight thickening of the interstitial septum in cats infected with irradiated parasites. Mucous exudates, which result from irritation caused by a foreign body, were not observed in the bronchus and bronchioles of most of the cats observed. In the cat infected with B. pahangi irradiated with 25 krads. there

was hyperplasia of mucous glands around the bronchioles. In this cat and cats inoculated with parasites irradiated with 10 krads, areas of emphysema (alveolar inflation with gas) and alveolar collapse were seen. No focal reaction was detected. Both the arterial and venous walls were normal. Haemorrhage was seen. No parasites were seen.

In cats inoculated with non-irradiated parasites there was no focal reaction in the lungs. The interstitial septa were thickened and the alveoles were distended (emphysema). Blood vessel walls were thickened. Microfilariae were found in the interstitial spaces of the lungs of cats M79, M87 and M89 (see Plates 18 and 19). There was no cellular reaction around the microfilariae suggesting that they may be non-pathogenic. Microfilariae were found frequently in the blood capillaries. In all these cases, the epithelial linings of the bronchus and bronchioles were normal. In the lung of cat M87, which had become amicrofilaraemic, microfilariae were found in the lungs, trapped between the interstitial cells. These parasites were dead and undergoing degeneration.

No microscopic changes were observed in the heart or kidney.

Lymph nodes and vessels

Sections of lymph nodes and vessels of the infected and non-infected legs of cat A77, which was inferted with parasites irradiated with 10 krads., were examined microscopically. In the popliteal node of the infected limb there was an intense cellular reaction and many germinal centres were found (see Plate 20). No regions of focal

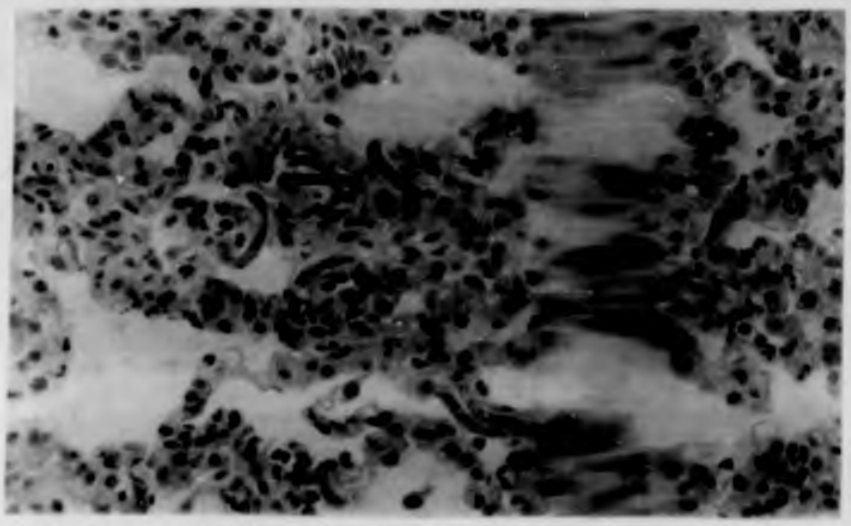


Plate 18. Microfilariae in interstitial [illegible]ues of lungs of cats infected with B.pahang[illegible]1200)(HE)

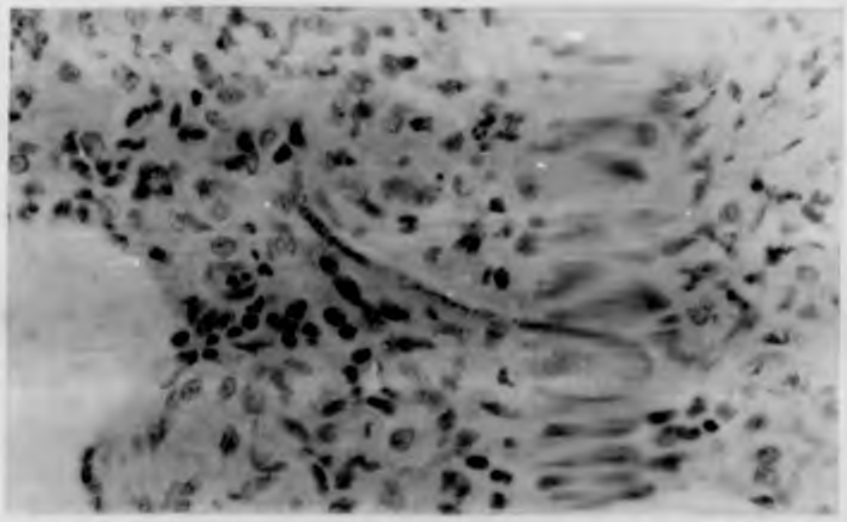


Plate 19. Microfilariae in interstitial [illegible]ues of lungs of cats infected with B.pahang[illegible]1200) (HE)

Plate 20. Popliteal lymph node from a cat limb infected with non-irradiated B.pahangi. (x220) (HE)

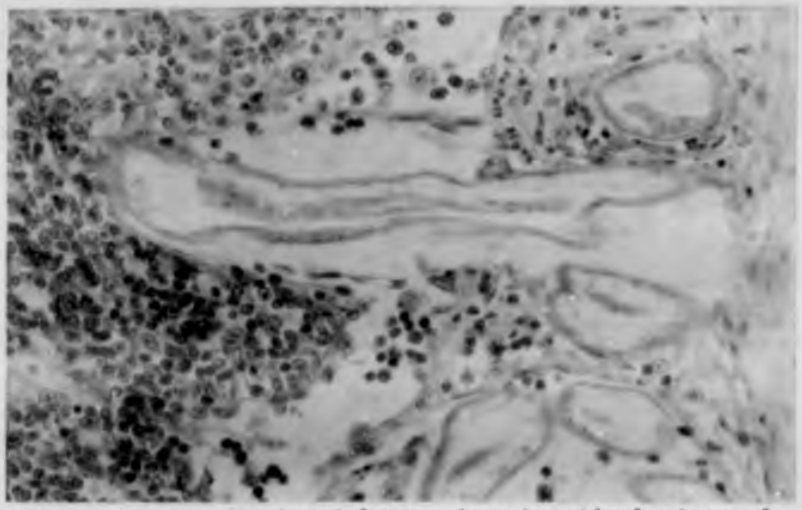


Plate 21. Live B.pahangi larvae in subcortical sinus of a popliteal lymph node. (x875) (HE)

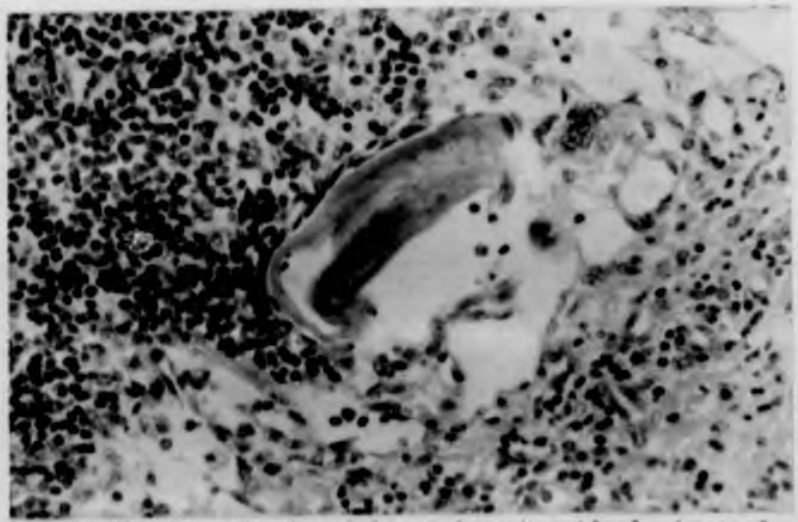


Plate 22. Live B.pahangi larva in subcortical sinus of a cervical lymph node. (x875) (HE)

Plate 23. B.pahangilarvae in different stage of degeneration in a lymphatic nodule. (x220) (HE)

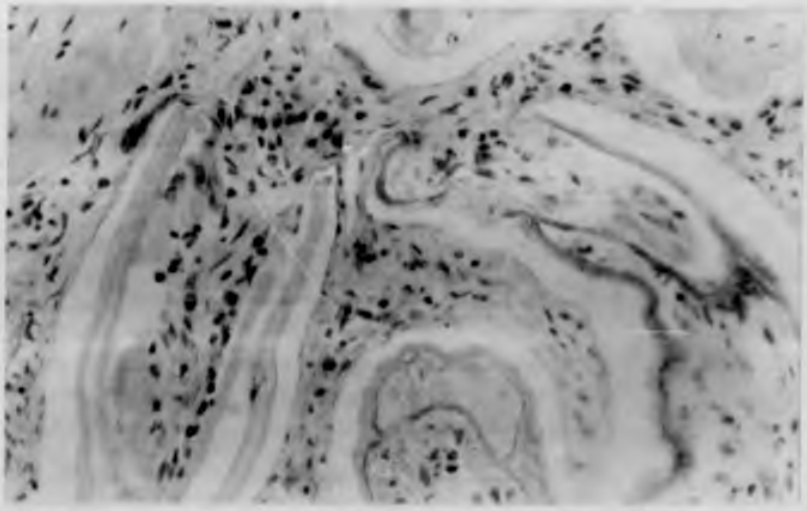


Plate 24. Sections of B.pahangi in lymphatic nodule with host cellular reaction (x875) (HE)

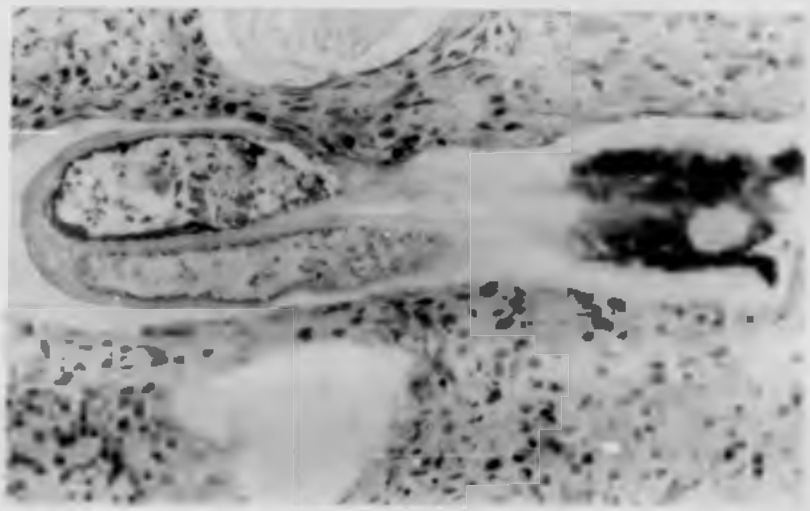


Plate 25. Partially necrosed B.pahangi larvae in a lymphatic nodule. (x875) (HE)

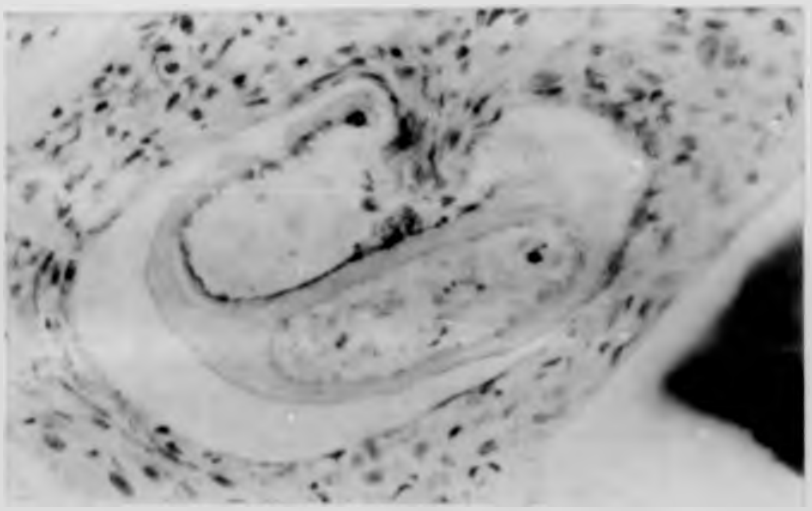


Plate 26. Section of B.pahangi larva in a lymphatic nodule with host cellular reaction. (x875) (HE)

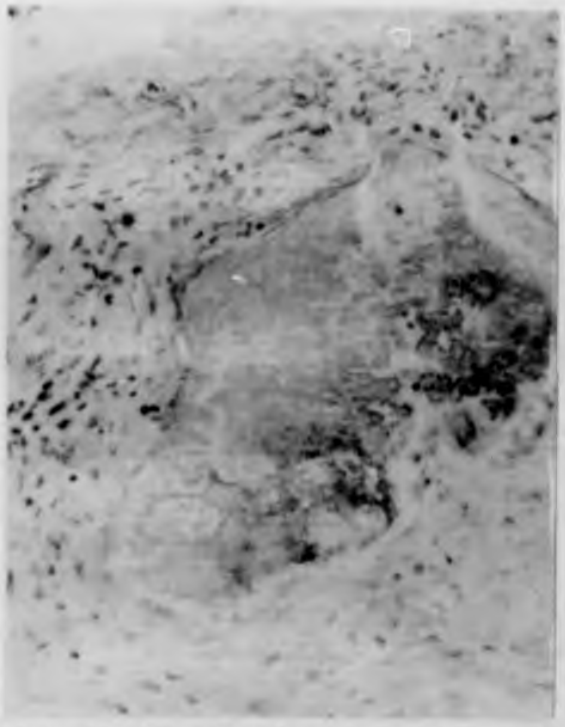


Plate 27. A completely necrosed B.pahangi in a lymphatic nodule of an inf-infected cat. (x875) (HE)

reaction could be detected. Eosinophils were absent in the node and mitosis was not observed. In sections with parasites in the subcortical sinus (Plates 21 and 22) there was no focal reaction.

In the lymph vessel of the infected leg, a mild reaction was observed around the region where the parasite was found. No cellular reaction was observed elsewhere in the lymphatics.

In sections of afferent lymphatics of a cat repeatedly infected with non-irradiated B. pahangi (cat M89), smaller vessels were found to join into the main vessel. This indicated the proliferation of collateral lymph vessels. A fibrous reaction was seen around the wall of the afferent vessel.

Serial sections of the nodular tissue obtained from the lymphatic of cat M76 were examined. This cat had been inoculated with parasites irradiated with 25 krads. Parasites were found in different stages of degeneration (see Plates 23 to 27). There was cellular reaction around the degenerated parasites with infiltration of polymorphonuclear cells and lymphocytes. Plate 25 shows a partially calcified parasite, and Plate 27 a necrosed parasite. The double uteri of female worms were observed in many of the sections, but there were no microfilariae within. This lends support to the earlier findings (Chapter 3) that irradiated parasites migrated to the nodes but did not return to the afferent vessels.

