PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST TUBULIN FROM INTESTINAL AND TISSUE NEMATODES

(*Ascaris suum* & *Brugia pahangi*)

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SUGGESTED SHORT TITLE

Characterization of anti-nematode tubulin monoclonal antibodies

TO THE MEMORY OF MY FATHER INAYAT ALI BUGHIO

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PREFACE

The work described in this thesis was carried out in Dr. G. M. Faubert and Dr. R. K. Prichard's laboratories, at the Institute of Parasitology, McGill University, Montréal, between September 1986 and March 1991.

The purpose of the study was to produce monoclonal antibodies (MAbs) against tubulin from body wall muscle, intestine and reproductive tract of *A. suum* and *B. pahangi*, respectively. Anti-*A. suum* MAbs were used to identify and compare the tubulin content, isoforms, and proteolytic peptides from various tissues of *A. suum*. The specific binding of mebendazole drug in extracts from body wall muscle, intestine and reproductive tract of *A. suum* was also examined and compared. The binding data were analyzed using computer programs EBDA and LIGAND, based on the fundamental principles of receptor-interactions. Tubulin was characterized in various species of intestinal and tissue nematodes. β-tubulin was detected in the sera of infected human, and sera and peritoneal fluid of infected animals. The effects of anti-*B. pahangi* tubulin MAbs on the adult *B. pahangi* were also examined.

The accounts of the studies have been arranged in six chapters. Chapter one gives a general introduction of the subject and a review of the literature relating to the area of study. Chapters two to five describe the various studies undertaken. Chapter six gives a general discussion and conclusions of the studies.

The references are cited in the text by first author where there are three or more authors, or by both authors where there are only two, and are arranged in alphabetical order in the References section. The abbreviations used in the text are given in full in Appendix i. Appendix ii gives a list of the publications that have resulted from work described in this thesis.

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CLAIMS OF ORIGINALITY

Several aspects of the studies reported in this thesis are original contributions to scientific knowledge of the biochemistry of parasitic nematodes:

1. Production of monoclonal antibodies against β-tubulin of parasitic nematodes *Ascaris suum* and *Brugia pahangi*, is reported for the first time.
2. The tubulin content of body wall muscle, intestine and reproductive tract of *A. suum* are characterized and compared for the first time.
3. The presence of β_1 and β_2 tubulin peptides in the body wall muscle, intestine and reproductive tract of *A. suum* is demonstrated for the first time.
4. It is demonstrated for the first time that α- and β-tubulin isoforms are expressed differentially in the body wall muscle, intestine and reproductive tract of *A. suum*.
5. It is reported for the first time that total binding per mg protein decreases from intestine, followed by body wall muscle to reproductive tract extracts in the *A. suum*. No specific binding was detected in the reproductive tract. This coincides with the parallel decrease in tubulin per mg protein in the various tissues.
6. For the first time, the specific and non-specific binding of mebendazole to the tubulins of body wall muscle, intestine and reproductive tract of *A. suum*, is demonstrated.
7. The presence of tubulin in the cuticular layers, such as median and basal, of *B. pahangi* is shown for the first time.
8. Anti-tubulin antibodies are cytotoxic, and they induce significant reduction in the viability of *B. pahangi;* this is demonstrated for the first time.
9. Circulating β-tubulin was detected in the sera of infected animals by anti-*B.*

pahangi tubulin specific monoclonal antibodies; this is demonstrated for the first time.

10. Species-specificity of anti-*B. pahangi* or anti-*A. suum* tubulin specific monoclonal antibodies has not been reported before.
11. β-tubulin isoforms in the intestinal extract of *A. suum* are different from those of body wall muscle, and reproductive tract, in number and electrophoretic mobilities; this is reported for the first time. It is suggested that these differences may account for the sensitivity of intestinal tissue to mebendazole attack.
12. The numbers of mebendazole receptors in the tubulin of *A. suum* body wall muscle and intestine are reported for the first time.
13. This is the first report of differences in the peptide patterns from tubulins in the body wall muscle, intestine and reproductive tract of *A. suum*, following proteolytic digestion.

ABSTRACT

Monoclonal antibodies (MAbs) have been raised against β-tubulin of *B. pahangi* and *A. suum*. Anti-*B. pahangi* MAbs were used to investigate the heterogeneity of tubulins from nematodes and mammals. One-dimensional SDS-PAGE showed that MAbs P3D and 1B6 react with β-tubulin from a number of filarial and intestinal nematodes, but not with tubulin from protozoan and mammalian cells. Two-dimensional SDS-PAGE demonstrated that MAb P3D recognizes two isoforms of β-tubulin and 1B6 recognizes one. Limited proteolysis showed that MAb 1B6 reacted with the amino-terminal fragments and MAb P3D with the carboxyl-terminal fragments of β-tubulin. The effect of anti-*B. pahangi* MAbs on the viability of adult *B. pahangi* was assessed using MTT assay. It was found that MAbs P3D and 1B6 caused an 80% and 40% reduction respectively, in worm viability, whereas anti-chick MAb 357 or mebendazole drug had no effect. Immunogold labelling of *B. pahangi* demonstrated the presence of tubulin in the median and basal layers of the cuticle, hypodermal layer and somatic muscle blocks, as well as the uterus of *B. pahangi*. The reduction in the viability of worms may, therefore, be due to the disruption of microtubules in the body wall muscle of *B. pahangi*. The total MBZ binding was highest in the intestine followed by the body wall muscle and in the reproductive tract extracts of *A. suum*. Electron microscopy of *A. suum* tissues demonstrated that the tubulin content decreased from the intestine through the body wall muscle to the reproductive tract. One dimensional SDS-PAGE revealed the presence of α, β_1 and β_2 tubulin subunits in all tissues of *A. suum*. This data confirmed the reduction of tubulin from the intestine through the body wall muscle to the reproductive tract. Two dimensional SDS-PAGE followed by Western blotting demonstrated that α and β tubulin isoform patterns are dissimilar in different tissues of *A. suum*. Body wall muscle, intestine and reproductive tract tubulins were found to differ in their isoelectric points, number of isoforms and peptide maps. These data indicate that different tubulins are found in different tissues of adult *A. suum*. Differences in tubulin isoforms from different tissues may be important determinants in selective sensitivity of these tissues to benzimidazole attack.

Résumé

Des anticorps monoclonaux (AcM) ont été produits contre la β-tubuline de *B. pahangi* et de *A. suum*. L'hétérogénéité des tubulines de nématodes et de mammifères a été étudiée à l'aide des AcM dirigés contre *B. pahangi*. L'électrophorèse dans des gels de polyacrylamide contenant du SDS (SDS-PAGE) a démontrée que les AcM P3D et 1B6 ont réagi avec la β-tubuline d'un certain nombre de nématodes filariens et intestinaux, mais non avec la tubuline de protozoaires et de mammifères. De plus, un SDS-PAGE bidimensionnel a établi que le AcM P3D reconnaît les deux isoformes de la β-tubuline mais que le AcM 1B6 n'en reconnaît qu'une. Suite à une protéolyse partielle, l' AcM 1B6 a réagi avec les fragments terminaux aminés et l' AcM P3D avec les fragments terminaux carboxyles de la β-tubuline. L'effet des AcM P3D et 1B6 dirigés contre *B. pahangi* sur la viabilité des vers adultes a été déterminé par un test utilisant du MTT. Cette viabilité a été réduite de 80% par le AcM P3D et de 40% par l' AcM 1B6. Aucun effet n'a été enregistré avec le AcM 357 dirigé contre le poulet et le mébendazole. Le marquage de *B. pahangi* avec des particules d'or (*immunogold labelling*) a révélé la présence de tubuline dans la couche intermédiaire et la couche de fond du cuticule, dans l'épiderme, dans les cellules somatiques des muscles et dans l'utérus. Cette diminution de viabilité des vers pourrait ainsi être expliquée par la dépolymérisation des microtubules dans le tégument musculaire de *B. pahangi*. La tubuline des intestins d'*ascaris* a démontré une fixation totale plus élevée de mebendazole que dans les autres tissus et une plus haute affinité de fixation que le tégument musculaire Comparativement aux tissus intestinaux, la fixation maximale de la tubuline du tégument musculaire a été réduite mais son affinité de fixation n'a pas changé. Les tissus de *A. suum* observés au microscope électronique ont démontré que le contenu en tubuline diminuait successivement de l'intestin aux muscles et des muscles aux organes reproducteurs. Un SDS-PAGE a révélé la présence des sous-unités de la tubuline α, β_1 et β_2 dans tous les tissus de *A suum*. Ces résultats ont confirmé que la concentration en tubuline est plus élevée dans les intestins que dans le tégument musculaire et que les organes reproducteurs contiennent la plus faible concentration. Un SDS-PAGE bidimensionnel a démontré que les isoformes de la tubuline α et β sont différentes dans les

divers tissus de *A. suum*. La tubuline du tégument musculaire, des intestins et des organes reproducteurs diffèrent dans leurs points isoélectriques, leurs nombres d'isoformes et la localisation des polypeptides Ces résultats ont indiquént que les différentes tubulines se retrouvent dans les tissus de l'adulte *A. suum*. La connaissance de ces différentes isoformes de la tubuline dans les tissus de nématodes peut être un facteur déterminant de leur sensibilité à l'attaque des benzimidazoles.