Conclusion

Only mild pathological changes occurred in the lungs of cats infected with irradiated B. pahangi. In cats inoculated with irradiated parasites, the only histological change observed in the

lungs was the thickening of the interstitial septa. However, in the lungs of cats that were infected with non-irradiated parasites, there were emphysematous areas and alveolar collapse in some regions. Microfilariae were found trapped in the interstitial septa of the lungs. The thickening of the interstitial septum observed in lungs of cats infected with non-irradiated parasites may have been due to the presence of microfilariae. Microfilariae have been reported in tissues of filarial patients (Webb et al., 1960; Ratnam and Pradasundarasar, 1961; Danaraj et al., 1966). Microfilariae of Loa loa are destroyed in the spleen of infected drills (Duke, 1960).

It appears that healthy microfilariae in the lungs of infected cats do not stimulate intense reactions. The exaggerated response in the lungs of patients suffering from TPE, may be in response to animal microfilariae (van der Sar, 1945; Webb et al., 1960). Ah et al. (1974a) reported histopathological observations of the lung of dogs infected with irradiated and non-irradiated D. pahangi. They found focal pulmonary endarteritis and thromboembolic endarteritis resulting from localised filariae.

In the lymph nodes of cat M76 immunised with irradiated parasites there was an increase in the number of germinal centres. In the same animal, the popliteal node of the uninfected leg did not have as many germinal centres. Ah et al. [illegible] to detail the histological changes in the affected lymph nodes of cats infected with D. pahangi. As the infection progressed, there was proliferation of lymphocytes and enlargement of germinal centres. This indicated the antibody type response in the lymph node due to the infection.

Degenerative and calcified D. pahangi worms in the lymph nodes

were surrounded by intense host reaction, with proliferation of cells. Fibrous reaction was also seen around the calcified parasites. Schacher et al. (1967) described the histopathological changes in lymph nodes of cats and dogs infected with D. pahangi, in relation to the life-cycle stages of the parasite.

Chapter 9

ANALYSIS OF SERUM COMPONENTS OF CATS INFECTED WITH IRRADIATED AND NON-IRRADIATED B. PAHANGI

Serum samples were collected from cats that were infected with parasites irradiated with 25 krads. and with non-irradiated parasites, as in Chapter 4. Two uninfected cats were used as controls. Samples of serum were collected from experimental cats before infection and fortnightly subsequently. They were bled early in the morning, before being fed.

In cats, the marginal ear vein was found to be very convenient for collecting blood samples, as much as 5 ml. of blood being easily obtained from cats anaesthetised with Saffan. Occasionally blood for serum was withdrawn from the splenic vein. Fur around the elbow joint was clipped off and the region disinfected with 70% alcohol. An elastic band was tied above this region to make the veins more prominent. A 21 G 1½ inch needle was introduced into the vein and a test tube held directly beneath the open end of the needle to collect the blood. The blood samples were kept at 37°C for 20 minutes and were spun at 2,000 r.p.m. for 15 minutes. Occasionally the serum samples were stored at -20°C.

The serum samples were analysed in the Department of Pathology, St. Helier Hospital, Surrey, on a sequential multiple autoanalyser on SMA 12/60.

Findings and comments

Figs. 31 and 32 show the mean values of the different serum components of the infected and control cats. Bilirubin was not

Fig. 31 SERUM ANALYSIS OF CATS INFECTED WITH B. PAHANGI

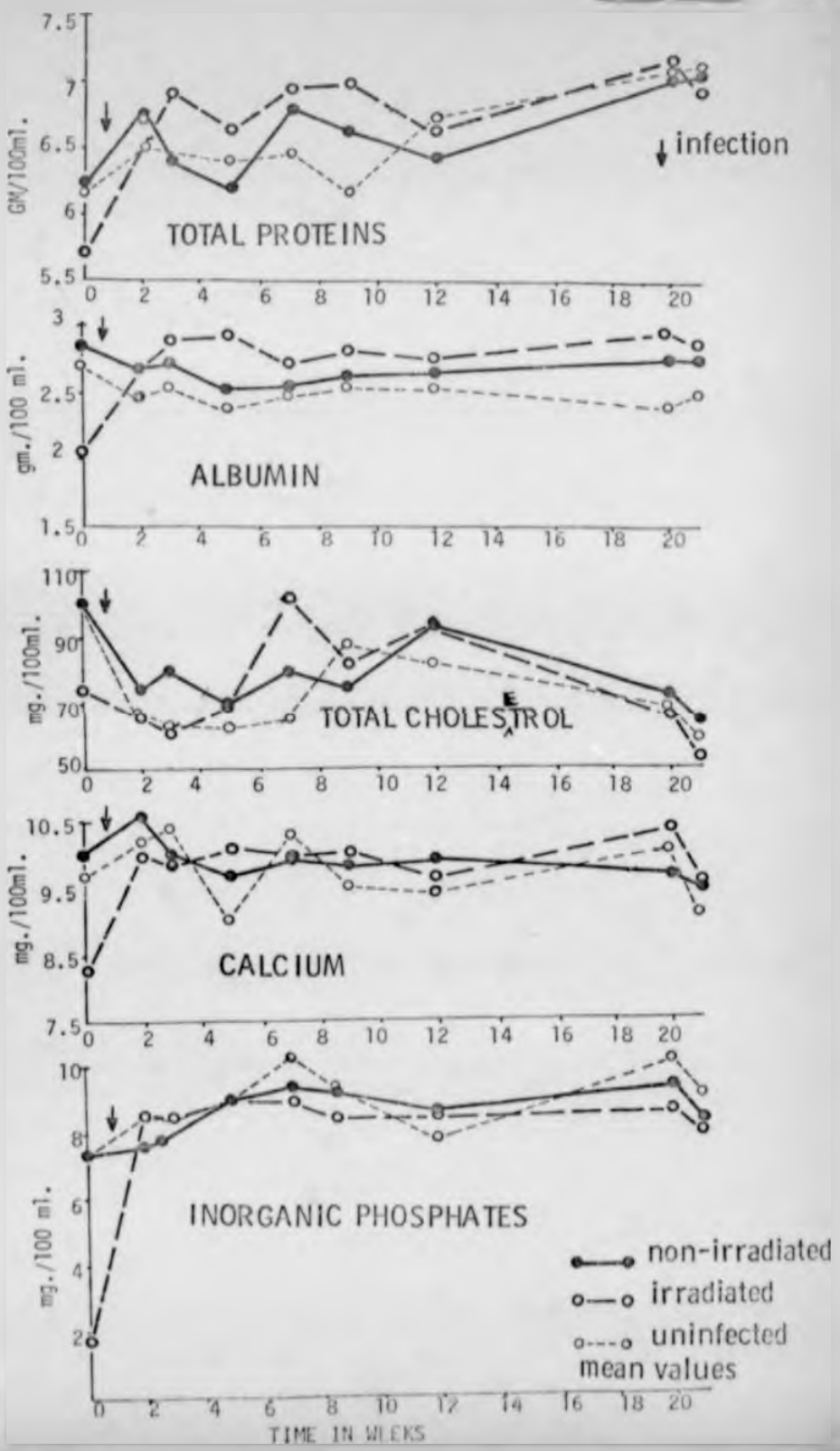
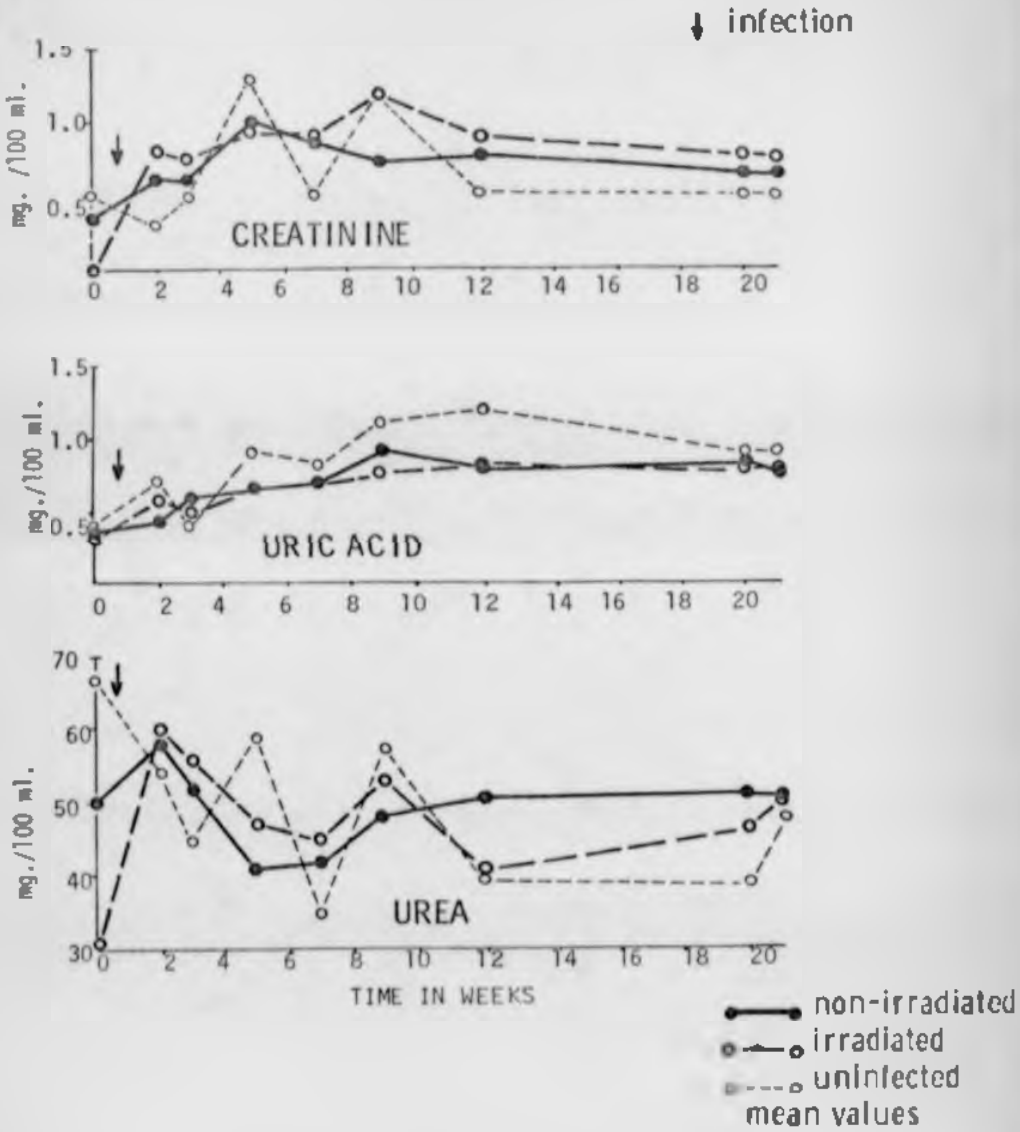


Fig. 32

SERUM ANALYSIS OF CATS INFECTED WITH B. PAHANGI



detected.

Quantities of other serum components did not differ significantly between the two groups of infected cats and the control cats.

The occasional disparities obtained in the estimation of some serum components may have been due to the sera samples being stored at -20°C.

There has been no report of any work on changes in serum components in filarial infections. This preliminary work indicated that despite repeated infection with irradiated and non-irradiated *B. pahangi*, no changes could be detected in the serum components.

Chapter 10

RESISTANCE OF JIRDS (MERIONES UNGUICULATUS) REPEATEDLY INOCULATED WITH IRRADIATED AND NON-IRRADIATED D. PAPILLOSUS

Introduction

Ash and Riley (1970a) found that D. papillosus developed in the peritoneal cavity of the mongolian jird, M. unguiculatus, and that the male of the species was more susceptible to infection (Ash, 1971). If the parasite was inoculated subcutaneously, adult worms were retrieved from the heart, pulmonary arteries, lymphatics and testes (Ash and Riley, 1970a, b; Ash, 1971; Ah and Thompson, 1973; El Dihari and Ewert, 1971). However, if the parasites were inoculated intraperitoneally, they developed to the adult stage free in the peritoneal cavity. In some males, adult worms were also found in the lymphatics of the spermatic cord or peritoneum (Ah and Thompson, 1973; McCall et al., 1973).

Szawillo (1974) and Kowalski and Ash (1975) repeatedly infected jirds in an attempt to produce resistance against challenge.

Experiment 8

In this experiment, an attempt was made to test the immunogenicity of irradiated and non-irradiated parasites in jirds.

Materials and Methods

Jirds were inoculated intraperitoneally with 75 infective larvae on 6 occasions. Five groups of 6 jirds each were inoculated at

fortnightly intervals as follows:

Group 1 infected with parasites irradiated with 25 krads.

Group 2 " " " " " 45 "

Group 3 infected with non-irradiated larvae.

Group 4 challenge controls, also used for control eosinophil readings.

Blood samples were obtained by bleeding jirds from the tail and eosinophil numbers counted as described in Chapter 4.

After 6 repeat infections with irradiated or non-irradiated B. pahangi, the jirds were challenged on two occasions with 50 infective larvae. At the time of recovery the challenge worms were 25 days and 7 days old. Six uninfected jirds were challenged along with the experimental animals in order to establish the normal recovery rates. Two animals from each group were killed to ascertain the number of parasites remaining from the immunising dose.

Method of autopsy of jirds

The jirds were anaesthetized with Nembutal and exsanguinated without opening the thoracic cavity. Peritoneal washings were examined for the presence of microfilariae. The peritoneal cavity was examined for worms and large worms were transferred into petri dishes containing 199 medium. Some 199 medium was then pipetted into the peritoneal cavity and the entrails of the animal moved about to dislodge the worms into the medium. This fluid was transferred into a marked petri dish. This procedure was repeated several times. The digestive system was separated, soaked in a

petri dish containing agar medium, and examined for parasites. The animal was then soaked in PBS to collect any remaining parasites from the peritoneal cavity.

The motility of worms recovered from groups 1 and 2 was checked as these were worms remaining from the immunizing dose that consisted of irradiated larvae. The two groups of challenge worms were collected and [illegible]. [illegible] from jirds repeatedly inoculated with non-irradiated parasites [illegible].

Results

Microfilariae were found only in the peritoneal cavities of animals inoculated with non-irradiated parasites.

The mean percentage recoveries of parasites used as immunizing doses in the different groups are as follows:

Group 1 1.9%

Group 2 1.0%

Group 3 24.9%

Tables 24-27 show the number of challenge worms recovered from the different groups. Separate figures are given for the recovery of worms from the two challenges. Percentage resistance from the different groups is summarised in Table 28. Jirds inoculated with non-irradiated B. pahangi did not resist challenges. Table 25 compares the numbers of worms recovered from this group of animals with those recovered from challenge control animals. There was no statistically significant difference between immunised and normal jirds. Jirds inoculated with parasites irradiated with 25 krads

TABLE 24. CHALLENGE WORM RECOVERIES FROM UNIMMUNIZED JIRDS.

Jird No / challenge dose No.	J1	J2	J3	J4	J5	mean recovery
1.		19	27	13	26	21.3 (3.28)
2	32	24	4	28	16	20.8 (4.96)

TABLE 25. CHALLENGE RECOVERIES FROM JIRDS REPEATEDLY INOCULATED WITH NON-IRRADIATED B.PAHANGI.

Jird No. / challenge dose No.	J1	J2	J3	J4	mean recovery	significance compared with challenge control
1.	31	27	27	30	28.6 (1.0)	NS
2.	20	16	26	32	23.5 (3.5)	NS

TABLE 26. CHALLENGE RECOVERIES FROM JIRDS REPEATEDLY IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS.

Jird No. / challenge dose No.	J1	J2	J3	J4	mean recovery	significance compared with challenge control
1.	22	27	16	27	23.0 (5.2)	$P < 0.20$
2.	6	6	4	16	8.0 (5.4)	$P < 0.0005$

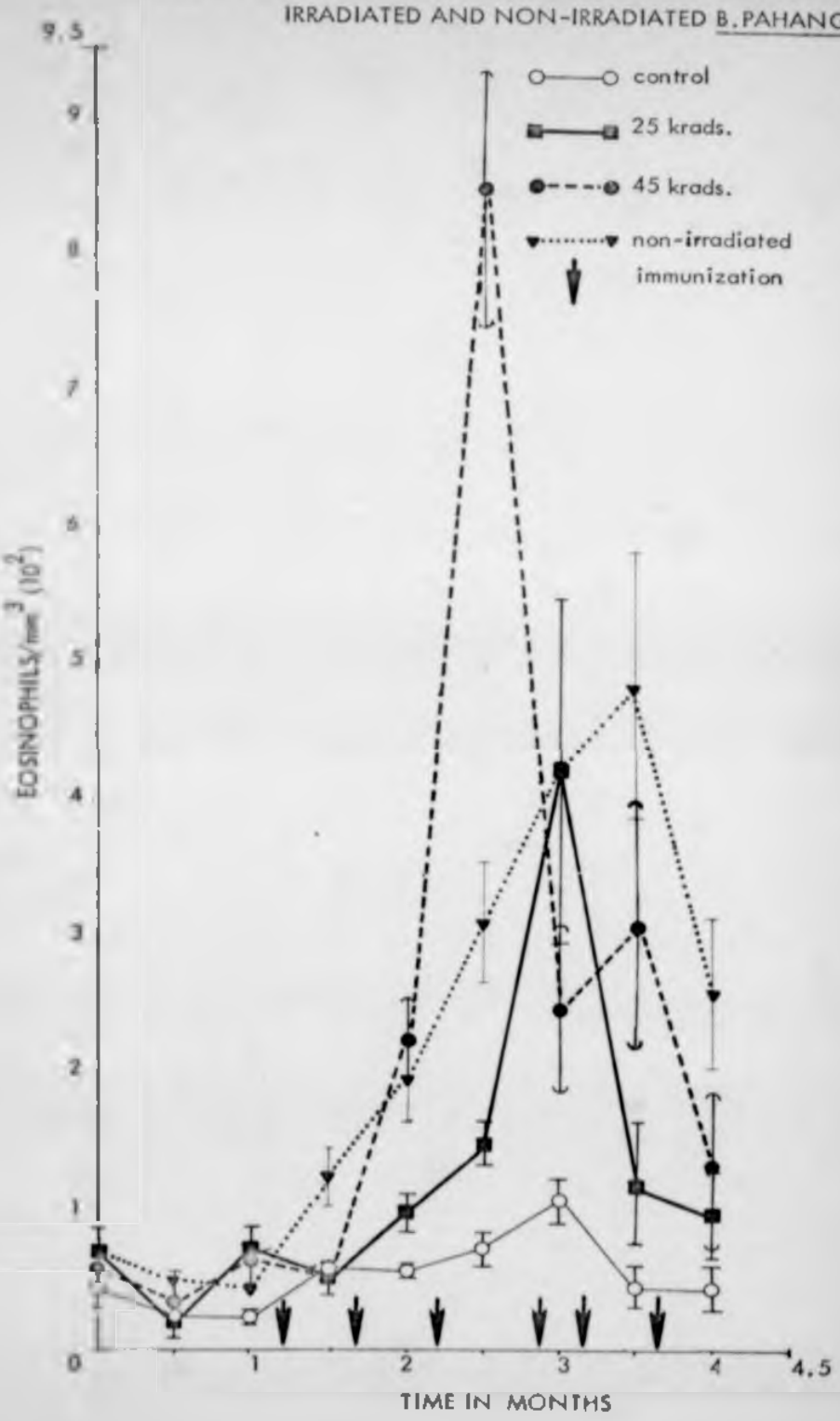
TABLE 27. CHALLENGE RECOVERIES FROM JIRDS REPEATEDLY IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 45 KRADS.

Jird No. / challenge dose No.	J1	J2	J3	J4	mean recovery	significance compared with challenge control
1.	1	7	20	8	9.0 (3.98)	P > 0.0005
2.	4	16	14	22	14.0 (3.74)	P > 0.01

TABLE 28. % RESISTANCE OF JIRDS FROM DIFFERENT GROUPS TO CHALLENGES WITH NORMAL WORMS.

	1st challenge	2nd.challenge
Non-irradiated infections (GROUP 3)	-34%	-13%
Infections with B.pahangi irradiated with 25 krads. (GROUP 1)	-8%	61.5
Infections with B.PAHANGI irradiated with 45 krads (GROUP 2)	57.7	32.6

Fig.33 EOSINOPHIL LEVELS IN JIRDS REPEATEDLY INFECTED WITH IRRADIATED AND NON-IRRADIATED B.PAHANGI



partially resisted the challenges, i.e. -8% and 61.5%. The resistance against the second challenge was statistically significant when compared with the parasites recovered from the challenge control animals (see Table 26).

Jirds infected with B. pahangi irradiated with 45 krads. resisted 57.7% and 32.6% of the first and second challenges respectively (see Table 28).

Eosinophil levels of jirds infected with irradiated and non-irradiated parasites increased after infection, compared with control animal eosinophil levels (see Fig. 33). The eosinophil levels did not correlate with the moulting periods of the different life-cycle stages of the parasite, or the onset of microfilaraemia. The very high levels of eosinophilia observed in jirds infected with B. pahangi irradiated with 45 krads. could not be explained.

Discussion

Since Ash and Riley (1970a, b; Ash, 1973) demonstrated that B. malayi, B. pahangi and B. patei developed in jirds, workers have attempted to use these systems for parasitological and immunological investigations. Only limited success has been achieved. This exploratory experiment demonstrated that jirds can be used as hosts for immunological experiments.

Jirds repeatedly infected intraperitoneally with non-irradiated B. pahangi did not develop protective immunity to challenges. These findings were in agreement with Sunwillo (unpublished) who repeatedly inoculated jirds intraperitoneally with 50 larvae inoculated at weekly intervals for 5, 10 and 14 weeks. The percentage recovery

of adult worms was the same for each group. However, Kowalski and Ash (1975) found that the percentage of worms recovered after 4 subcutaneous, repeat infections with 75 infective larvae of B. pahangi, was lower than when jirds were given a single infection.

It is encouraging to find that D. pahangi irradiated with 45 krads. could be immunogenic in jirds. McCall (1975) reported Ash et al.'s work with irradiated larvae. They found that jirds immunised subcutaneously with irradiated D. pahangi partially resisted the challenge larvae inoculated in the same manner. However, they did not report the amount of irradiation used to attenuate the parasite.

McCall and Thompson (1975) found that transfer of spleen and lymph node cells from patent jirds conferred partial protection to donors. Raised eosinophil responses were observed after infection with D. pahangi. The changes in the eosinophil levels in the different groups of animals did not differ greatly. Jirds infected with 50 infective larvae of D. witae had peak levels of circulating eosinophils approximately 14 days later, raised levels persisting for over 3 months (Bianco, personal communications). This indicates that jirds [illegible] rodent hosts for experimental filariasis.

CONCLUSIONS

Filarial infections of the lymphatic system are very long lived and repeated infection appears to be quite usual (Wilson and Ramachandran, 1971; Denham et al., 1972a). This suggests that immunity does not play a crucial role in these infections in the same way as it does in nematode infections of rodents (Denham, 1966; Ogilvie and Jones, 1971). Denham et al. (1972b and unpublished observations) showed that cats can become highly resistant to infection after repeated infection. This combined with the observation that infective larvae which have been irradiated are often more immunogenic (Jarrett et al., 1959; Miller, 1964) indicated that resistance to reinfection may develop after repeated infection with irradiated B. pahangi.

In order to obtain a vaccine using an attenuated parasite, the development of the parasite must be modified to produce the maximum amount of antigenic stimulus and the minimum of pathology. This may be achieved either by arresting development of the parasite at its most immunogenic stage, or by arresting its migration at an immunogenically competent site in the host. In experiments 1-4, changes in the development of the parasite caused by irradiation and altered pattern of migration were considered. Parasites irradiated with 25 and 45 krads. were arrested at the fourth and third life stages respectively and failed to leave the subcortical sinus of the lymph nodes. Irradiation with 10 krads. produced sexually sterile juvenile fifth stage worms which did migrate back into the afferent lymphatics.

Experimental immunisation of cats with irradiated parasites indicated that these parasites were much more immunogenic than were

normal, i.e. non-irradiated larvae.

Vaccination with parasites which had been irradiated at 10 krads. produced 79% resistance to challenge. Increasing the level of irradiation to 25 krads. did not significantly interfere with the immunogenicity of the larvae. This suggests that the main immunogenic phase of the parasite is not the adult. It seems that prolonging the earlier part of the life cycle by irradiation allows these early stages to stimulate much more immunity than they do during the brief period that they exist in the normal infection. The fact that larvae irradiated with 45 krads. stimulate immunity in jirds (Chapter 10) and that these worms do not develop past the third stage suggests that the third stage might well be the most immunogenic.

It is recognised, however, that resistance was induced only by repeated infections with large numbers of larvae (the total number of irradiated larvae injected ranging from 1,426 and 2,494). For other than experimental purposes it is not practical to use such high numbers of larvae in a vaccine dose. Some degree of success has also been obtained with B. malayi infections in monkeys (Wong et al., 1969), with attenuated D. immitis in dogs (Ah et al., 1972; Wong, 1974) and with attenuated D. pahangi in dogs (Ah et al., 1974a).

Resistance against heterologous challenges with B. patei was also observed, although it was not as great as against the homologous challenges. This may be due to the presence of shared antigens between D. pahangi and B. patei. Antibodies produced in cats infected with D. pahangi cross reacted against B. patei antigens.

It is difficult to control filariasis by any one known method. Vector control is bedevilled by insurmountable problems. The only

drug which has been used with considerable success is DEC, which is a microfilaricide. A solution might be achieved by a combination of different methods, especially vector control supported by chemotherapy and vaccination. The successful production of helminthic vaccines such as DICTOL, DIFIL, and canine hookworm vaccine gives hope for successful vaccination against filarial parasites.

The present investigation indicated that resistance to reinfection with *D. immitis* can be acquired after immunization with irradiated parasites, although, whether the same effect can be produced in humans with attenuated parasites can only be speculated. These results certainly suggest that further experiments with irradiated larval vaccines against filarial worms are justified, even if there seems to be little immunity in normal infections.

Absolute protection against helminth parasites has yet to be achieved. In these experiments, only one animal failed to show microfilaraemia after challenge. However, the substantial degree of resistance shown in the experimental animals is a hopeful sign.

As in the studies of Wong and Guest (1969) and Ponnudurai *et al.* (1974) the IFAT proved very useful in studying the antibody response (Chapter 7). Comparative studies using the various antigens indicated that the third stage larvae are more reactive than the fourth stage larvae or the adult stage. Whether this is because these stages are more immunogenic or because, for technical reasons, they give higher titres cannot yet be said with any confidence. However, the fact that larvae, which fail to mature, stimulate strong resistance supports the suggestion that they are basically more immunogenic than the fourth or fifth stage worms. Antibodies against microfilariae could not

be detected as long as the infected cats retained their circulating microfilariae. Once the microfilarial production was suppressed, free antibodies were detected using the IFAT method. Antibodies against the B. pahangi antigens cross-reacted against closely allied parasites, B. malayi, B. patei and W. bancrofti, but not against D. witmae antigens.

It is encouraging that repeated infections with irradiated parasites did not alter the architecture of the lymphatic system or its functioning. This was probably because irradiated parasites fail to return to the afferent vessels as happens after infection with non-irradiated parasites. The lymph nodes enlarged, due to the initial CMI response, but their size did not increase greatly later. These changes were noted using the xeroradiographic method (Chapter 5). Little or no reaction was observed around the parasites located in the subcorticular nodal sinuses of the infected cats.

Histological examinations of the lung tissue of cats infected with irradiated parasites showed less thickening of the interstitial septa than was seen in the lungs of cats infected with non-irradiated parasites. Microfilariae were detected in the lung tissues of cats infected with non-irradiated parasites and the absence of host reactions around them suggested they are non-pathogenic in cats.

After infection with B. pahangi, changes in the blood of infected cats restricted to increased numbers of circulating eosinophils. In infections with the normal parasite, there were three phases noted: a period of gradual increase, followed by a peak value and then the eosinophil levels continuing at a plateau level. The peak value of eosinophils recorded in 6 cats consistently concurred with the onset of microfilaraemia in these animals. The levels of eosinophils were

lower in cats immunized with irradiated parasites than those in cats immunized with non-irradiated parasites.

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APPENDIX 1.