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CHAPTER 1

LITERATURE REVIEW

1.0 ASCARIS AND ASCARIASIS

Ascariasis, caused by the nematode *Ascaris lumbricoides*, is the most common human parasitic infection in the world. It has a worldwide distribution and approximately 1.3 billion people are infected with this parasite (Crompton, 1988) *A lumbricoides*, which lives in the upper part of the small intestine, is often referred to as the giant intestinal worm because it grows to a length of more than 30 cm *Ascaris* eggs survive best in warm, moist soil however, they are highly resistant to a variety of environmental conditions and can survive even in the sub-arctic regions Although the mortality rate for ascariasis appears low, the absolute number of deaths due to this disease is high in relation to other infections because of the high prevalence (Crompton, 1985)

1.1 MORPHOLOGY OF ASCARIS

The adult worms are cylindrical, with a tapering anterior end Females are 20-49 cm long and 3-6 mm in diameter; males are 15-31 cm long and 2-4 mm in diameter with a curved posterior end (Garcia & Buckner, 1988) The body of *Ascaris* is rounded and tense because the body cavity (pseudocoelom) is filled with fluid at a high pressure. The cuticular surface of *Ascaris* is striated and cream coloured with two whitish lateral cords in which the excretory canals are situated There are three prominent lips in the anterior part, which are the characteristic of this group The vulvar opening is on the ventral surface between the anterior and middle third of the body length in the females. In males there are two copulatory spicules and numerous papillae at the ventrally curved posterior end Most of the pseudocoelom of the *Ascaris*

is taken up by an alimentary canal and a tubular reproductive tract, which is double in females and single in males (Pawlowski, 1990). *A. lumbricoides* is specific for humans but it has also been found accidentally in other hosts such as orangutan, dog, cat and sheep (Pawlowski, 1982) *A. lumbricoides* is most closely related to *A. suum*, the round worm that infects pigs. The larvae of *A. suum* can develop in humans but without reaching to maturity (Phills *et al.*, 1972).

Although infections due to *A. lumbricoides* have been experimentally induced in humans (Gelpi & Mustafa, 1967), much of the knowledge about human ascariasis derives from infections experimentally induced with *A. suum* in pigs. Such infections in pigs provide a good experimental model for biochemical, immunological and pathological studies (Stephenson *et al.*, 1980).

1.2 LIFE CYCLE

A. lumbricoides has a relatively simple life cycle (Fig. 1.1) with humans as the only host for both the larval and adult stages and soil as the environment for development and a natural reservoir of eggs (Pawlowski, 1990) The adult worms live in the lumen of the upper small intestine, where they consume predigested food. The reproductive potential of the female worm is extremely high (up to 240 000 eggs per day) (Brown & Cert, 1927) and thus, counterbalances the heavy losses in viability and infectivity of the eggs in the environment The eggs laid by female worms are fertilized but non-embryonated. They become incorporated into the feces in the large intestine Embryogenesis (development of the first stage larva in fertilized egg) of the fertilized eggs is temperature dependent and takes place in the soil. If the eggs are deposited in the soil directly, the embryogenesis is completed within 2-4 weeks, depending on the ambient temperature and moisture (Pawlowski, 1987).

Infection in humans is acquired through ingestion of the embryonated eggs from contaminated soil, food and water. On ingestion, the second stage larvae leave the egg shell; hatching of the larvae is stimulated in the small intestine by the combined action of bile salts and alkalinity of the enteric juice. The second-stage larvae then penetrate the intestinal wall and enter the lamina propria, where they

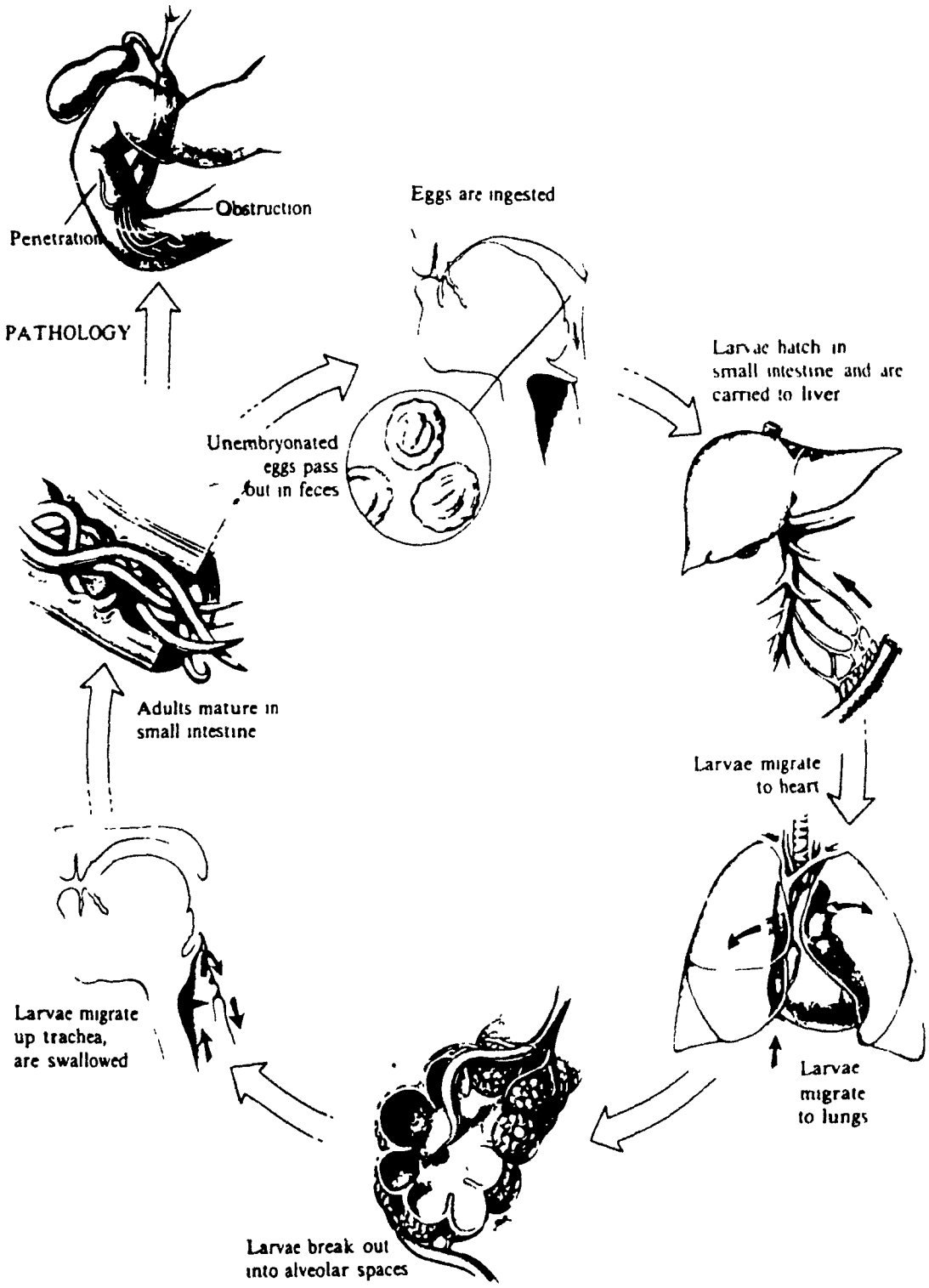


Fig. 1.1. The life cycle of *A. lumbricoides*.

(Katz *et al*, 1988)

enter into a capillary and are carried passively to the liver. From there, they migrate to the right heart chamber via the hepatic portal circulation. On reaching the lung through the pulmonary circulation, the now third-stage larvae reside in alveolar capillaries and break out into the alveolar spaces.

After approximately 10 days in the lungs, the larvae migrate actively up the bronchi into the trachea, across the epiglottis, and are then swallowed, finally reaching the lumen of the small intestine. After two molts, the worms mature to adulthood. Occasionally, egg production may precede mating. When this occurs, infertile eggs are passed by the worm. Worms reach sexual maturity within 6 weeks after the final molt and cease growing. The entire developmental process, from ingestion of eggs by the host, to the production of eggs by female worm, may take from 10-12 weeks (Grove 1990; Schwartzman, 1991). The life cycles of *A. lumbricoides* in humans and *A. suum* in pigs are virtually identical in terms of developmental stages. The time necessary to complete each stage of development may vary slightly between species as well as between worms of the same species. However, the life cycle described for *A. lumbricoides* applies also to *A. suum* infection in pigs, and this is one of the reasons why *A. suum* infection in pigs is such a useful animal model for the study of human ascariasis.