EXPERIMENT 5. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A42 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	892	97	89	adults	0.6	43.0	98.6
		65	75	adults			
		49	48	third stage	2.0	32.0	93.8
Lf1	881	98	100	adults	0.0	19.0	100
		44	42	third stage	0.0	71.0	100
Rf1	892	74	69	adults	0.0	40.0	100
		44	43	third stage	0.0	55.0	100
Rh1	--	100	97	adults	0.6	46.0	98.7
		65	74	adults			
		50	49	third stage	2.0	34.0	94.1

APPENDIX 2

EXPERIMENT 5. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A42 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lhl	834	98	100	adults	5.1	40.0	87.3
		45	46	young adults	17.7	41.3	57.1
		50	50	third stage	6.0	46.0	87.0
Lfl	837	50	49	adults	6.0	14.3	58.0
		50	49	third stage	2.0	16.0	87.5
Rfl	823	49	48	young adults	14.3	29.0	50.7
		50	49	third stage	4.0	38.0	89.5
Rhl	--	49	49	adults	22.4	36.6	38.8
		98	100	young adults	6.1	17.0	64.1
		49	49	third stage	6.2	46.0	86.5

APPENDIX 3.

EXPERIMENT 5. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A44 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	774	100	96	adults	5.1	31.2	83.7
		50	46	young adults	8.0	41.3	80.6
		48	50	third stage	25.0	46.0	45.7
Lf1	788	50	44	adults	0.0	29.0	100
		50	49	third stage	24.0	23.0	0
Rf1	786	49	48	young adults	1.0	29.0	96.6
		50	49	third stage	12.0	38.0	68.4
Rh1	--	46	49	adults	6.1	24.5	75.1
		99	100	young adults	5.6	17.0	67.1
		46	49	third stage	36.0	28.0	0

APPENDIX 4

EXPERIMENT 5.DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A50 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.pahangi

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	842	98	99	adults	2.7	25.1	89.2
		48	48	adults			
		48	45	third stage	12.5	55.6	77.5
Lf1	836	99	95	adults	0.0	22.1	100
		48	50	third stage	4.2	56.0	92.5
Rf1	838	47	43	adults	4.3	34.6	87.6
		46	50	third stage	6.5	58.0	88.8
Rh1	--	100	100	adults	2.6	40.8	93.6
		49	42	adults			
		46	49	third stage	2.2	61.2	96.4

APPENDIX 5

EXPERIMENT 5. DETAILS OF INOCULATIONS AND WOPM RECOVERIES FROM CAT A53 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	791	100	92	adults	3.0	22.4	86.6
		99	100	fourth stage	9.1	15.0	39.3
		99	96	third stage	11.1	21.9	45.2
Lf1	739	94	95	adults	2.0	13.7	85.4
		100	97	third stage	23.0	20.6	0
Rf1	789	50	49	fourth stage	5.0	16.3	69.3
		100	100	third stage	6.0	22.0	72.7
Rh1	--	100	100	adults	0.0	23.5	100
		50	45	fourth stage	3.0	53.5	94.4
		96	98	third stage	3.13	43.9	92.9

APPENDIX 6

EXPERIMENT 5 DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A60 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	780	99	98	adults	16.8	36.7	54.2
		50	50	fourth stage	26.0	8.0	0
		49	50	third stage	2.0	16.0	87.5
Lf1	787	98	96	adults	4.0	17.7	77.4
		50	50	third stage	6.0	32.0	81.3
Rf1	789	50	50	fourth stage	2.0	38.0	94.7
		50	50	third stage	6.0	24.0	75.0
Rh1	--	99	100	adults	17.2	20.0	14.0
		47	47	fourth stage	18.1	44.7	59.5
		49	50	third stage	0	32.0	100

APPENDIX 7

EXPERIMENT 5 DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A56 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PATEI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	744	99	100	adults	5.5	14.0	60.7
		100	100	fourth stage	15.5	38.5	59.7
		100	96	third stage	0	25.0	100
Lf1	736	90	94	adults	0	4.3	100
		99	99	third stage	1.0	33.0	97.0
Rf1		50	48	fourth stage	2.0	22.0	90.9
		100	99	third stage	1.0	35.0	97.1
Rh1	--	98	97	adults	11.5	16.5	30.3
		99	75	fourth stage	10.5	16.0	34.4
		100	100	third stage	1.0	20.0	96.0

APPENDIX 8

EXPERIMENT 5. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT A59 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH KRADS. AND CHALLENGED WITH NORMAL B.PATEI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	784	97	100	adults	2.6	4.0	35.0
		100	96	fourth stage	17.5	40.1	56.3
		100	97	third stage	34.0	52.6	35.4
Lf1	780	99	94	adults	1.1	4.26	74.2
		100	99	third stage	5.0	13.1	61.8
Rf1	792	50	48	fourth stage	2.0	33.3	94.9
		100	97	third stage	3.0	46.4	93.5
Rh1	--	100	97	adults	8.5	16.5	48.5
		100	75	fourth stage	24.5	29.3	16.4
		100	100	third stage	9.0	69.0	87.0

APPENDIX 9

EXPERIMENT 6. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M73 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI:

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lhl	673	100	96	adults	2.0	33.3	94.0
Lfl	667	100	96	adults	9.0	43.8	79.5
Rfl							
Rhl		98	100	adults	21.9	29.0	24.5

APPENDIX 10

EXPERIMENT 6. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M74 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	471	100	98	adults	2.0	42.0	95.2
		99	97	fourth stage	5.0	35.0	85.7
		49	49	third stage	0.0	27.0	100
Lf1	493	99	100	adults	0.0	4.0	100
		49	49	third stage	2.0	18.0	88.9
Rf1	498	100	98	fourth stage	1.0	25.0	96.0
		50	50	third stage	0.0	20.0	100
Rh1	--	100	100	adults	0.0	40.0	100
		98	97	fourth stage	5.0	32.0	84.8
		48	48	third stage	2.0	21.0	90.5

APPENDIX 11.

EXPERIMENT6. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M75 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	657	49	50	third stage	2.0	16.0	87.5
Lf1	660	49	50	third stage	4.5	14.0	67.9
Rf1	673	48	50	third stage	3.0	25.0	88.0
Rh1							

APPENDIX 12.

EXPERIMENT 6. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M77 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lhl	673	50	50	adults	0.6	28.0	78.6
		100	100	fourth stage	19.5	25.3	22.9
		100	100	third stage	17.0	43.0	60.7
Lfl	686	50	50	adults	0.0	6.0	100
		100	100	third stage	3.0	12.0	75.0
Rfl	672	100	100	fourth stage	5.5	13.0	57.6
		100	100	third stage	17.5	33.0	47.0
Rhl	--	49	49	adults	6.0	12.0	5.0
		98	99	fourth stage	19.5	53.0	63.2
		100	100	third stage	17.0	48.0	64.6

APPENDIX 13.

EXPERIMENT 6. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M86 IMMUNIZED WITH B.PAHANGI IRRADIATED WITH 25 KRADS. AND CHALLENGED WITH NORMAL B.PAHANGI

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lhl	482	100	100	adults	1.0	25.0	96.0
		99	99	adults			
		100	100	third stage	2.0	27.0	92.6
Lfl	495	99	98	adults	1.0	28.0	96.4
		100	94	third stage	4.0	38.0	89.0
Rfl	482	100	100	adults	8.0	34.0	76.5
		100	99	third stage	6.0	58.0	89.7
Rhl	--	100	99	adults	2.0	8.0	75.0
		100	99	adults			
		100	100	third stage	0.0	12.0	100

APPENDIX 14

EXPERIMENT 7 DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M79 INOCULATED WITH NON-IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	687	95	99	adults	4.2	29.8	85.9
		100	99	fourth stage	4.0	35.4	88.7
		93	100	third stage	12.9	37.6	65.1
Lf1	681	98	99	adults	20.4	17.1	0
		100	100	third stage	16.0	46.0	65.2
Rf1	679	100	97	fourth stage	23.0	18.6	0
		100	99	third stage	22.0	50.5	56.4
Rh1	--	99	94	adults	25.2	21.3	0
		96	93	fourth stage	17.7	25.8	33.7
		100	100	third stage	22.0	84.8	73.8

APPENDIX 15

EXPERIMENT 7. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CATM85 INOCULATED WITH NON-IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	682	96	98	adults	0	36.7	100
		50	50	fourth stage	0	8.0	100
		50	50	third stage	0	16.0	100
Lf1	671	98	96	adults	0	17.7	100
		50	50	third stage	0	32.0	100
Rf1	676	50	50	fourth stage	0	38.0	100
		47	50	third stage	0	24.0	100
Rh1	--	100	100	adults	0	20.0	100
		48	47	fourth stage	0	44.7	100
		49	50	third stage	0	32.0	100

APPENDIX 16

EXPERIMENT 7. DETAILS OF INOCULATIONS AND WORM RECOVERIES FROM CAT M87 INOCULATED WITH NON-IRRADIATED B.PAHANGI AND CHALLENGED WITH NORMAL B.PAHANGI.

LIMB	TOTAL No.OF LARVAE			%RECOVERY			% protection
	immunization	CHALLENGE		stage of challenge worms	exptl.	control	
		exptl.	control				
Lh1	687	100	99	adults	1.0	12.0	91.7
		97	93	fourth stage	1.4	35.0	96.0
		100	100	third stage	2.0	30.2	93.0
Lf1	679	100	100	adults	8.3	21.0	60.5
		98	99	third stage	4.0	40.2	90.0
Rf1	673	98	95	fourth stage	0.0	47.0	100
		99	100	third stage	2.0	33.0	93.9
Rh1	--	99	99	adults	2.1	12.0	82.5
		92	93	fourth stage	7.2	62.0	88.4
		98	100	third stage	8.0	23.1	65.7

Appendix 17

Abnormal development of a filarial worm, *Brugia patei* (Buckley, Nelson and Heisch), in a mosquito host, *Anopheles labranchiae atroparvus* van Thiel

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ABSTRACT

The morphology of abnormal development of the filarial worm *Brugia patei* in a mosquito host, *Anopheles labranchiae atroparvus*, is described. Development was very variable, from little growth beyond the microfilarial stage to the complete development of two mosquito-stage larvae in one mosquito. The majority of larvae developed beyond the microfilarial stage but integrated differentiation did not occur. In the most bizarre larval development, the intestinal and the [illegible] cells prolapsed and then attempted to differentiate outside the body of the filarial larva. It is concluded that the abnormal development of the filarial larvae in the mosquito host is due to the host reaction elicited by the developing larvae, apparently an melanisation 48–78 hours after ingestion by the mosquito, often localised specifically over the excretory and anal vesicles

Normal development of filarial worms in the mosquito host has been described many times in the past. Little has been recorded of the morphology of abnormal development in the "wrong" host although, here again, there have been many records of "aberrations" or melanisation reactions around filarial larvae developing in mosquitoes. Recently Brambles and Bruckes (1972) have described normal and abnormal development of *Wuchereria kansasi* Caldwell in *Anopheles quadrimaculatus* Say and *Mansonia uniformis* Theobald. In this paper, we describe the morphology of abnormal development of *Anopheles labranchiae atroparvus* Nelson & Heisch in the indirect thoracic flight muscles of *Anopheles labranchiae atroparvus* van Thiel and *Aedes* sp. Normal development of *Brugia patei* in mosquitoes has been described in earlier papers (Laurence and Pester, 1961; Laurence and Simpson, 1971).

MATERIALS AND METHODS

The mosquito colony used in these experiments was originally obtained from wild-caught mosquitoes in 1931 (Hamilton, Maldonado and Ito) and has been kept in laboratory culture continuously since then. Females less than one week old were fed on anaesthetised cats infected by *Brugia patei* (Laurence and Pester, 1961) showing 25 or more microfilariae in 20 cu. mm. of peripheral blood. Three cats were used in these experiments (cats 11, 80, F 90), which were carried out in 1965, 1967 and 1971. Similar results were obtained in all three experiments over this 10 year period.

After infection, the blood-fed females were maintained in an incubator at 26°C. Mosquitoes were dissected in saline at intervals up to 10 days after the blood meal and any developing filarial larvae were measured and drawn. Other mosquitoes were fixed directly in

Abnormal development of a filarial worm, *Brugia patei* (Buckley, Nelson and Heisch), in a mosquito host, *Anopheles labranchiae atroparvus* van Thiel

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ABSTRACT

The morphology of abnormal development of the filarial worm *Brugia patei* in a mosquito host, *Anopheles labranchiae atroparvus* is described. Development [illegible] the microfilarial stage to the complete development of two [illegible] in one mosquito. The majority of larvae developed beyond the microfilarial stage but integrated differentiation did not occur. In the most bizarre larval development, the intestinal and the [illegible] prolapsed and then attempted to differentiate outside the body of the filarial larva. It is concluded that the abnormal development of the filarial larvae in this mosquito host is due to the host reaction elicited by the developing larvae, apparent as melanisation 48–78 hours after ingestion by the mosquito, often localised specifically over the excretory and anal vesicle.

Normal development of filarial worms in the mosquito host has been described many times in the past. Little has been recorded of the morphology of abnormal development in the "wrong" host although, here again, there have been many records of "chitinisation" or melanisation reactions around filarial larvae developing in mosquitoes. Recently Brunhes and Brunhes (1972) have described normal and abnormal development of *Wuchereria bancrofti* Cobbold in *Anopheles gambiae* Giles and *Mansonia uniformis* Theobald. In this paper, we describe the morphology of abnormal development of *Brugia patei* (Buckley, Nelson & Heisch) in the indirect thoracic flight muscles of *Anopheles labranchiae atroparvus* van Thiel and discuss its significance. Normal development of *Brugia patei* in mosquitoes has been described in earlier papers (Laurence and Pester, 1961; Laurence and Simpson, 1971).

MATERIALS AND METHODS

The mosquito colony used in these experiments was originally obtained from wild-caught mosquitoes in 1931 (Harefield, Middlesex) and has been kept in laboratory culture continuously since then. Females less than one week old were fed on anaesthetised cats infected by *Brugia patei* (Laurence and Pester, 1961) showing 27 or more microfilariae in 20 cu. mm of peripheral blood. Three cats were used in these experiments (cats 55, 80, P 90), which were carried out in 1963, 1967 and 1973. Similar results were obtained in all three experiments over this 10 year period.

After infection, the blood-fed females were maintained in an incubator at 26°C. Mosquitoes were dissected in saline at intervals up to 10 days after the blood meal and any developing filarial larvae were measured and drawn. Other mosquitoes were fixed directly in

Bouin-Dubosq at intervals after the blood meal, sectioned at 5 μm, and stained in chrome haematoxylin phloxin or in other stains (Simpson and Laurence, 1972), or were fixed directly in 80% methanol, stained in Mayers acid haemalum (Nelson, 1958), and dissected in glycerine. Serial sections of infected mosquitoes were examined and photographed under the oil immersion of a Zeiss Photomicroscope.

RESULTS

Migration of the microfilariae to the thoracic musculature

Dissection of female mosquitoes 2–24 hours after the blood meal showed that most of the microfilariae ingested (in the three experiments) had migrated successfully from the stomach into the thorax of the mosquitoes. Of 558 microfilariae recovered at this time from 17 mosquitoes, 82·6% were found in the thorax, and the remainder of the microfilariae were found in the head, abdomen and in the blood meal in the stomach.

Development in the thorax

24 hours—Examination of serial sections of larvae in the thoracic muscles of mosquitoes killed 21–24 hours after the blood meal showed that the inner body (Innenkörper) had broken down and the Ci cell divided (Plate I, fig. 3) or in the process of dividing. Small cells immediately anterior and posterior to the divided Ci cell were growing together. These observations indicate the commencement of normal development (Laurence and Simpson, 1971). One larva, however, was noted with abnormal distension of the anal vesicle.

48 hours—Melanization of larvae was found in saline dissections of mosquitoes, from total melanisation of a microfilaria to discrete melanin reactions over the excretory and anal vesicles. Some larvae contained swollen vesicles below the melanin reactions. In sections, some larvae showed a reaction (positive with periodic-acid-Schiff staining) over the excretory pore, or over and within the anal vesicle. Other larvae in the same mosquito at this time showed a normal appearance with no melanisation (Plate I, fig. 4). The cells appeared to be normal for this stage of development, with nuclei containing prominent nucleoli, but little differentiation was observed in the intestinal region.

72 hours—The majority of larvae showed melanisation around the tail and anal region and also anteriorly. Although some larvae were partially encapsulated, they were still capable of movement. Sections of larvae showed that the intima of the pharynx (oesophagus) of the second larval stage was differentiated normally (Plate II, fig. 7) and the pharyngeal thread of the microfilaria had formed a buccal knot (Laurence and Simpson, 1971). The anal vesicle of most larvae appeared to be abnormal, with little growth of the anal membrane or with vacuolation of the cytoplasm. Prolapse of cells through the anal membrane was observed in some larvae although differentiation of the intestine was seen in others.

96 hours—There was considerable variation in the size of the larvae. Most did not progress beyond a length of 200 μm but one larva was found, free of melanisation, that measured 375μ × 28μm. In sections, several larvae were observed with an anal prolapse (Plate II, fig. 6) and the inner membranated surface of the excretory vesicle in one larva was completely and locally melanised.

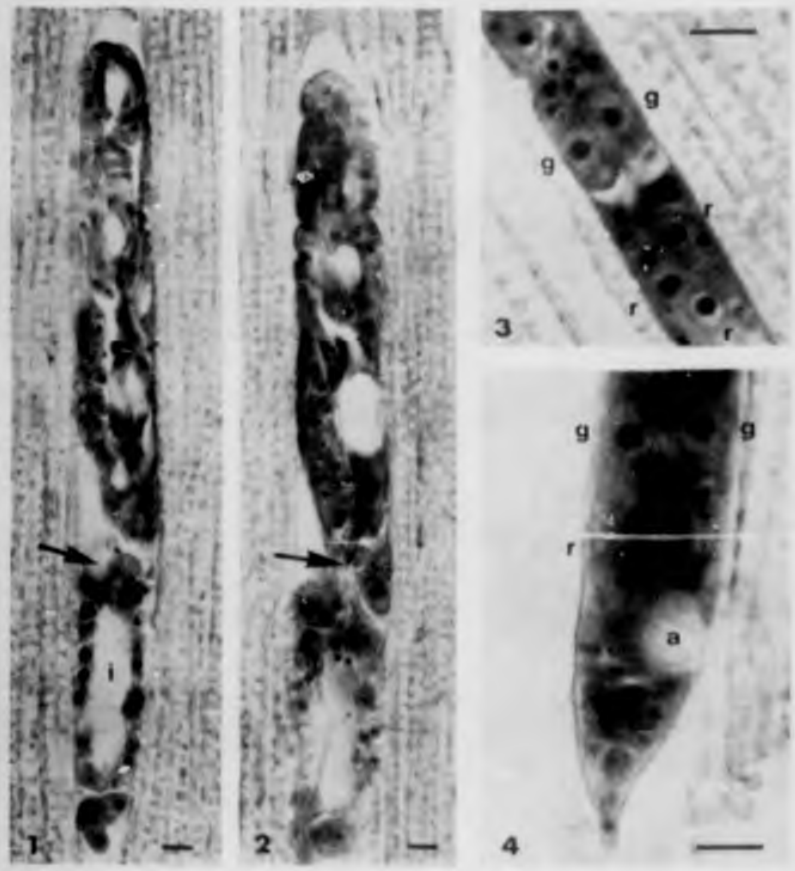

PLATE 1

FIGURES 1–4. *Brugia patei* in *Amythelcs labranchiae arraparvus* (scale = 10 μm). 1. 10 days, to show anal vesicle (arrowed) in intestine (i) outside body of larva. 2. 10 days, to show pharynx (arrowed) extending to anus of larva. 3. 21 hours, G cell divided (g), rectal cells (r). 4. 48 hours, G cell (g) divided again, rectal cells (r) and anal vesicle (a) normal.

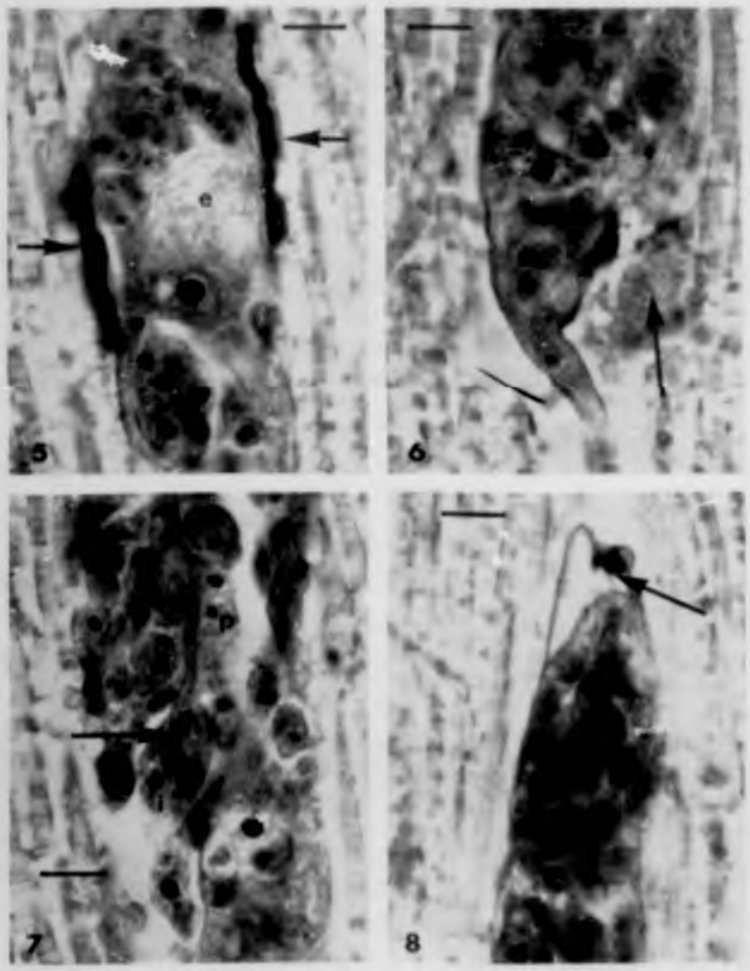

PLATE II

FIGURES 5–8. *B. patei* in *A. l. atroparvus* (scale = 10 μm). 5, 6 days, excretory vesicle (e) surrounded by melanin reaction (arrowed). 6, 6 days, anal prolapse of cells (arrowed). 7, 6 days, pharynx of second stage larva differentiated around end of pharyngeal thread (arrowed), pharyngeal cells (p), intestinal cells (i). 8, 8 days, cuticle of microfilaria moulted at anterior end of larva, cephalic and pharyngeal structures arrowed.

5 days One larva with an anal prolapse, in section, showed a small discretely melanised microfilarial anal membrane flexed back by the prolapse Other larvae were found with a completely melanised anal vesicle or with melanin around a distended excretory vesicle (Plate II, fig 5). Larvae with necrotic cells were found at this stage

6 days the cuticle of the first stage larva was detached from new underlying cuticle but the larvae had not moulted (Plate II, fig. 8) The pharynx and its intima extended back to the anal region and some cells in the anal prolapse were connected to the chrome haematoxylin positive end of the pharyngeal thread (Laurence and Simpson, 1968) This represents the position of the pharyngo-intestinal valve in the microfilaria (Plate II, fig 7) Larvae varied in size from 120 μ to 760 μm in length and the larger larvae showed greater differentiation

7–9 days Most larvae did not grow beyond 300 μm in length The most advanced larvae showed a differentiated pharyngeal intima of the second larval stage, and the first stage larval cuticle detached but not moulted from the cuticle of the second larval stage The pharynx extended back to, and sometimes beyond, the anal area into the anal prolapse of cells (Plate I, fig 2). Other larvae showed a differentiated alimentary canal comparable to normal development at 96 hours but no growth in length since then Completely necrotic larvae were also observed

10 days Two apparently normal infective stage larvae were found in the head and in the abdomen of one female mosquito in the experiment in 1967 (Laurence, 1970) Seven other abnormal larvae (less than 200 μm long) were also present in the thorax of this mosquito One further female was found with an active but abnormally short filarial larva (450 μm long) in the abdomen, within the previous unmoulted larval cuticle This female also had 10 larvae less than 200 μm long in the thorax Otherwise the most advanced larval stages found in the thoracic flight muscles of this species of mosquito were larvae with a differentiated pharyngeal region and with the rectal and intestinal cells attempting to form an anal vesicle and an intestine outside the body of the larva, in the anal prolapse lying behind the larva in the mosquito flight muscle (Plate I, fig 1).

DISCUSSION

The microfilaria of *Brugia patei* commences development in the flight muscles of *Anopheles labranchiae atroparvus* However, by 48 hours, there is evidence of a host reaction by the mosquito in the form of a melanin reaction around some filarial larvae, specifically over the excretory and anal vesicles Other larvae in the thorax appear to be normal at this time By 72 hours many larvae show a localised or more extensive melanisation and most larvae evidently develop abnormally from 48 to 72 hours The excretory cell complex is known to be metabolically active in the microfilaria (Simpson and Laurence, 1972) and it appears to be homologous with the hypodermal gland or bacillary cells of other nematodes (McLaren, 1972) The function of the excretory cell complex is not known and the effect of blockage of the excretory pore or excretory atrium, seen in many abnormal larvae, cannot be predicted although fluid accumulates in the blocked gland Prolapse of cells through the anus is visible by 72 hours, possibly due to pressure and to the weakness of the anal membrane at this point, and the prolapse may be hastened by the growth backwards of the pharynx or oesophagus which reaches the anal region by 6 days Discrete melanisation of the microfilarial anal membrane, seen in one prolapsed larva at 5 days, may also lead to the prolapse of the intestinal cells outside the body of the larva The anal vesicle of the microfilaria of *Brugia* contains a syncytium of villi from the three rectal or R cells (Laurence &

Simpson, in press) which probably have an absorptive or secretory function (McLaren, 1972). Blockage of the anal membrane and anal pore would prevent the function of the anal vesicle and this is associated with the failure in organisation of the intestinal region. Where an anal vesicle has been formed by the rectal cells in the anal prolapse of abnormal larvae, some organisation of the intestinal cells has been possible (Plate I, fig. 1). The hypodermal cells are capable of forming a new cuticle below the microfilarial cuticle but the hypodermal and muscle cells composing the body wall do not grow normally so that most larvae rarely attain a length of 300 μm. This lack of growth is not explained by our observations. In contrast, the new pharyngeal intima of the second stage larva is formed around the microfilarial pharyngeal thread and in many larvae the pharyngeal cells continue to grow back to the anal region (Plate I, fig. 2). Cell division continues even in grossly melanised larvae and the larvae are still capable of movement, showing the continued function of the muscle cells. Autoradiographic studies have also shown that abnormal melanised larvae at 4 to 5 days continue to incorporate radioactive amino acids into cells of both the body wall and the alimentary canal (including the cells of the anal prolapse) unless these cells are obviously necrotic histologically (Laurence and Simpson, 1974).

The variation in filarial growth and differentiation found in this mosquito can be associated with the localisation over individual larvae of the melanin reaction. Very few larvae may escape this reaction and attain the infective stage, although others are prevented from doing so in the same mosquito host. Our observations suggest that the melanisation of the developing filarial larvae, first visible at 48 hours, is the primary response of the mosquito host and that the observed abnormal development from 48 hours to 9 days is a consequence of the melanin reaction. Glass fibres implanted into the thorax of this mosquito also elicit a melanin reaction over a period of 5 days (Oothuman, unpublished M.Sc. thesis). However, localised melanin reactions do not explain the absence of normal growth of the hypodermal and muscle cells unless these cells are dependent on the normal function of the excretory and anal complexes. It is not known what triggers off the melanin deposition but the marked reaction over the excretory and anal pores suggests an interaction here between the metabolic products of the parasite and the host. The melanin reaction in *Anopheles l. atroparvus* is complex, periodic acid-Schiff positive, resistant to diastase digestion, and staining in solochrome cyanine R. The origin of the melanin reaction in this mosquito is not known but, from histological sections, it appears to be a humoral rather than a cellular response, as Pomar and Lutenegger (1971) and Salt (1963) have indicated may be found in the nematocerous Diptera, with few free blood cells.

The host reaction of *Anopheles l. atroparvus* appears around the developing filarial larva. This is in contrast to the resistance to filarial infection shown by other mosquito species. In *Aedes aegypti* L., where resistance and susceptibility to infection is known to have a genetic basis (Macdonald, 1962), there is no melanisation of *B. patei* in the thoracic muscles. In resistant females, the microfilariae of *B. patei* show no further development in the thoracic muscles, some are necrotic at 24 hours, the G1 cell does not divide, and the microfilariae become progressively more necrotic from 24 to 72 hours (Laurence, 1970). In other species of mosquito, well-developed cibarial and pharyngeal armatures damage microfilariae mechanically as they pass into the stomach with the blood meal (Colluzzi and Trabucchi, 1968; Bryan, Oothuman, McGreevy and Andrews, 1974). Recently Brunhes and Brunhes (1972) have described the development of *Wuchereria bancrofti* in *Anopheles gambiae* and in *Mansonia uniformis* in Madagascar. Their observations on the abnormal development of *Wuchereria* in *Mansonia* parallel some of our observations on the development of *Brugia* in *Anopheles l. atroparvus*. *Mansonia*, however, shows no reaction to infection by the larvae of *Brugia* (Uyeger, 1938; Laurence and Pester, 1961). This raises the possibility that the

specificity of filarial infection to certain species of mosquito could include mechanisms that protect the filarial larvae during normal development from the potential host reactions of their mosquito intermediate hosts. Otherwise the programming of normal development by the parasite breaks down.