1.3 PATHOGENESIS AND DIAGNOSIS

The pathogenesis caused by *Ascaris* infections is attributed to: (1) the host's immune response; (2) effects of larval migration; (3) mechanical effects of the adult worms; and (4) nutritional deficiencies due to the presence of the adult worms (Pawlowski, 1990). In ascariasis the human host responses and the clinical picture differ in the tissue phase of infection, caused by migrating larvae, and in the intestinal infections with pre-adult and adult worms (Garcia & Buckner, 1988). The initial passage of larvae through the liver and lungs usually elicits no symptoms. The intensity of the systemic response to the migratory phase of *Ascaris* is related directly to the number of larvae migrating simultaneously. If the infection is light and only a few larvae pass through the tissues, the host response is negligible and clinically

inapparent. If the infection is heavy, the patients experience intense pneumonitis, enlargement of the liver and generalized toxicity. When the larvae break out of the lung tissue and into the alveoli, there may be some bronchial epithelium damage. With reinfection and subsequent larval migration, there may be intense tissue reaction, even with small numbers of larvae. There may be pronounced tissue reaction around the larvae in the liver and lung with infiltration of eosinophils, macrophages and epitheloid cells. This has been called *Ascaris* pneumonitis and is accompanied by an allergic reaction consisting of dyspnea, transient eosinophilia and pneumonia (Gelpi & Mustafa, 1967). This transient pulmonary infiltrate, clears within a few weeks and is associated with general hypersensitivity reactions such as asthma and peripheral eosinophilia called Loeffler's syndrome. However, these reactions can also be mediated by contact with *Ascaris* allergen without any actual infections (Pawlowski, 1978; 1985; 1990).

The intestinal phase of the disease is generally asymptomatic and the presence of the adult worm in the intestine usually causes no difficulties unless the worm burden is very heavy. The most serious complications occur due to the migration of the adult worms and intestinal obstruction by a bolus of adult worms (in the intestine the worms may become entangled and form a bolus obstructing the lumen) Such migrations may also cause the obstruction of the hepatic duct, appendicitis, intestinal perforation, including penetration of intestinal incisions and pancreatic duct obstruction (Maki, 1972). Certain irritants, such as fever, a diet rich in pepper, anesthesia or improper treatment tend to stimulate migration of the adult worms (Pawlowski, 1978). *Ascaris* may affect nutrition of infected children, especially those already in a state of marginal adequate nutrition. Nesheim (1985) demonstrated that the severe nutritional impairment related to the worm burden contributes to growth retardation in young children. It has been suggested that *Ascaris* induces malabsorption of fats and carbohydrates.

The diagnosis of ascariasis is usually made by stool examination. Characteristic eggs may be seen on direct examination or may be concentrated by centrifugation. Infections consisting of only male worms will produce no eggs; if such infections are

symptomatic, the worms may sometimes be detected radiologically (Grove, 1990).

1.4 ASCARIS ALLERGEN

The *Ascaris* allergens are the most potent allergen of parasitic origin, which are present in all stages of the *Ascaris* life cycle (Strejan, 1975). The *Ascaris* allergens can cause a hypersensitivity reaction in the lungs, skin, conjunctiva, gastrointestinal tract, anaphylactic shock and degradation of visceral organs (Strejan, 1978). Such reactions have been observed in infected individuals and in uninfected researchers who were in contact with the *Ascaris* allergen (Coles, 1985).

Three types of allergies are recognized in researchers handling *Ascaris* worms: (1) respiratory allergies, (2) skin rashes and (3) gastrointestinal disorders. For example, one month after being in contact with the parasite, irritation of eyes and lacrimation leading to fits of sneezing, pronounced dyspnoea, asthmatic attacks and painful headaches were observed in these workers. However, prolonged contact with this nematode seemed to lead to reduced sensitivity (Lloyd Jones & Kingscote, 1935; Pawlowski, 1985, 1990). In another researcher, a transient irritating rash on the hands was observed about one year after handling the worms. Severe nasal symptoms developed when dried worm tissues were inhaled and these were followed by temporary asthmatic breathing. Respiratory allergies to *Ascaris* are associated with high levels of IgE (O'Donnel & Mitchell, 1978). Presumably sensitization is by inhalation of aerosols of the worm products, thereby swallowing the antigens. The IgE produced may, however, be specific for *Ascaris*. The role of this IgE in the control and expulsion of *Ascaris* from host remains to be clarified (Ogilvie & Parratt, 1977; Pawlowski, 1990).

2.0 CHEMOTHERAPY OF ASCARIASIS

No specific treatment for pulmonary ascariasis is available. In severe cases most of the symptoms, which are of a hypersensitivity type, respond well to corticosteroid therapy (Phills *et* al., 1972). Whereas, the treatment of intestinal ascariasis can be accomplished by several effective drugs (Pawlowski, 1990) as

mentioned below.

2.1 Piperazine

Piperazine derivatives temporarily paralyse the *Ascaris* worms by producing neuromuscular blockage through an anti-cholinergic action at the myoneural junction; the paralyzed worms are easily evacuated by the peristaltic movement of the intestine (Pawlowski, 1982). Piperazine, widely used for over 25 years in ascariasis is now being withdrawn from the market in the developed countries because of sporadic hypersensitive and neurotoxic reactions and also because better drugs have been introduced. However, in developing countries piperazine is still used because it is one of the cheapest drugs available. The efficacy of a single dose treatment is 70-80%; treatment for two consecutive days is effective in over 90% of ascariasis cases (Janssens, 1985).

2.2 Levamisole

Levamisole acts as a cholinergic agent, causing a spastic paralysis of nematodes. Levamisole shows non-specific activation of macrophages and some immuno-modulating activities. When used in a single dose, it is effective in 80-100% of cases of ascariasis (Pawlowski, 1978; Janssens, 1985).

2.3 Pyrantel pamoate

Pyrantal acts on the neuromuscular transmission in *Ascaris* and causes paralysis of the worm. The efficacy of a single-dose treatment in ascariasis is over 90% (Pawlowski, 1978; Janssens, 1985).

2.4 Benzimidazole carbamates (BZs)

Mebendazole, a benzimidazole carbamate, inhibits phosphorylation in the *Ascaris* mitochondria and damages the microtubule system of the intestinal cells, leading to the slow death of the worm. It has been observed that *Ascaris* are rather frequently expelled by mouth during therapy. Several regimens have been devised for

effective single-dose therapy using benzimidazole derivatives. In one study, a single dose of mebendazole was given to patients and was about 90% effective in eradicating *Ascaris* (Pawlowski, 1978). The efficacy of mebendazole is between 84-100% (Janssens, 1985). The other BZs such as fenbendazole, flubendazole, and albendazole, show a similar efficacy and good tolerance. Albendazole, given in a single dose has resulted in cure rates of 100% against *Ascaris* infections (Anderson & May, 1982).

3.0 FILARIA AND FILARIASIS

Lymphatic filariasis is caused by infections of the human parasites *Wucheraria bancrofti*, *Brugia malayi*, and *Brugia timori*. Filarial worms are thread-like nematodes, whose adults live within lymphatic vessels. The female worms are ovoviviparous, and their larvae are called microfilariae (Mf). Approximately 400 million people are infected with *W. bancrofti* alone (WHO, 1989) in Africa, South East Asia and to a lesser extent in Latin America (Grove, 1990). Lymphatic filariasis is rarely life-threatening, but causes severe chronic suffering and disability.

Depending on the species, Mf may exhibit periodicity in the blood circulation. A circadian fluctuation in which the highest number of Mf occur in the blood at night is called nocturnal periodicity. Some species may be non-periodic or diurnal. In sub-periodic or nocturnally subperiodic species, the Mf can be detected in the blood throughout the day but are found at higher levels during the night. Nocturnal periodicity can be the result of the Mf's penchant for low oxygen tension. In experiments in which sleep habits of infected volunteers were reversed, the periodicity of Mf also reversed (Hawking, 1966). Nevertheless, the diurnal periodicity pattern has not been satisfactorily explained yet.

3.1 MORPHOLOGY OF BRUGIA

B. malayi is closely related to *W. bancrofti*, however, there are important differences in their biology, epidemiology and clinopathologic characteristics of infection. *B. malayi* is similar to *W. bancrofti* in morphology, but the adult worms are about half the size of those of *W. bancrofti*. The adult *B. malayi* also closely resembles

B. pahangi which is found in wild cats, tigers and monkeys

Female *B. malayi* are 40 cm long and 0.17 mm wide and the male worms are 20 cm long and 0.08 mm wide and have copulatory spicules and caudal papillae that distinguish them from *W. bancrofti*. Both female and male *B malayi* are creamy white, cylindrical, bluntly tapering thread-like nematodes with smooth cuticular surfaces and unarmed mouth. The vulva of the female opens just 0 8-0 9 mm from the anterior tip of the worm but extends posteriorly to the vagina and the bilateral coiled uterus that runs the full length of the adult female. Mf measure 177-260 µm long and 5-6 µm wide, and have a column of nuclei that extends into the caudal region with two terminal nuclei. These caudal nuclei along with a long cephalic space, are the most helpful characteristics distinguishing Mf of *B. malayi* from those of other filarial Mf The developmental stages of *B. malayi* and *B. pahangi* are morphologically similar. The Mf of these two species can be distinguished by the distinct distribution of acid phosphatase activity on their surface (Redington *et al.*, 1975).