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Appendix 18

Effects of the Pinworm and Pharyngeal Apparatuses of [illegible] on the Motility of [illegible]

by

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Summary

The intensity and rate of infection of B. [illegible] were high in Ae. aegypti and Ae. togoi but low in An. [illegible] species A, An. gambiae species B and An. farauti No. 1 when they were fed on patent cats. The following evidence indicates that refractoriness in An. gambiae species A is caused by the cibarial armature in the foregut: 1) B. pahangi developed normally when exsheathed microfilariae were injected into the thorax indicating that the thoracic muscles are susceptible to infection; 2) A very small proportion of ingested microfilariae penetrated the midgut epithelium suggesting that refractoriness to Brugia infection was expressed in the gut; 3) Large proportions of freshly ingested microfilariae in the midgut were motile and probably dead; 4) Large proportions of freshly ingested microfilariae had cuticular abrasions which appeared to be of mechanical origin and the probable cause of death. These microfilariae could have been damaged during ingestion by the papillae, spines of the pharyngeal armature and teeth of the cibarial armature which protrude into the lumen of the foregut. The Aedes spp. have papillae and a pharyngeal armature but they damaged only small proportions of ingested microfilariae. In contrast, the Anopheles spp. have papillae, a pharyngeal armature and a cibarial armature and they [illegible]. It is likely that the cibarial armature is the most lethal structure in the foregut. However, there are interspecific variations in the structure of the cibarial armature which account for variations in the proportion of microfilariae killed by different species of mosquitoes. An. gambiae species A has a well [illegible] and kills [illegible] of

ingested W. bancrofti while C. p. fatigans has a less developed armature and kills only 5.8% when fed on patent carriers. There is also variation in the ability of different species of microfilariae to evade injury from the cibarial armature. While An. gambiae species A kills 89.5% of ingested Brugia microfilariae, it kills only 45.2% of ingested Wuchereria. The cibarial armature may represent an adaptation of mosquitoes to the selection pressure of filarial infection while the ability of microfilariae to evade injury may be a filarial adaptation to selection pressure by the armature. The cibarial armature is not found in natural vectors of Brugia and in some vectors of Wuchereria. At least one of these mosquitoes, An. polynesiensis, can transmit W. bancrofti from people treated with diethylcarbamazine (= DEC) and presenting ultra low microfilaraemias. We suggest that vectors with well developed cibarial armatures such as An. gambiae are less likely to become infected with W. bancrofti from treated carriers and that mass chemotherapy with DEC may break transmission.

The successful transmission of filarial nematodes depends on the susceptibility of their vectors, a characteristic which varies both within and between species. Under laboratory conditions, the most susceptible vectors support the development of large numbers of filarial larvae and often suffer pathological changes such as damage to their flight muscles (Beckett 1971, Hockmeyer et al. 1975), reduced fecundity (Javadian and Macdonald 1974) and premature death (Townson 1971). Other vectors minimize these pathological changes by limiting the intensity of their infection through various refractory mechanisms. In mosquitoes these mechanisms may affect filarial larvae in the gut (Obiamiwe and Macdonald 1973, Bain and Chabaud 1975), in the haemocoel or at their developmental site (Macdonald 1976, Denham and McGreevy 1976). In the present study we have compared the susceptibility of various species of mosquitoes to infection with Brugia pahangi and Wuchereria bancrofti and have given special attention to the effects of the cibarial and pharyngeal armatures on the viability of ingested microfilariae.

The cibarial armature is present in the foregut of female mosquitoes in the subgenera Cellia and Myzorhynchus of the genus Anopheles and in the genus Culex. It occurs at the junction of the cibarial and pharyngeal pumps and is composed of one or two rows of teeth which project from the posterior end of the ventral plate into the lumen of the foregut (Fig. 1) (Sinton and Covell [illegible] Covell 1928).

In addition to the cibarial armature there may be up to 18 small papillae which protrude from the walls of the cibarium into its lumen (Fig. 1). In the pharynx there may be two types of spines. The most

prominant are the spines of the pharyngeal armature which protrude from the posterior wall (Fig. 7). Smaller more delicate spines are found on the anterior wall and these have been called 'spines of the bucco-pharyngeal ridges' by Sinton and Covell (1927) and 'spines of the post-armature ridges' by Christophers (1933).

The functions of the spines, papillae and teeth in the foregut of insects are generally unknown (Lewis, 1975). However, Coluzzi and Trabucchi (1968) have shown that microfilariae are injured as they pass through the foregut by the teeth of the cibarial armature. Bain et al. (1974) and Omar and Garms (1975) have extended these observations to the Simulium ochraceum-Onchocerca volvulus system. They demonstrated injured microfilariae of O. volvulus entangled in the teeth of the cibarial armature, described the type of damage inflicted on ingested microfilariae and the proportion of ingested parasites which suffer this damage.

Our observations on B. pahangi in mosquitoes provide experimental data which confirm the original observations of Coluzzi and Trabucchi (1968). We have also determined the proportions of microfilariae of W. bancrofti which are killed by the cibarial armature of its natural vectors, Anopheles [illegible] species A and Culex pipiens fatigans.

The nomenclature of the foregut is confused with excessive synonomy and the terms we use are taken from Snodgrass (1943) and [illegible] pharyngeal armature' of Sinton and Covell (1927) and Coluzzi and [illegible] and the 'pharyngeal armature' of Christophers (1933). Our term 'pharyngeal armature' is applied only to the larger spines in the posterior portion of pharyngeal pump (Figs. 1, 7).

Material and Methods

B. pahangi was maintained in cats at the London School of Hygiene and Tropical Medicine (= LSHTM) by the method of Denham et al. (1972). The density of microfilariae in peripheral blood was determined from 20 ul samples of blood from the marginal vein of the ear using the counting chamber technique (Denham et al. 1971). The density varied between 2 and 294 microfilariae per 20 ul (100 and 14,700 mf/ml). Cats were anaesthetized with sodium pentobarbitone and the hair was clipped from their sides before the mosquitoes were allowed to feed. The laboratory studies on the lethal effects of the armatures on microfilariae of B. pahangi were carried out on 5 species of mosquitoes: An. gambiae species A from Upper Volta, Anopheles gambiae species B from Nigeria, Anopheles farauti No. 1 from Papua, New Guinea, the LSHTM strain of Aedes togoi and a Brugia-Wuchereria susceptible (fm/fm) strain of Aedes aegypti.

Studies on W. bancrofti microfilariae were conducted at the WHO/MRC/Tanzania laboratory in Tanga. Three species of mosquitoes were studied: An. gambiae species A obtained from the Tropical Pesticide Research Institute, Arusha, Tanzania, C. p. fatigans isolated from Tanga and the fm/fm strain of Ae. aegypti. The mosquitoes were fed on [illegible] at night. The density of microfilariae was [illegible] 20 ul samples of finger prick blood examined in counting chambers. The densities ranged from 1 to 1,393 microfilariae per ml.

Experimental Procedure and Results

1. Distribution of the Cibarial and Pharyngeal Armatures in Various Mosquitoes

The morphological diversity of the armatures was studied in 25 species of mosquitoes maintained at the LSHTM and the Liverpool School of Tropical Medicine (Table 1). After the mosquitoes were killed they were cleared for 4 hours at room temperature in a mixture containing 1 part of a 5% solution of KOH and 1 part of 70% ethyl alcohol. The armatures were removed with fine needles, mounted in glycerol and examined under a compound microscope.

All of the mosquitoes had pharyngeal armatures and this structure was present in both sexes, but only 14 of the 25 species had a cibarial armature and this structure was limited to females (Table 1). There were interspecific variations in the size, shape and number of teeth of the cibarial armature and in the spines of the pharyngeal armature (Figs. 2-7).

2. Effects of the Armatures on Ingested Microfilariae

Two criteria were used to determine the effects of the armatures [illegible] changes in motility. The mosquitoes were fed to repletion on infected cats or human donors, immediately anaesthetized by placing them in a freezer held at -10°C for 30 seconds, and maintained on ice until their [illegible] were examined. This procedure had no effect on the

motility of normal microfilariae.

To determine the effects of the armatures on motility the midguts of freshly engorged mosquitoes were teased apart in tap water in counting chambers to lyse the red blood corpuscles. The microfilariae were examined under the dissecting microscope for movement and individuals that did not move for at least 15 seconds were scored as amotile. To determine the proportion of microfilariae with cuticular abrasions, the blood meals were expelled from the stomachs into small pools of water on slides. Blood clots were teased apart and the smears and midgut epithelia left overnight to dry. They were deheamaglobinized by briefly dipping them in water, fixed in 70% methanol, stained and examined under the compound microscope. The B. pahangi microfilariae were stained with Giemsa and scored as damaged if lesions were observed in either their sheaths or cuticles. The W. bancrofti microfilariae were stained with haematoxylin heated to 50°C. The sheaths did not stain at this temperature and microfilariae were scored as damaged only when nicks were seen in their cuticles. Microfilariae were sometimes obscured by the midgut epithelia and if they could not be seen clearly they were excluded from calculations on the proportion of damaged microfilariae but were included in calculations on the overall number of microfilariae ingested by mosquitoes.

damage on microfilariae was used as a control in each experiment to check that microfilarial damage in other species of mosquitoes was

The [illegible] of mosquitoes and microfilariae used to study the

effects of the armature on Brugia and Wuchereria are presented in Table 2. Most of the damaged Brugia microfilariae had small nicks in their sheaths and holes in their cuticles through which the nuclei exuded and some of the microfilariae were completely severed (Figs. 8-10). These lesions were distributed throughout the bodies and sheaths of the microfilariae.

Between 92 and 96% of the Brugia microfilariae ingested by Anopheles spp. were damaged while only 9 and 22% of those ingested by Aedes spp. were damaged (Fig. 12). This trend was also seen in studies on the motility of ingested microfilariae determined from blood meal examinations in counting chambers. Large proportions of microfilariae from the anophelines were amotile, but only small proportions from the aedines were amotile (Fig. 12). The Aedes spp. have pharyngeal armatures, spines and papillae in the foregut but lack cibarial armatures which are present in the anophelines. This experiment indicates that the cibarial armature is capable of damaging large numbers of ingested Brugia microfilariae while the other structures are relatively harmless.

Damaged, amotile Wuchereria microfilariae were more prevalent in An. gambiae species A which has a cibarial armature than in Ae. aegypti which lacks this structure (Fig. 13) and this observation agrees with the results obtained with Brugia. Damage to Wuchereria was similar to that of Brugia [illegible] in the cuticle to the complete bisection of microfilariae (Fig. 11). However, the degree of damage inflicted on the two species of filariae by the same mosquito was different. An. gambiae damaged twice as many Brugia as Wuchereria while Ae. aegypti damaged four times as many Brugia as Wuchereria.

The observations on C. p. fatigans, which has a cibarial armature but damaged only 5.5% of ingested Wuchereria microfilariae, suggest that it is the structure of the armature and not merely its presence which determines the degree of lethality to microfilariae.

The exact proportion of ingested microfilariae that are actually killed by the armatures is difficult to determine because of technical problems. It was hoped that the proportion of damaged microfilariae in stained smears would match the proportion of amotile microfilariae in counting chambers and that these two values would give the true degree of lethality. Amotile microfilaria are rarely seen in counting chambers containing blood taken directly from the vascular system of infected vertebrates and amotile individuals seen in midgut preparations are probably dead. Unfortunately, amotile microfilariae are difficult to detect in counting chambers and the proportion of dead microfilariae found in chambers was usually less than the proportion found in smears. Analysis of the Brugia data using 2X2 contingency tables showed that the proportions of amotile microfilariae in chambers was less than the proportions on smears in all mosquitoes ($P < 0.01$) except An. farauti ($P < 0.1$) (Fig. 12). The difference between the two techniques was usually greater in mosquitoes which killed large proportions of Brugia microfilariae and it is possible that many of these amotile worms were ov[illegible]. [illegible]t for this conclusion was provided by Fain et al. (1974) who [illegible] that dead B. malayi microfilariae are often overlooked in wet mount preparations.

Analysis of the [illegible] data using 2X2 contingency tables showed that the proportions of amotile microfilariae in chambers and

A. aegypti and C. p. fatigans were examined (Fig. 13). However, in An. [illegible] species A the proportion of amotile microfilariae found in chambers was marginally less than the proportion of damaged worms found in smears ($P < 0.025$). The numbers of amotile microfilariae that were overlooked in chambers must have been minimal because regression techniques showed that the overall numbers of Wuchereria found in chambers and smears fall within normal variation. It is clear that the chamber technique was more efficient in detecting amotile microfilariae of Wuchereria than Brugia. Wuchereria microfilariae might be easier to detect in chambers because they are larger than Brugia.

While the proportion of amotile microfilariae is often erroneously low, the proportion of microfilariae scored as damaged is probably erroneously high. The proportion of damaged microfilariae is a subjective value that was determined from stained smears of infected midguts and does not necessarily reflect the actual proportion of microfilariae killed by the armature. This is particularly relevant to Brugia because these microfilariae were scored as damaged even if injury was limited to the sheath. As injury to the sheath is unlikely to affect microfilarial survival, our estimate of the proportion of damaged [illegible] was probably higher than the proportion that was actually killed. Re-examination of a sample of thick smears indicated that 1/5 [illegible] to [illegible] proportion of [illegible] that were actually killed by the armatures is between the 'damaged' and 'amotile' values (Figs. 12, 13), and it is suggested that the means of these [illegible] from Table 2 be used for the sake of

3. In Vitro Migration of Brugia Microfilariae from the Midguts of An. gambiae and Ae. aegypti

The migration of B. pahangi microfilariae from the stomach was studied to determine if the damaged, immobilized microfilariae seen in previous experiments were capable of penetrating the midgut epithelium. Midguts were removed from An. gambiae species A and Ae. aegypti immediately after feeding and placed in counting chambers containing insect saline at room temperature. The emergence of microfilariae through the gut wall into the media was observed under the dissecting microscope.

A total of 630 microfilariae were observed in 17 An. gambiae species A and 2,172 microfilariae in 14 Ae. aegypti. Only 2.4% of the microfilariae ingested by An. gambiae migrated from the blood meal through the gut epithelia into the culture media whereas 61.7% of the microfilariae in Ae. aegypti migrated to the media (Fig. 14). Microfilariae migrated from all parts of the stomachs of Aedes and Anopheles and there was no preferred 'penetration' site.