3.2 LIFE CYCLE

Filarial worms have a complex life cycle (Fig 1.2). Adult *B. malayi* live in the lumen of lymphatic vessels of vertebrate hosts. The females produce partially embryonated eggs that develop and then uncoil to become snake-like Mf, the first stage larvae. The microfilarial embryos develop within individual membranes or shells. These are retained after the Mf have been discharged from the female and are recognized as the Mf sheath. Transmission is effected by the uptake of the first stage larvae (Mf) into blood sucking arthropods (Anopheles, Mansonia) as they feed on the infected host. Mf lose their sheath in the insect's stomach, penetrate the gut of the host and migrate to the thoracic muscles. The larvae develop, without multiplication into second- and later into infective third-stage larvae that migrate throughout the hemocoel and eventually reaching the labium. They escape from there when the arthropod host is feeding and enter the skin. After migrating through the peripheral lymphatics, the worms reside either in the lymph nodes and adjacent lymphatics or in the subcutaneous tissues (Nelson, 1979).

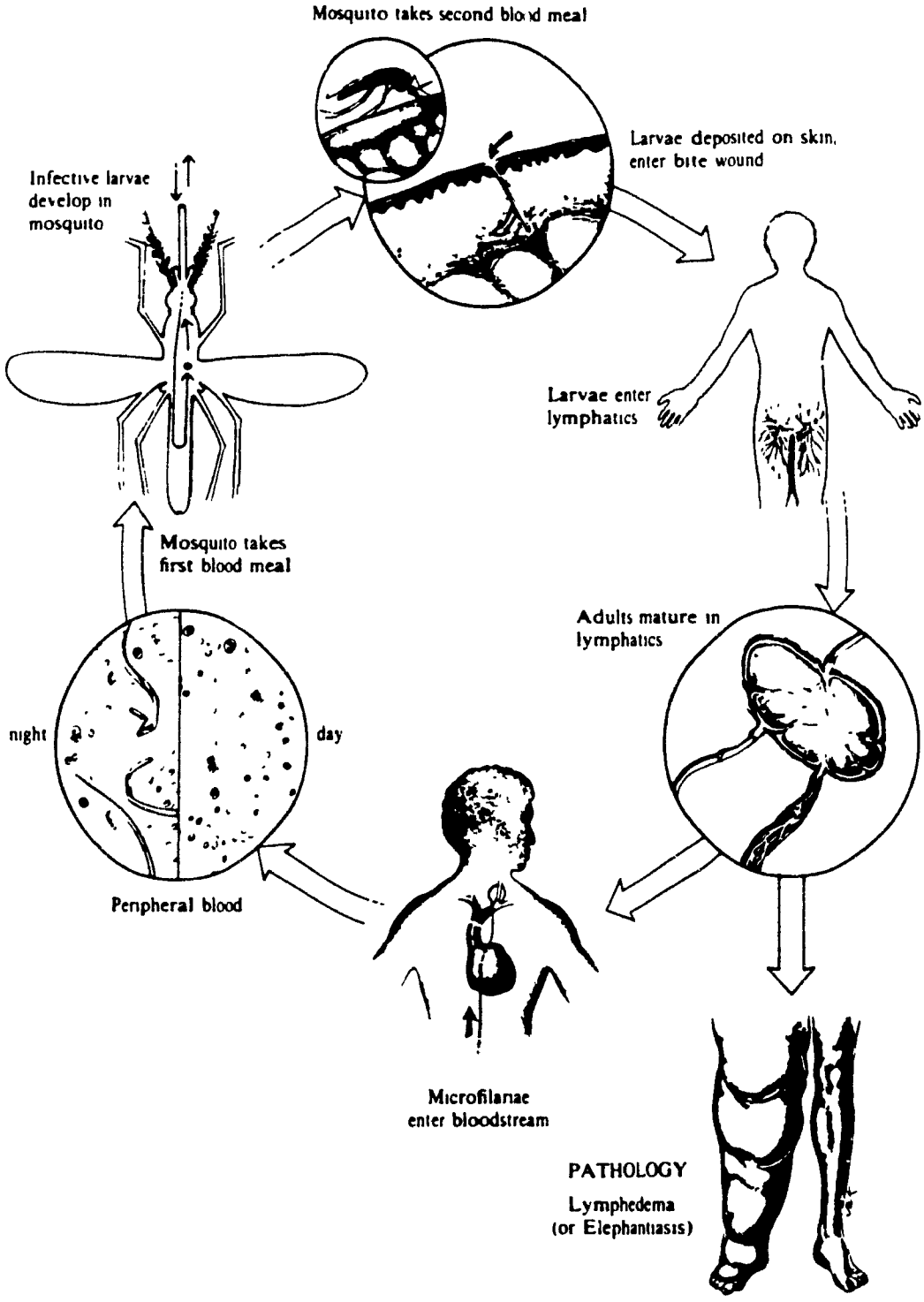


Fig. 1.2. The life cycle of *B malayi*

(Katz *et al.*, 1988)

W. bancrofti does not infect any laboratory rodent, and limited parasite material can be obtained from humans, natural mosquito vectors or experimental infections of the silvered leaf monkey (Palmieri *et al.*, 1982) On the other hand, *B malayi* can be maintained in the laboratory in jirds and sufficient parasite material can be obtained for immunochemical analysis. However, this model does not mimic the disease observed in human. The closely related animal parasite *B. pahangi*, is the species most readily maintained in the laboratory, either in dogs, cats or in jirds and infection of cats with *B. pahangi* mimics many of the features of lymphatic filariasis in human, and thus serves as a useful model system (Denham *et al.*, 1972).

3.3 TRANSPORT MECHANISMS IN FILARIAL WORMS

The surface of the filarial worm is entirely covered by a complex, proteinaceous exoskeleton, the cuticle. This extracellular layer, is composed of three principal zones, basal, median and cortical layers. No clear demarcation exists between each zone, and the structure and complexity of each vary greatly, both between species and between the different life cycle stages of the same species (Howells, 1980). Several studies indicate that the nematode cuticle may not be simply an inert, non-immunogenic exoskeleton. The surface of the cuticle expresses antigenic protein molecules which alter qualitatively after the moulting process, change quantitatively as the worms grow within each stage and are released from the worms *in vitro* (Phillip *et al.*, 1980; Selkirk *et al.*, 1986).

With the exception of the Mf and the third-stage infective larvae, filarial worms have a functional gut. However, although oral feeding has been demonstrated *in vivo* (Howells & Chen, 1981) no one has yet succeeded in demonstrating it *in vitro* (Howells, 1980; Chen & Howells, 1979a, 1979b; 1981b). The absence of oral feeding *in vitro* points to the requirement for a specific feeding stimulus, and this has implications in relation to the route of uptake of nutrients or anthelmintic drugs during *in vitro* incubations.

Comparison of an adult filarial nematode and a gastrointestinal species shows that filarial worm have a much smaller intestine and less musculature in the body

wall, whilst the lateral cords of hypodermal tissue are expanded to subtent a large proportion of the sub-cuticular area (Howells, 1987). The outer plasma membrane of the hypodermis, which forms a basal membrane to the cuticle, is much folded in filarial worms and possesses characteristics of an absorptive surface (Howells, 1980). Correlated with the absorptive function of the cuticle, acid phosphatase and naphthylamidase activity is present in the hypodermal tissues of adult *B. pahangi*, but not in the intesine (Howells & Chen, 1981). High levels of acid phosphatase have also been noted in the body wall of *Litomosoides carinii*, *B. pahangi*, *Dirofilaria immitis* and *Setaria* species (Yanagisawa & Koyama, 1970).