4. Susceptibility of the Thoracic Muscles of An. gambiae and [illegible]

To determine if refractoriness to B. pahangi in An. gambiae species A was expressed only in the gut or if it was also expressed in the haemocoel and thorax, exsheathed microfilariae were inoculated

directly into the thoracic muscles. The exsheathed microfilariae were obtained from Ae. aegypti that had fed on cats with high parasitaemias. The engorged midguts were removed intact from the mosquitoes and were placed in counting chambers containing insect saline. The [illegible] microfilariae migrated through the stomach epithelium into the media and were aspirated into fine injection needles which were made from glass capillary tubes. The needles were inserted into the thorax of recipient mosquitoes which had been anaesthetized by cold and the microfilariae were introduced into the thorax by gently blowing into a connecting rubber hose. The mosquitoes were maintained at 28°C and 80% RH and supplied with a 20% sucrose solution. Individuals that died after day 4 of infection were examined for developing stages of Brugia and all surviving mosquitoes were examined on day 11. The head, thorax and abdomen of individual mosquitoes were placed in separate pools of tap water and teased apart. The pools were examined for filarial larvae under the dissecting microscope. The development of injected microfilariae in An. gambiae species A was compared with their development in susceptible Ae. aegypti that were infected in a similar fashion.

The susceptibility of the thoracic muscles of An. gambiae was comparable to that of the susceptible fm/fm strain Ae. aegypti (Table 3). As [illegible] which survive their journey through the gut of Anopheles will develop to the infective stage.

Conclusions

Refractoriness in the [illegible]

There have been a number of studies on the migration of microfilariae in their mosquito vectors and it has often been noted that large proportions of the ingested parasites fail to migrate from the stomach to their developmental site in the thoracic muscles, fat body or Malpighian tubules (Kartman 1953, Laurence and Foster 1961, Jordien and Goatly 1962, Ewert 1965, Goonaratne 1970, Obiamiwe and Macdonald 1973, Bain and Chabaud 1975). These observations have led to a number of speculations on the mechanisms by which mosquitoes kill microfilariae in the gut, but these hypotheses have rarely been subjected to experimental analysis (Denham and McGreevy 1976). However, there is now substantial evidence that the armatures in the foregut inflict lethal damage on microfilariae and that clotting of the blood meal in the midgut inhibits microfilarial migration to the haemocoel (Kartman 1953, Ewert 1965, Obiamiwe and Macdonald 1973).

The effects of the cibarial and pharyngeal armatures on microfilariae were first described by Caluzzi and Trabucchi (1968) who examined midguts from mosquitoes that had fed through membranes on blood containing Dirofilaria [illegible]. Large proportions of the ingested microfilariae were injured in mosquitoes that have cibarial [illegible] and few of the worms migrated to the thorax to complete their development. In other experiments the effects of the cibarial armature were avoided by introducing microfilariae through the anus of mosquitoes. These worms were not damaged in the midguts and developed normally in

the thorax. Microfilariae were not injured when incubated in intestinal and salivary gland extracts. It was concluded that microfilarial damage was inflicted by the teeth of the cibarial armature and was not the result of digestive enzymes or putative antifilarial toxins.

In our experiments, the degree of microfilarial damage and immobilization was compared in species of mosquitoes which have cibarial and pharyngeal armatures. In anophelines which have both armatures, refractoriness to infection by B. pahangi was expressed largely in the midgut because few of the ingested microfilariae penetrated the stomach wall (Fig. 14).

Examinations of microfilariae from the gut of freshly fed anophelines showed that large proportions of the ingested microfilariae of Brugia and Wuchereria were damaged and motile (Figs. 12, 13). This damage was unlikely to be caused by digestive enzymes or antifilarial toxins because the mosquitoes were anaesthetized with cold shortly after feeding and maintained on ice until they were dissected. The dissections were performed as quickly as possible and were completed 5-15 minutes after feeding. The severity of the damage could not have been caused chemically in such a short time at such low temperatures. The damage appeared to be of mechanical origin caused by the teeth, spines and papillae of the foregut which operate in a chewing fashion [illegible].

Armature Variations and Microfilarial Mortality

It is likely that all the papillae, spines and teeth in the foregut of mosquitoes present a hazard to ingested microfilariae. However, the worst damage was seen in microfilariae ingested by species of mosquitoes which have a cibarial armature and there can be little doubt that this is the most lethal structure in the foregut (Figs. 12, 13). However, there are interspecific variations in the shape of the cibarial armature and these differences are reflected in the degree of damage inflicted on microfilariae by a particular mosquito species. For example, An. gambiae species A has a well developed cibarial armature and kills an average of 45.2% of ingested Wuchereria microfilariae while C. p. fatigans has a 'weak' cibarial armature and kills only 5.8% of the microfilariae. The damage to microfilariae in Culex is roughly equal to that in Ae. aegypti which lacks a cibarial armature and it is questionable if the cibarial armature of Culex is lethal at all.

In addition to the presence and structure of the cibarial armature, the degree of damage inflicted on microfilariae may also be related to the size of the mosquito. Coluzzi and Trabucchi (1968) found that [illegible], a very large mosquito, inflicted less damage to the microfilariae of D. [illegible] than smaller An. [illegible] spp. They [illegible] mosquitoes with a large aperture between the cibarial and pharyngeal pumps. Although this speculation may be correct, there is no reason to believe that the cibarial armature of An. [illegible] is simply less lethal [illegible]. [illegible] normal and [illegible] An. [illegible] would clarify the relative importance of the diameter

of the foregut and the shape of the cibarial armature in killing microfilariae.

As mentioned above, hyperinfection with filarial larvae causes pathological changes in mosquitoes which often results in reduced fecundity and premature death. Since the cibarial armature limits the intensity of infection in mosquitoes, its evolutionary development could be an adaptive response to the selection pressure of filarial infection. In terms of mosquito survival, the cibarial armature is a very efficient refractory mechanism because it acts in the foregut immediately after microfilarial ingestion and before any damage to the midgut and thoracic muscles can occur. It is interesting that the thoracic muscles of An. gambiae species A are susceptible to filarial infection. As so few microfilariae reach the thorax of An. gambiae, the selection of refractory genes similar to Rm of Ae. aegypti may not have occurred.

Microfilarial Variations and Survival

The severity of damage to filarial worms may not depend entirely on the physical features of the mosquito foregut, but may also be related to characteristics of the microfilariae. Bafutsi and Trpiowski (1[illegible]) found that longer microfilariae were more likely to be damaged [illegible] which is 31[illegible] u long in wet mount preparations, always suffered more damage than Lufilaria aegypti, which is only 120 u. In contrast to these results we found that An. gambiae species A damaged a higher proportion of Brugia than W. bancrofti even though the latter species

is the longer (Figs. 12, 13). The maximum width X length measurements of the bodies of 20 microfilariae of each species was determined from thick smears of mosquito blood meals and Wuchereria averaged 6 x 355 u while Brugia averaged 7 x 271 u.

The mechanics that results in differential damage to Wuchereria and Brugia in the same species of mosquito is unknown. An Upper Volta strain of An. gambiae species A was used to study B. pahangi and an East African strain was used to study W. bancrofti, but major differences in the structures of their armatures were not detected. This observation is supported by Chwatt (1945) who could not find differences in the structure of the cibarial armatures between fresh and salt water species of An. gambiae (s.l.). Although Coluzzi and Trabucchi (1968) suggested that microfilariae survived better in larger mosquitoes, there were no obvious differences in the size between the East and West African strains of An. gambiae species A that we used.

Differential damage between two species of microfilariae also occurs in Culex. Coluzzi and Trabucchi (1968) found that large proportions of B. rogersi were damaged while we found that only 5.0% of W. bancrofti were killed. Differences in the size of these microfilariae do not appear to be critical because W. bancrofti is about 355 u long in stained smears while B. rogersi is 340 u. W. bancrofti

Coluzzi and Trabucchi (1968). If the sheath protects W. bancrofti in Culex, it provides less protection in An. gambiae species A where 45.0% of the ingested microfilariae are killed and it does not protect Dipetalonema [illegible] microfilariae may [illegible] to avoid injury during their passage through the cibarial

armature.

The ability of some species of microfilariae to avoid injury may represent adaptations in response to selection pressure by the armatures of natural vectors. It is interesting that many of the natural vectors of Wuchereria have cibarial armatures. It is tempting to speculate that during evolutionary time Wuchereria has developed some mechanism to minimize damage by the armature. Since the natural vectors of B. pahangi and B. malayi do not have cibarial armatures, these parasites may not have been exposed to comparable selection pressure and have not developed evasion mechanisms.

The Cibarial Armature and Facilitation

Brengues and Bain (1972) studied the migration of W. bancrofti from the stomach to the thorax of An. gambiae species A. They found that the proportion of microfilariae which leave the stomach increases with the number of microfilariae that are ingested - a phenomenon they called facilitation. The mechanism of facilitation is unknown but Bain and Brengues (1972) and Bain and Chabaud (1975) believe that the local hyperplasia of the midgut epithelium which follows the penetration of the first microfilariae provides a site which is conducive to the penetration of further microfilariae.

of tissue sections of the stomach. They noted that microfilarial penetration and the resulting tissue changes were largely confined to the anterior and posterior poles of the engorged midgut where the epithelium [illegible]. In our in vitro observations

of infected stomachs, we found that B. pahangi microfilariae migrated equally well from all parts of the stomach of Ae. aegypti and [illegible] species A including the stretched 'squamous' shaped epithelium of the midportion. Since hyperplastic reactions rarely occur in the midportion of the stomach (Bain and Chabaud 1975), they could not facilitate the penetration of microfilariae from this point.

Although stomach hyperplasia may play a role in facilitation we feel that the cibarial armature may also be important and supporting evidence comes from studies on the Simulium-Onchocerca system. Bain et al. (1974) found that the proportion of O. volvulus microfilariae which migrate from the gut of S. ochraceum increased as the number of microfilariae ingested increased (= facilitation). They also showed that the proportion of microfilariae that were damaged by the cibarial armature decreased as the number of ingested microfilariae increased. The mechanism of facilitation in S. ochraceum could lie in the clumping of the cibarial teeth with debris from damaged microfilariae when large numbers of parasites are ingested. Omar and Garms (1975) have presented photographs which clearly show that O. volvulus microfilariae do become entangled in the cibarial teeth of S. ochraceum.

To clarify the role of the cibarial armature in facilitation in mosquitoes, the proportion of microfilariae that are damaged by the cibarial armature and the proportion that migrate from the midgut should [illegible] correlation could not be made in the present study because the mean number of microfilariae that were ingested by mosquitoes was far too low.

Practical Application

In filariasis control campaigns based on mass chemotherapy with diethylcarbamazine (= DEC), transmission from treated carriers depends in part on the susceptibility of the mosquitoes to infection by Wuchereria. In the Pacific Islands, transmission probably continues because the vector Aedes, which lack well developed armatures, are very susceptible to infection (Rosen 1955, Symes 1960). When Aedes polynesiensis feeds on carriers presenting ultra low parasitaemias it is capable of ingesting a few microfilariae which in the absence of strong refractory mechanisms develop to the infective stage. Bryan and Southgate (1976) fed Ae. polynesiensis on a donor with 6 mf/ml and found that 10% of the mosquitoes were infected with a mean of 1.3 worms.

It is likely that the dynamics of Wuchereria transmission in the Pacific Islands may be similar to that in areas where C. p. fatigans is a vector but may be different in areas where An. gambiae species A is a vector. Like Ae. polynesiensis, East African C. p. fatigans ingested microfilariae from carriers with ultra low parasitaemias and supports their development to the infective stage. In contrast, An. gambiae species A ingests fewer microfilariae than either Aedes or Culex when fed on carriers with ultra low parasitaemias (McGreevy et al. [illegible] killed by the [illegible] needed to interrupt transmission is higher in areas where Anophelines are vectors relative to areas where Aedes and Culex are vectors.

Table 1. Distribution of the cibarial armature in selected Culicids

Species with cibarial armatures	Species without cibarial armatures
Anopheles (Cellia)	Anopheles (Anopheles)
[illegible], funesti No. 1,	[illegible], [illegible],
funesti No. 2, funestus,	labranchiae, quadrimaculatus
gambiae species A,B,C,D,	Aedes
maculatus, [illegible], pharoensis,	aegypti, cooki, malayensis,
stephensi	polynesiensis, tabu, togoi
Anopheles (Nyssorhynchus)	Mansonia
albimanus	uniformis
Culex	
pipiens fatigans	

Table 3. Development of *B. pahangi* in *An. gambiae* species A and *Ae. aegypti* after intrathoracic injection with exsheathed microfilariae.

Mosquito spp.	No. mosquitoes dissected	No. mosquitoes infected	Normal larvae	Abnormal larvae
An. gambiae species A	56	38	148	6
Ae. aegypti	42	20	98	6

Table 2. *B. pahangi* and *W. bancrofti* in blood meals of mosquitoes: Number of microfilariae with normal, damaged and unscored* morphology found in stained smears and the number of motile and amotile microfilariae found in counting chambers.

Filarial species	Mosquito species	Microfilarial Morphology				Microfilarial motility		
		Infected mosquitoes	Normal	Damaged	unscored*	Infected mosquitoes	Motile	Amotile
B. pahangi	*An. [illegible]rti*	37	1446	135	8	37	2386	141
	An. [illegible]i	12	135	38	1	17	203	24
	An. [illegible]uti No.1	10	17	194	4	11	8	40
	An. [illegible]iae A	53	49	974	31	23	84	161
	An. [illegible]iae D	28	42	965	50	19	28	43
W. bancrofti	*Ae. [illegible]ti*	178	817	17	114	63	485	11
	C. p. fatigans	152	835	49	200	40	412	27
	An. [illegible]iae A	117	151	148	21	23	91	52

* A clear view of these microfilariae was obscured by pieces of mosquito stomach and they could not be scored as normal or damaged.

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Legends for Figures

Fig. 1. Schematic longitudinal section of the head of a mosquito showing the armature of the foregut. Cibarial pump (A), palatal papillae (B), dorsal papillae (C), ventral papillae (D), posterior hard palate (E), cibarial armature (F), pharyngeal pump (G), pharyngeal armature (H).

Figs. 2-7. Cibarial armatures of An. gambiae species A (2), An. albimanus (3), C. p. fatigans (4), An. balabacensis (5), and An. farauti (6). Pharyngeal armature of An. gambiae species B (7).

Figs. 8-11. Microfilariae. Normal B. pahangi with intact sheath, cuticle and nuclear column X1600. (8). Damaged B. pahangi with nuclei protruding through abrasions in cuticle and sheath X1800. (9). B. pahangi with tears in sheath X570. (10). Anterior end of W. bancrofti with disrupted nuclear column X1700. (11).

Fig. 12. Proportion of microfilariae of B. pahangi in midguts of mosquitoes that were damaged in stained smears and the proportion that were motile in counting chambers.

Fig. 13. Proportion of microfilariae of W. bancrofti in midguts of [illegible] were motile in counting chambers.

Fig. 14. Proportion of microfilariae of W. bancrofti that migrated from the opposite ends of [illegible] in vitro.

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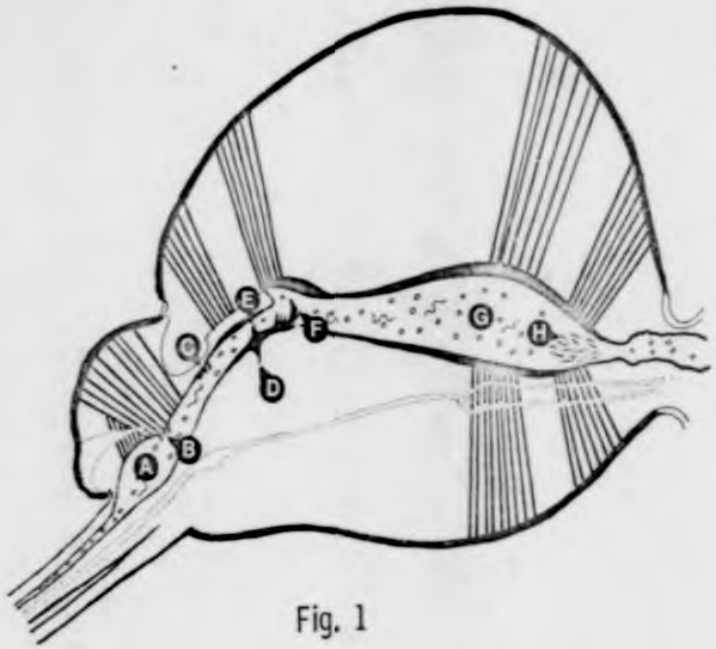

Fig. 1

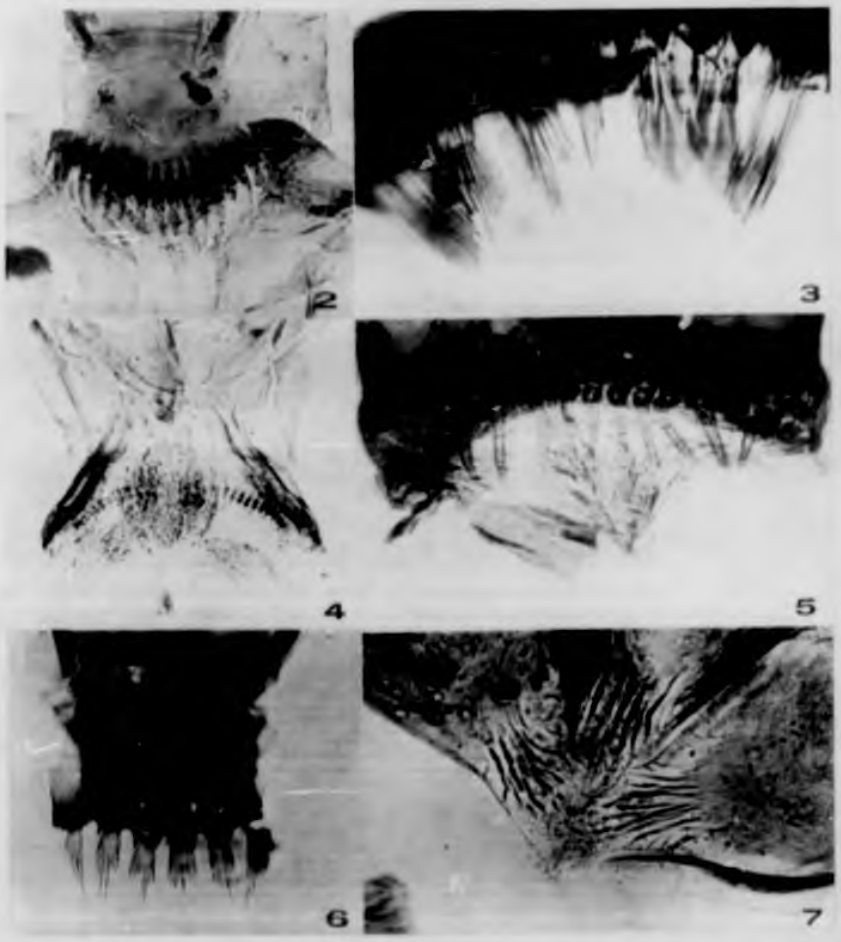

Fig.2－7

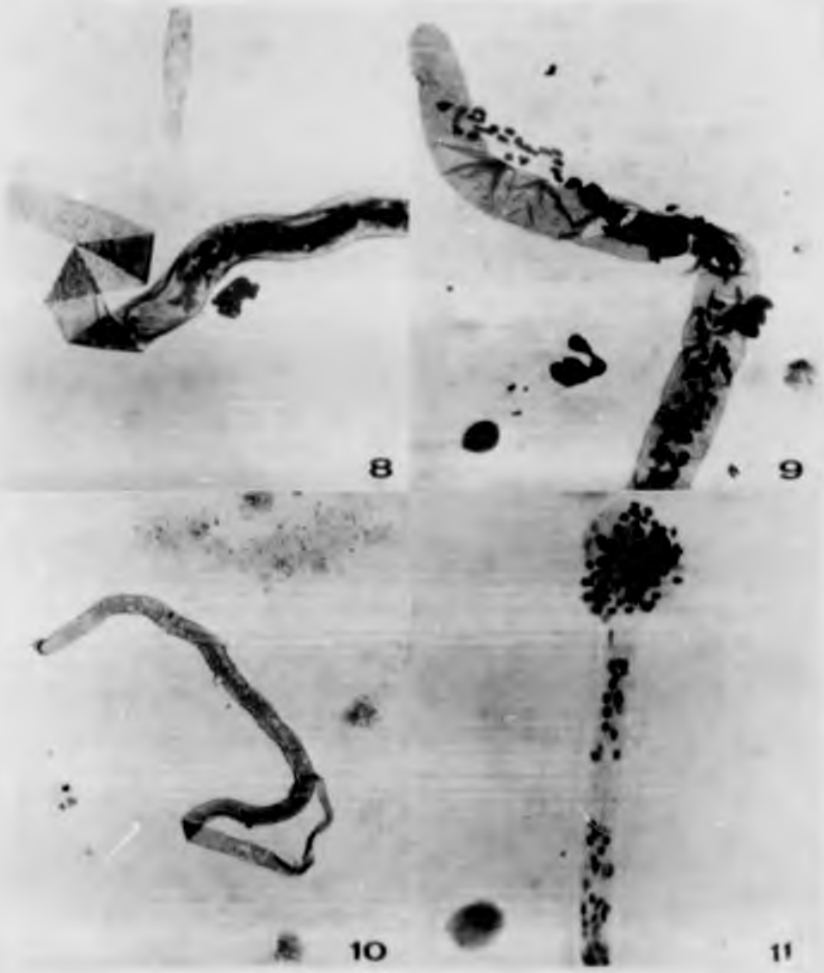

Fig.8 - II

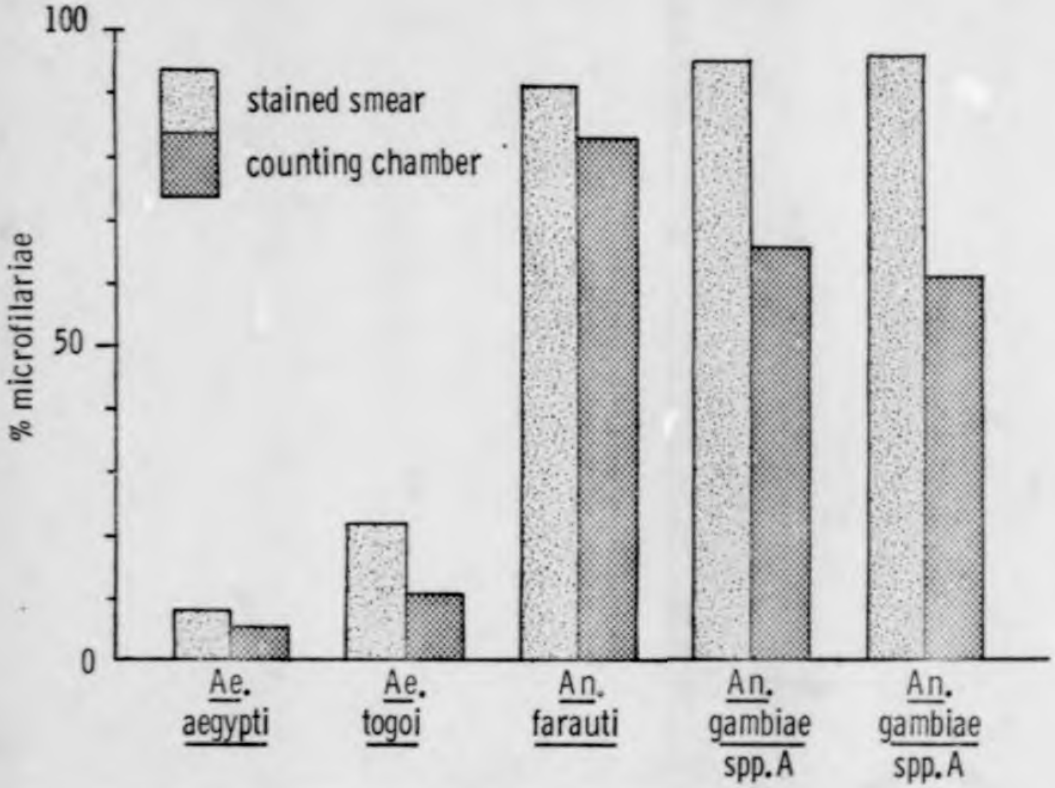
100
50
0
% microfilariae
stained smear
counting chamber
Ae. aegypti
Ae. togoi
An. farauti
An. gambiae spp.A
An. gambiae spp.A
MOSQUITO SPECIES

Fig. 12

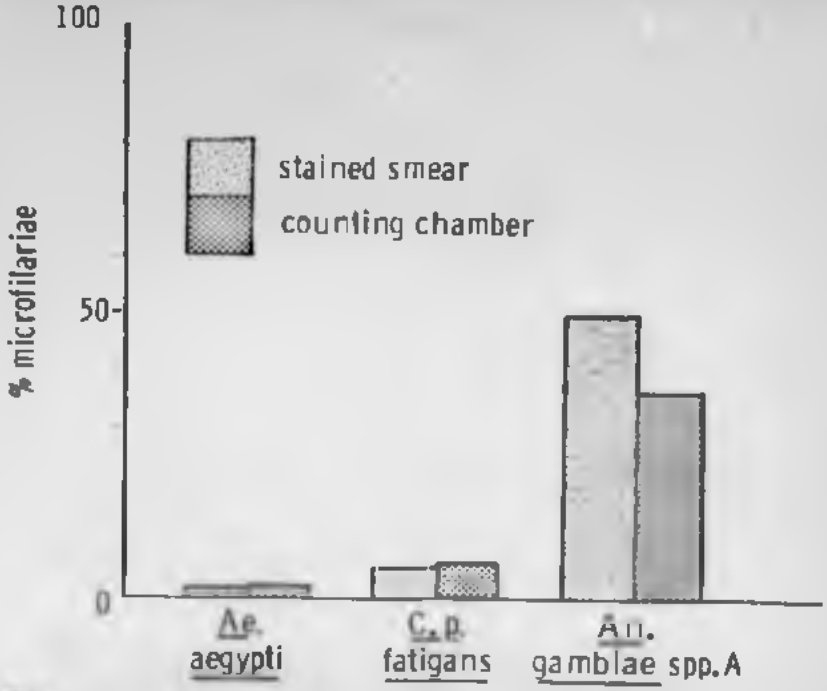
100
50
0
% microfilariae
stained smear
counting chamber
Ae. aegypti
C. p. fatigans
An. gamblae spp. A
MOSQUITO SPECIES

Fig. 13

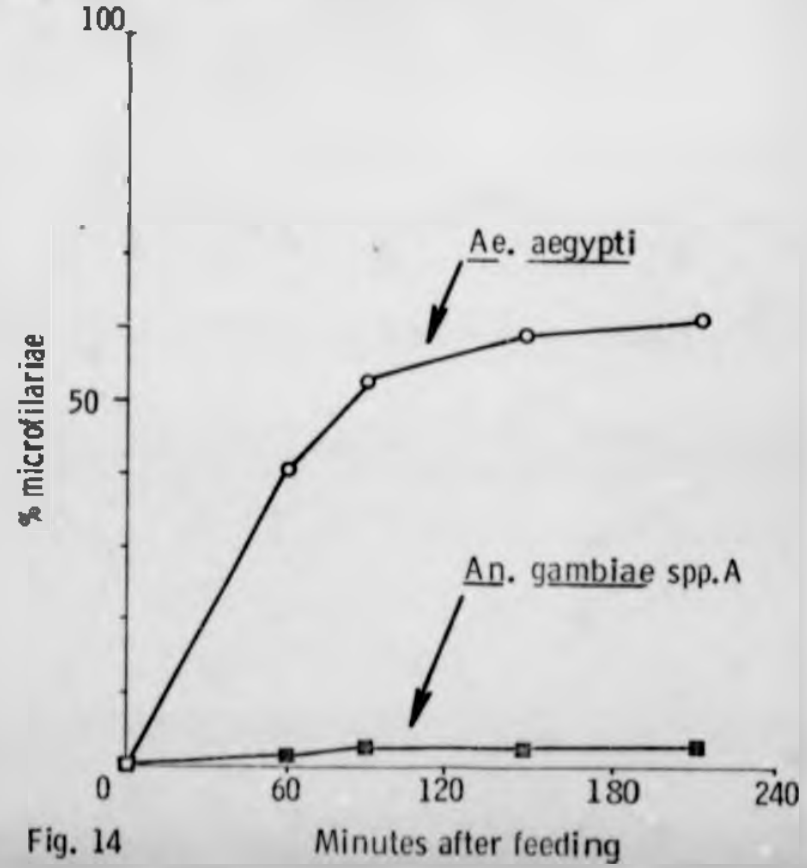
100
50
0
% microfilariae
Ae. aegypti
An. gambiae spp. A
60
120
180
240
Minutes after feeding

Fig. 14