Cuticular transport is not a usual feature of parasitic nematodes. Adult *B. pahangi* has been shown to be able to take up D-glucose, L-leucine, glycine, cycloleucine and adenosine across the cuticle (Chen & Howells, 1979a,b; Howells & Chen, 1981), whilst *D. immitis* adults have been shown to take up D-glucose and adenosine by this route (Yanagisawa & Koyoma, 1970; Chen & Howells, 1981a). The transcuticular uptake of glycine has also been demonstrated in *Onchocerca guttuerosa* (Howells, 1980). Cuticular transport in *D. immitis* and *B. pahangi* appears to be selective because L-glucose, sucrose or thymidine, are not taken up. The kinetics of the transcuticular uptake of glycine and cyloleucine by *B. pahangi* suggests simple diffusion. Howells *et al.* (1984) observed that the transcuticular uptake of glucose in *B. pahangi* occurs in physiologically significant amounts and involves a saturable component and diffusion The glucose transport locus differs from that in the tissue of the mammalian host The difference in the specificity of the glucose transport locus in the worms and in mammalian tissues suggests that appropriate glucose analogues might serve as selective agents for transporting therapeutic compounds into filarial worms. The transcuticular uptake of amino acids by *B. pahangi* consists of a saturable and a diffusion component (Howells *et al.*, 1983), and the incorporation of nucleic acid precursors after transcuticular uptake in *D. immitis* and *B. pahangi* has been reported by Chen & Howells (1981a,b). In filarial worms it is likely that the transport of glucose and amino acids occurs at the hypodermal plasma membrane (Howells, 1987). The easy accessibility of the cuticle to chemical agents makes it an attractive target

for chemotherapy. The final assembly of the nematode cuticle occurs extracellularly and is essentially collagenous, but unlike vertebrate collagen, the nematode cuticle is stabilized by sulphhydral links. Damage to the cuticle could render the worm more permeable, but this might upset the ionic balance of the tissues, render the parasite more vulnerable to the host's immune response and increase the penetration of anthelmintic drugs (Ottesen, 1987) Most studies have been done on *B. pahangi* and *B. malayi* but still many questions about the dynamics of the filarial surface remain unanswered.

3.4 PATHOGENESIS AND DIAGNOSIS OF LYMPHATIC FILARIASIS

3.4.1 Clinical Spectrum

The most intriguing aspects of filarial infections caused by lymphatic dwelling parasites, is the extremely broad spectrum of clinical presentation found among individuals in endemic regions. At one extreme there are individuals with neither detectable parasitaemia nor symptoms or history of disease, despite their constant exposure to infective larvae. This group is probably heterogeneous, containing individuals who may be innately resistant to larval development, with those who may have previously sustained, or may currently harbour a subclinical infection (Piessens *et al.*, 1982; Maizels *et al.*, 1983a, Freedman *et al.*, 1989).

A second clinical presentation is also asymptomatic but is characterized by the presence of Mf circulating in the peripheral blood. People with this manifestation of filariasis have been called asymptomatic microfilaremics These patients are characteristically hyporesponsive, relative to endemic "normal" groups, both in terms of anti-filarial antibody and lymphocyte proliferation (Ottesen *et al.*, 1977; Piessens *et al.*, 1980a,b,c; Ottesen *et al.*, 1982). Whilst this condition may persist for years, some individuals are subject to recurrent attacks of lymphadenitis and lymphangitis, and constitute a third group with manifestations of acute filariasis. This symptomatic clinical syndrome is characterized by the recurrent episodes of "filarial fevers" seen in many infected individuals. Patients with these filarial fevers may or may not be microfilaremic. A minority of these patients develop the characteristic symptoms such

as hydrocoele or chyluria, of chronic obstruction filariasis, called elephantiasis, and are classified as a fourth group. People with these manifestations are generally amicrofilaremic, and have very high levels of anti-filarial antibodies (Ottesen 1984; Ottesen, 1990). A final relatively rare syndrome generally regarded as a form of occult filariasis, is that of tropical pulmonary eosinophilia (TPE), characterized by asthmatic symptoms, hypereosinophilia and elevated IgE levels, and thought to result from immediate type hypersensitivity reactions to microfilarial antigens in the lungs (Ottesen, 1984). Mf are never found in the peripheral circulation.

These different clinical manifestations of filarial infection reflect different types of immunologic responses among infected individuals. Lymphatic filariasis elicit immune responses of all types such as, humoral as well as cellular from their human hosts, but there are at least three distinctive features of this response which should be recognized before one considers the specific immunopathology associated with these diseases.

First is the fact that chronic filariasis is characterized by a state of marked immunologic hypo-responsiveness to parasite antigens. The immunosuppression has been demonstrated by *in vitro* studies of lymphocyte function. Patients with chronic infection respond poorly or not at all to filarial antigens, but their responses to other antigens and to mitogens remain normal. Early in the exposure to infection, individuals may develop normal lymphocyte responses to parasite antigens, but with time and the establishment of infection in the host these responses are modulated or damped by various immunologic suppressor mechanisms (Partono *et al.*, 1977; Weiss & Tanner, 1979; Ottesen, 1980).

A second characteristic feature of the immunologic profile of these patients derives from the prolonged persistance of the living parasites within the host. Antigens are shed or secreted almost continuously by the parasites, and in the presence of antibody, immune complexes are formed. Several groups have documented the fact that a large percentage of patients with filariasis have either circulating antigens or circulating immune complexes (Lambert, 1978). These complexes play an important role in initiating or modulating numerous aspects of the overall host

responses to these parasites.

The third distinctive feature of the host response in filariasis is the prominent involvement of immediate hypersensitivity immune mechanisms. Serum IgE levels are elevated, eosinophils are prominent, and basophils and mast cells can be shown to be sensitized with specific antibody (Ottesen, 1980). How these immediate hypersensitivity mechanisms function during chronic filarial infection is still a mystery. The fact that most infected individuals with high levels of IgE, sensitized basophils, and circulating parasite antigens are not allergically symptomatic is quite remarkable and indicates the likelihood that suppressive regulatory mechanisms act on the immediate hypersensitivity responsiveness of the individuals just as such mechanisms have been shown to do for delayed-type hypersensitivity responses (Ottesen, 1990).

3.4.2 Mf-related pathology

Most of the recognized pathology associated with the Mf results from tissue reactions around parasites that have been cleared from the blood. In microfilaremic patients where there is continual production of Mf, clearance of these worms presumably takes place constantly in the lungs, liver and spleen but this clearance appears to be not associated with any definable clinical symptoms, the attrition of Mf from the blood takes place silently (Saxena *et al.*, 1975). Even in infected patients who have developed antibodies to the surface of the Mf and who then become amicrofilaremic, clearance of Mf occurs with no clinical expression.

The situation, however, is entirely different in patients with the tropical eosinophilia syndrome in which patients appear to be immunologically hyper-reactive to all filarial antigens, especially to those antigens which are derived from Mf. Anti-filarial antibodies of all classes are markedly elevated (Neva & Ottesen, 1978). It has become clear that all filariasis patients have large amounts of IgE antibody directed against adult worm antigens, patients with tropical eosinophilia show a special hyper-sensitization, not found in individuals with other forms of filariasis, to antigens derived from the microfilarial stage of the parasite (Ottesen *et al.*, 1979). These

observations strongly support the original speculations of Danaraj *et al.* (1966) and Wong & Guest (1969) that tropical eosinophilia is a form of occult filariasis in which the absence of circulating Mf reflects an immunologic hyper-responsiveness on the part of the host which results in effective clearance of this stage of the parasite from the blood Much of this clearance is probably mediated by IgG antibodies and effected preferentially by the lungs; the asthmatic symptoms are likely the result of pulmonary allergic responses mediated by the specific IgE antibodies directed against the Mf. It is not clear what predisposes certain individuals to react to the parasite in such a hyper-responsive way that they become severely symptomatic from the clearance of Mf, in contrast to the almost complete lack of clinical expression which follows microfilarial clearance in the majority of patients with other forms of filariasis.

3.4.3 Adult-related pathology

Most of the pathology associated with bancroftian and brugian filariasis is limited to the lymphatic system. Incompetence of the draining lymphatics lead, first, to lymphedema and then to either elephantiasis of the limbs, breasts, or genitalia or to leakage of chyle into the urine (chyluria). The adult worms reside within the lymphatic vessels, generally in the afferent approaches or cortical sinuses of the lymph nodes. Secreted metabolic products of the adult worms induce immunological reactions causing alterations of the walls of the lymphatics. As a direct result of hypersensitization, the walls thicken and become distended, but the drainage of lymph remains largely unaffected.

When the adult worms die, the sudden release of antigenic material from the disintegrating worms exacerbates the inflammatory response in the wall of the lymphatic vessel (O'Conner, 1932). An area of necrosis develops around the parasite, resulting both from degeneration of inflammatory cells and dissolution of parasite material. A granulomatous reaction ensues, with a concomitant infiltration of plasma cells and eosinophils. Collagen deposition is seen and the parasite, after fragmentation, either becomes completely absorbed or partially calcified. The blockage of lymphatic circulation continues in heavily infected individuals until most major

lymph channels are occluded, causing lymphedema in the affected region of the body It is during these pathologic reactions within the lymphatic vessel that lymphatic obstructions occurs. Subsequently, colateral vessels develop to aid the passage of lymph but recanalization of the obstructed tracts can also be seen, especially during the early phases of lymphatic obstruction (Denham & McGreavy, 1977; Schacher *et al.*, 1973).

The local lymphatic inflammation and distortion occur primarily when the infecting parasites are molting and releasing the highly antigenic material which is shed at this time (Schacher & Sahyoun, 1967). Death of adult worm could intensify the local inflammation but death of the parasites alone is incapable of inducing the same levels of inflammatory response that the molting products do. Funiculitis, epididymitis and orchitis are also frequent concomitants of filarial infection in the male patients. The inflammation at each of these sites occurs as a direct result of parasites in the associated lymphatics. It was clear that the marked lymphatic inflammation observed in these patients was being caused both by reactions to dying parasites (O'Conner, 1932) and by responses to living adult worms of both sexes (Ottesen, 1990). The identity of the specific immune mechanisms underlying the lymphatics inflammatory episodes is as yet undetermined

Definitive diagnosis of lymphatic filariasis can be made only by demonstration of the adult worms associated with the lymphatics (very rarely observed), microfilariae trapped in the tissues (as in the tropical eosinophilia syndrome), or microfilariae found in blood, hydrocoele fluid, or urine (Ottesen, 1990).

3.5 ANTIBODIES TO FILARIAL WORMS

Filarial nematodes elicit strong humoral responses in human hosts (Ottesen *et al.*, 1981). Antibody levels increase with disease severity (Piessens *et al* 1980a; Ottesen *et al.*, 1981; Maizels *et al.*, 1983a,b). Asymptomatic microfilaraemic patients tend to have relatively low titres of antibody, whereas people showing lymphatic disease and edema have intermediate titres and elephantiasis patients have high titres. In some studies, Mf carriers have lower antibody levels than controls from the

endemic area (Ottesen *et al.*, 1982; Weil, 1990), which suggests that microfilaremic individuals are specifically hypo-responsive to filarial antigens (Ottesen, 1984).

A series of monoclonal antibodies (MAbs) have been generated in the laboratory to Mf, infective larvae and adult worms of *B. malayi*. Analysis of these MAbs indicated the existence of stage-specific antigens of *B. malayi*, as well as of antigens shared by different stages of this parasite (Canlas *et al.*, 1984; Aggarwal *et al.*, 1985). Canlas & Piessens (1985) showed that an anti-*B. malayi* MAb recognized antigenic determinants in extracts of *B. malayi* Mf and promoted the cell adherence of peripheral cells to the surface of Mf *in vitro* and reduced microfilaremia *in vivo*.

Parab *et al.* (1988) characterized a number of MAbs against infective larvae of *B. malayi*. One of these MAbs appears to be highly reactive to infective larvae in comparison to Mf and adult worm antigens. These MAbs enhanced the killing of the infective larvae of *B. malayi* and *W bancrofti*, respectively, by macrophages. These MAbs conferred 89% protection to jirds against challenge infection of *B. malayi* infective larvae. Tan *et al.* (1989) reported that two out of six MAbs against *B. malayi* infective larvae (L3) antigens, impaired *B. malayi* L3 motility. Scanning electron microscopic studies showed damage to L3 surface and loss of regular cuticular annulations. These MAbs had no effect on *B. malayi* Mf.

3.6 FILARIAL ANTIGENS

Filarial parasites present a diverse array of antigens that changes with time as a consequence of parasite maturation through different life cycle stages. The host's response against this diverse array of antigens is often complex and variable (Haque & Capron, 1986). There is increasing evidence that species and stage specific antigens occur in the secretions and on the surface of filarial worms. Kaushal *et al.* (1982) and Selkirk *et al.* (1986; 1989) have characterized the excretory/secretory products derived from adult *B. malayi* using cross-immuno-electrophoresis and radio-immunoprecipitation assays. Most of these antigens were antigenically similar to adult somatic antigens.

The cuticle of parasitic nematodes is the target of a variety of host immune

responses that may lead to the killing of the worms *in vitro* and *in vivo* (Ogilvie *et al*, 1980). Therefore, the cuticle may be a source of antigens capable of inducing resistance to circulating Mf. The antigenic components present on the surface of Mf frequently cross-react with antigenic determinants on the surface of adult worms or infective larvae of the same species and of the member of the same genus (Haque & Capron, 1986; Maizels *et al.*, 1983a,b). The initial characterization of the cuticular antigens of adult filariae demonstrated the presence of three major proteins on the surface of all *Brugia* species, of molecular weight 15 kDa, 20 kDa and 29 kDa (Maizels *et al.*, 1985; Kaushal *et al.*, 1982; Sutanto *et al.*, 1985), and two minor bands of molecular weight 17 kDa and 50 kDa (Philipp *et al.*, 1986). Both the 29 kDa and 50 kDa protein are also prominent on adults of *W. bancrofti* (Morgan *et al.*, 1986). In situ precipitation and immunostaining with a monospecific antibody has confirmed that the 29 kDa molecule is localized to the epicuticle and contains exposed determinants (Maizels *et al.*, 1987). The proteins doublets of 60-70, 100-110 and 160 kDa represent the major structural proteins of the adult cuticle (Selkirk *et al.*, 1986). Both the kinetics and specificity of antibody recognition are critical to understanding the development of disease and also to constructing reliable immunodiagnostic tests

If an antibody can confer resistance toward circulating Mf in the host, and/or induce cell mediated killing of Mf in the host, it should aid in the identification and isolation of the relevant target antigens. Aggarwal *et al.* (1985) produced several MAbs against the infective larvae of *B. malayi* One of these MAbs gives a positive immunofluorescence reaction on the surface of *B. malayi* Mf and was able to mediate mouse peritoneal macrophage adherence to, and killing of, *B. malayi* Mf *in vitro*. Adherence and killing were enhanced by fresh normal mouse serum suggesting a role for complement. When the same MAb was passively transferred to mice harboring Mf, a complete clearance of Mf was observed in about 70% animals. This MAb recognized antigenic determinants with a molecular weight of 110 kDa on the surface of *B. malayi* Mf by radioiodination and immunoprecipitation followed by Western blotting. It seems possible that the antigenic determinants recognized by this MAb on the surface of Mf could be involved in effector mechanisms related to the development of

transmission-inhibiting immunity.

The application of new technologies such as, MAbs, biochemical separation methods, and recombinant DNA should enable the identification, isolation and production of antigens that are related to protection, immunodiagnosis, and pathology in filarial infections.

3.7 IMMUNO-DIAGNOSIS OF LYMPHATIC FILARIASIS

Lymphatic filariasis caused by *B. malayi* and *W. bancrofti* is a major public health problem in tropical countries manifesting a broad spectrum of acute and chronic clinical features (Partono, 1987), as described in the previous section. Improved methods for diagnosis of active filarial infections are needed to identify pre-clinical infections, monitor control efforts and to evaluate new drugs (WHO, 1984). Diagnostic methods based on Mf detection in peripheral blood have some practical and biological limitations. For example, diagnostic methods for filariasis based on Mf detection are insensitive and inconvenient, particularly in those areas of the world where Mf exhibit nocturnal periodicity; they are not useful for detection of amicrofilaremic infections.

Although host antibodies to filarial adult and microfilarial antigens of molecular weight in the range of 15-110 kDa are detected in most patients (Weil *et al.*, 1984; Dissanayake *et al.*, 1984), the serodiagnosis of filariasis based on the detection of antibodies is not always accurate and valid for the following reasons: (1) the presence of antibodies indicates exposure to the parasite but does not reflect the presence or extent of active infections; and (2) problems of specificity as a consequence of extensive antigenic cross-reactivity among nematode parasites (Ambroise-Thomas, 1984). In antibody determinations, one presumes a normal immune response in the host, but in endemic locations, environmental factors such as, undernutrition and other infections may modulate the host immune-responsiveness to filarial antigens. Such modulation may be one of the factors responsible for atypical filarial syndromes. If some of the important filarial antigens are not highly immunogenic or cause immune-unresponsiveness (Ottesen *et al.*, 1982; Piessens *et al.*, 1980a; 1981), certain

people will not show an antibody response. This could account for the so-called false negative reactions.

Difficulties with parasitological and antibody-based serological methods for diagnosis of active filarial infections have led to the development of immunodiagnostic tests based on the detection of circulating parasite antigens in the sera or other body fluids. Studies of human onchocerciasis and lymphatic filariasis, as well as animal lymphatic filariasis, have demonstrated the presence of circulating antigens and immune complexes during the patent stages of infection (Des Moutis *et al* , 1983; Weil *et al.*, 1984; Dissanayake *et al.*, 1982). Several studies have demonstrated the use of a parasite antigen detection for diagnosis and quantitation of filarial infections (Santhanam *et al.*, 1989; Weil & Liftis, 1987; Forsyth *et al.*, 1985; Weil *et al.*, 1990; Wenger *et al.*, 1988); and for monitoring the efficacy of therapy (Forsyth & Mitchell, 1984; Weil *et al.*, 1986; Weil, 1988; More & Copeman, 1991a,b; Chandrashekar *et al.*, 1991). Circulating filarial antigens were first demonstrated in lymphatic filariasis in 1946 by passive cutaneous anaphylaxis (Franks, 1946). The author reported the presence of soluble filarial antigens in the sera of patients with *W. bancrofti*, especially in those with raised microfilaremia. In the late 1970's, a variety of polyclonal antibody-based techniques were used to detect circulating parasite antigens in sera from humans infected with *W. bancrofti* and *B. malayi* (Weil, 1990). These tests were not sensitive enough to be practically useful. Recently, technical refinements such as, use of MAbs, Western blot for antigen analysis, pretreatment of sera to release antigen from immune complexes, have resulted in the development of more sensitive assays.

Soluble antigens have been detected by MAb in the sera of brugian filariasis patients or of multimammate rats. Circulating antigens can be detected in *B. malayi* infected animals as early as 15 days after infection, before the developing larvae become adults (Haque *et al.*, 1985). These results suggest that the naturally killed infective larvae, their excretory/secretory products, or their molting fluids, may be one of the sources of circulating antigens.

Several investigators have raised MAbs against different filarial nematodes

and used them as probes for the detection of target antigens. Most of these MAbs are phosphorylcholine (PC) specific and react with antigens of different molecular weights. Lal *et al.* (1987) raised three MAbs against a circulating filarial antigen of *W. bancrofti*, which bind to a PC epitope and react with a broad range of antigens in the microfilarial, infective larval and adult antigens of *B. malayi*. Another PC reactive MAb raised against the eggs of *O. gibsoni* identified antigens of 25-30, 57-90 and 200 kDa in *B. malayi* extracts. The sensitivity of PC assays for *W. bancrofti* infection had been very successful (85-93%) for sera from microfilaremic patients, depending on the population studied (Weil, 1988; 1990). This variability is believed to be related to the variable prevalence of high levels of anti-PC antibodies in different populations. PC antigenemia has also been detected in sera from patients infected with *B. malayi* (Maizels *et al.*, 1985).

A direct antigen capture enzyme-immunoassay (EIA) with a MAb that binds to a repeated, non-PC determinant on a 200-kDa circulating *W. bancrofti* antigen which is an excretion product of adult worms, has been developed (Weil *et al.*, 1987; Weil & Liftis, 1987; Ramzy *et al.*, 1991). Another promising filarial antigen assay is also based on a MAb to stage specific *W. bancrofti* excretory products with molecular weight of 55-63 kDa (Reddy *et al.*, 1989). This MAb has been used in several different assays to detect *W. bancrofti* antigen in sera from India and China. Sensitivity results have ranged from 68-96% for sera from microfilaremic subjects (Reddy *et al.*, 1989; Zheng *et al.*, 1987). The potential use of parasite antigen detection for diagnosis of human filariasis is also shown by studies of parasite antigenemia in canine dirofilariasis. Identification of heat stable adult worm products in sera from infected dogs led to development of a MAb-based EIA for parasite antigenemia that is specific for *D. immitis* infection and more sensitive than Mf detection tests (Weil *et al.*, 1985). Serum parasite antigen levels correlate significantly with the number of adult female worms in infected dogs, and antigen levels can be followed to monitor the success of anthelmintic therapy. Recently, parallel studies have demonstrated the value of antigen detection as a means of non-invasively monitoring *Brugia* infections in jirds (Wenger *et al.*, 1988; Weil *et al.*, 1990). Although most immunodiagnostic studies have

focused on detection of filarial antigens in sera, it should be noted that the antigens can also be found in other body liquids such as urine, hydrocoele fluids and breast milk (Zheng *et al.*, 1987; Dissanayake *et al.*, 1984).

All filarial antigen assays reported to date have shown relatively poor sensitivity for sera from amicrofilaremic patients with clinical filariasis. This low sensitivity may be caused by a lack of active infection in some patients with chronic lymphatic disease or by antibody-mediated clearance of antigen. The latter possibility is supported by Weil *et al.* (1987) who demonstrated that although antibodies to the epitope defined by MAb were not detected in antigen positive sera, such antibodies were present in over 70% of antigen-negative sera from clinical filariasis patients and endemic normals. Since antibodies to circulating antigens are usually absent in sera from subjects known to be actively infected and present in most sera from clinical filariasis patients and endemic normals, it is reasonable to consider whether such antibodies may be related to the pathogenesis of clinical filariasis or conversely, whether they may have protective activity. In a similar study, Kaliraj *et al.* (1981) reported the detection of circulating antigens in 23/30 microfilaremic patients and in only 1/30 amicrofilaraemic patients with lymphatic filariasis using counter immuno-electrophoresis. Dissanayake *et al.* (1984) using anti-*O. gibsoni* MAb reported the detection of antigen in 50% of the sera and urine from *W. bancrofti* infected patients The highest percentage of positives was seen in the microfilaremic group, using 2-site immuno-radio-metric assay Santhanam *et al.* (1989) reported a MAb-based enzyme immunoassay for the detection of *W. bancrofti* antigen in serum and finger prick blood samples by ELISA. In their study 97% of microfilaremic patients tested positive for antigen; conversely only 10.7% of amicrofilaremic endemic controls were considered positive.

The detection of circulating filarial antigens with polyclonal and monoclonal antibodies by counter immuno-electrophoresis, immunodiffusion, radioimmunoassay, enzyme immuno assay and sandwich ELISA have been reported previously (Kaliraj *et al.*, 1981; Weil, 1988; Dissanayake *et al.*, 1984). All these assays are cumbersome and require radioisotopes or sophisticated equipment. Often these materials and

equipment are unavailable in lymphatic filariasis endemic areas. Recently, Pappas (1983) and Zheng *et al.* (1987;1990) described a rapid, visually interpreted microassay called Dot-ELISA, for the serodiagnosis of major tropical diseases.

Antigen detection assays have several advantages over other diagnostic methods. They can identify individuals with pre-patent or occult infections which are undetected by classic parasitologic tests, they also give a more accurate indication of active infection than traditional serodiagnostic tests. Filarial antigen assays are clearly preferable to antibody tests for evaluation of drug therapy and prophylaxis trials in humans and animals, for vaccine studies in animals, and for epidemiological surveillance of bancroftian filariasis in humans (and dirofilariasis in dogs). However, there are a few limitations of this approach: (1) antigen detection is not sensitive for diagnosis of clinical filariasis in amicrofilaremic individuals. Species-specific antibody assays are needed for confirmatory testing of individual patients with clinical findings that are suggestive but not diagnostic for lymphatic filariasis; (2) antigen detection is complementary to and not a substitute for Mf detection. Microfilaria prevalence and density data are necessary for understanding the dynamics of filariasis transmission in populations.

3.8 CHEMOTHERAPY OF FILARIASIS

Chemotherapy of filariasis refers to the treatment of established infections using macrofilaricidal and/or microfilaricidal drugs. None of the drugs that have been used up to the present time are suitable for mass treatment of filariasis.

3.9.1 Diethylcarbamazine citrate (DEC)

DEC, a derivative of piperazine (1-diethylcarbamy-4-methylpiprazine), has been known to have filaricidal properties since 1947. DEC is an effective microfilaricidal drug in humans when administered daily at an oral dose of 200 mg for 12 days, however, comparable concentrations of the drug have no effect on the parasites *in vitro*. DEC kills all stages of brugian parasites and prolonged treatment may effect adult *W. bancrofti* (Mackenzie, 1985). Nathan *et al.* (1987) demonstrated that the use

of DEC at monthly intervals over 12 months reduced the number of microfilaremic patients with bancroftian filariasis by 90%.

DEC treatment is often accompanied by nausea, dizziness and febrile episodes, which can be severe in *Brugia* infected patients. In onchocerciasis, DEC induces Mazzotti reaction with intense pruritus and may cause retinal damage and anaphylactic shock (Subrahmanyam, 1987). It is believed that the side reactions are related to the release of pharmacological mediators in response to the massive toxic/antigenic products from dying parasites (Mak *et al.* 1991).

Although some elimination of Mf is caused by DEC administration alone, antibodies seem to have a synergistic effect. Thus, more rapid clearance of the circulating parasites was achieved when serum containing antibodies was injected intravenously along with the drug and parasites into a naive animal. Several investigators have indicated that ADCC to filarial larvae is enhanced by DEC *in vitro*. DEC seems to alter the parasite surface and makes it more amenable to ADCC, since prior treatment of the parasite with DEC increases such cytotoxicity. Eosinophils are not normally markedly cytotoxic to sheathed Mf of lymphatic parasites. De-sheathed Mf, however, are susceptible to eosinophil-mediated ADCC and such cytotoxicity is enhanced by DEC (Chandrasheker *et al.*, 1984). Vickery *et al.* (1985) reported that DEC cleared Mf from infected nude mice unable to produce antibodies to filarial antigen, which implies that DEC action is probably independent of the thymus. Instead, complement may be activated by the parasites (Zahner & Weidner, 1983) and may induce cellular cytotoxicity.

3.9.2 Levamisole

Levamisole is effective against the Mf and adults of *B. malayi* in cats (Mak *et* al. ,1974). It is microfilaricidal against human *W. bancrofti* and *B. malayi* (McMahon, 1979). However, side effects encountered were as severe as those seen with DEC. It would be advantageous to use a purely macrofilaricidal drug to kill the adults and allow the microfilariae to die off slowly or be removed through the judicious use of low doses of a microfilaricidal drug (Janssens, 1985).

3.9.3 Ivermectin

Ivermectin, a macrocyclic lactone, is a 22, 23-dihydro derivative of avermectin B1, and has been shown to have a wide spectrum of activity against nematodes, insects and acarine parasites but it does not show any effect on cestodes and trematodes (Campbell, 1985). Ivermectin is broadly used in veterinary medicine and it has been found to be effective against *O. volvulus* Mf and at a single dose was better tolerated and had a longer lasting effect on skin Mf than did DEC (Aziz *et al.*, 1982; Mak *et al.*, 1988). Its activity against the adult worms was poor. Ivermectin seems to have varied effects on filarial parasites depending on the species and the stage. The effect on Mf is high in *D. immitis* (Blair & Campbell, 1979), *Dipetalonema reconditum*, *Dipetalonema viteae* (Campbell, 1982), *Onchocerca cervialis* (Klei *et al.*, 1980), *Onchocerca volvulus* (Aziz *et al.*, 1982; Awadzi *et al.*, 1985) and *Litomosoides carinii* infections in jirds and cotton rats (Soffner & Wenk, 1985). In *Brugia* infections comparatively high doses are necessary to obtain at least moderate microfilaricidal effects *in vivo* (Campbell, 1982). Ivermectin acts chemo-prophylactically against all preadult stages of *D. immitis* but in the case of *D. viteae* marked activity is limited to third and fourth stage larvae and in *L. carinii* infections to the third stage larvae only (Campbell, 1982). It was shown to be ineffective against developing stages of *Brugia* species and of *Onchocerca* species in cattle although other avermectins had a limited effect on developing stages of *B. pahangi* (Denham, 1972).

Ivermectin was shown to clear *W. bancrofti* microfilaremia by the third post-treatment day with a single oral dose, patients remained amicrofilaremic until the third post-treatment week. At six months post-treatment, this was 13% of initial counts (Diallo *et al.*, 1987). The efficacy of ivermectin is comparable to that of DEC (Kumaraswami *et al.*, 1988). A dose ranging study was carried out in French Polynesia to determine its tolerability, biological and clinical safety and efficacy in *W. bancrofti* var pacifica carriers (Cartel *et al.*, 1990). The authors reported that ivermectin single-dose treatment was effective on lymphatic filariasis. The successful treatment rate was 100%. Preliminary results of the first large scale trials of this drug suggest that it is safe to be used for mass chemotherapy. Ivermectin is being investigated both for

individual and for large-scale treatment of onchocerciasis at yearly intervals (Mak *et al.*, 1991).

3.9.4 Isothiocyanates and derivatives

Among the new compunds the most promising agents are the isothiocyanate and their derivatives, amoscanate, CGP 6140, CGP 20376 and CGI 16343. Amoscanate treatment of rats infected with *B. pahangi* and *L. carinii* results in inhibition of glucose uptake and transport by the parasites. It also causes ultrastructural alterations in parasites isolated 12 h after therapy. Amoscanate is a potent inhibitor of cyclic-AMP phosphodiesterase of filarial worms, but the inhibition is not parasite specific. CGP 6140, is a methyl-piperazine derivative of amoscanate and is being tested against onchocerciasis in patients. CGP 6140, has been shown to inhibit the acetylcholinesterase of *O. volvulus* and *D immitis* (WHO, 1989). CGP 20376, was shown to have complete adulticidal activity against *B. malayi* and *B. pahangi*, as well as *L. carinii* (Zahner *et al.*, 1988a; Mak *et al.*, 1990) It was also microfilaricidal against *D. vanhoofi* in chimpanzees (Moysan *et al.*, 1988) Anjaneyulu (1987) demonstrated that CGP 20376, behaves as a prodrug, and is rapidly dissociated into its biologically active isothiocyanate precursor, CGP 20308, which has been shown to be an effective microfilaricidal and macrofilaricidal in experimental *Brugia* infections in rodents (Zahner *et al.*, 1988b). CGI 16343 affects the cuticular layer of filarial worms and perhaps exposes carbohydrate residues on the surface (Subrahmanyam, 1987). These compounds are currently in Phase I clinical trials in India.

3.9.5 Benzimidazoles carbamate (BZs)

Mebendazole (MBZ), flubendazole (FBZ) and CGI 13866 are representative filaricidal BZs (Dominguez-Vanquez *et al.*, 1983). When given orally at concentrations of 150 mg/kg per day for five days to infected *Mastomys*, MBZ and FBZ exert no significant effect on adults of *L. carinii* and *B. pahangi*. On subcutaneous administration, they do show macrofilaricidal activity, possibly because of depot formation and sustained release (Reddy *et al.*, 1983). However, CGI 13866 has 49%

macrofilaricidal activity when given orally, and completely clears Mf from the circulation of leaf monkeys infected with *B. malayi* (Subrahmanyam, 1987).

Denham *et al* (1978) indicated that MBZ was a potent microfilaricide against *B. pahangi* infections of cats and jirds and probably macrofilaricidal against *W. bancrofti*. Mak (1981a,b) demonstrated that both MBZ and FBZ have significant filaricidal properties against *B. boolıti* infection in white rats. FBZ was also shown to be lethal to *B. pahangi* in jirds and cats (Denham *et al.*, 1979) when administered subcutaneously. It also killed developing larvae in jirds. In *W. bancrofti* infection, MBZ at a dosage of 6 mg/kg daily for 10 days following an 8 day treatment with levamisole prevented the recurrence of microfilarial activity otherwise seen after withdrawal of levamisole, but was unable to produce zero counts (Narasimham *et al.*, 1978). Goldsmid & Rogers (1976) demonstrated two *D. perstans* infections which improved under 400 mg twice daily for 14 days. In one case the Mf disappeared, whereas the other patient relapsed during the follow-up period. Maertens & Wery (1975) observed disappearance of *D. perstans* Mf from the blood after a combination therapy of MBZ with levamisole.

MBZ interferes with embryogenesis and is microfilaricidal in humans when given at 30 mg/kg in three doses daily for three weeks in onchocerciasis. MBZ inhibits *in vitro* and *in vivo* glucose uptake by helminths. This decreased uptake is followed by an enhanced utilization of endogenous glycogen and reduced glycogenesis. The drug also diminishes ATP synthesis and/or the turnover of adenine nucleotides. Mitochondrial electron transport, especially by the fumarate reductase system, is inhibited by several BZ at concentrations of 10-100 µM in nematodes. The primary targets of BZs are tubulins; by binding to these proteins the drugs interfere with the assembly of MTs (Van Den Boosche *et al.*, 1982). High affinity MBZ binding to *B. malayi* and *B. pahangi* tubulin is reported by Tang & Prichard (1988). Franz *et* al. (1990a,b) showed that intestinal microtubules of *B. malayi* disappeared six hours post-treatment with FBZ *in vivo*. Furthermore, marked alterations of the oogonia and embryonic cells of *B. malayi* females were observed 24 hours post-treatment. MBZ may be a useful alternative to DEC in the treatment of onchocerciasis, because MBZ

showed similar or slightly greater reduction of Mf than DEC after 6 months and has fewer systemic side effects (Rivas-Alcala *et al.*, 1981a). However, poor oral absorption of MBZ and prolonged (2-3 weeks) extremely high dosages (1 5 g daily) may make it unsuitable for large-scale use in its present formulation (Bekhti *et al* , 1977; Vande Waa, 1991).

Amongst 20 different chemical classes of compounds synthesized and screened in different animal filarial models in recent years, the three most promising drugs for clinical application are: BZs, ivermectins and isothiocyanates and their derivatives. The potentiation of ADCC by DEC *in vitro* and the killing of larval stages of lymphatic and *Onchocerca* parasites should stimulate the search for drugs that collaborate with the immune responses of the host. Removal of the sheath from sheathed MF makes the parasites more susceptible to complement and antibody attack, so de-sheathing agents should promote microfilaricidal activity. The tubulins of intestinal and tissue nematodes need to be further characterized and specific binding agents developed. Compounds that damage the cuticle of the filarial parasite could render it more permeable, disturb the ionic balance and make it more vulnerable to the host's immune attack.

4.0 MICROTUBULES

Microtubules (MTs) are ubiquitous components of all eukaryotic cells and constitute a major structural element of spindles, the cytoskeleton and the axonemes of cilia and flagella (Dustin, 1984). MTs perform a number of diverse functions such as, segregation of chromosomes during meiosis and mitosis; the determination and maintenance of cell shape via dynamic changes in the organization of cytoplasmic MTs; intracellular transport, e.g the movement of organelles along nerve axons; and cell motility (Sullivan, 1988; MacRae & Langdon, 1989).

4.1 TUBULIN STRUCTURE

Microtubules are formed by the assembly of tubulin, an acidic protein heterodimer composed of α and β subunits of approximately 110 kDa (Fig. 1.3).

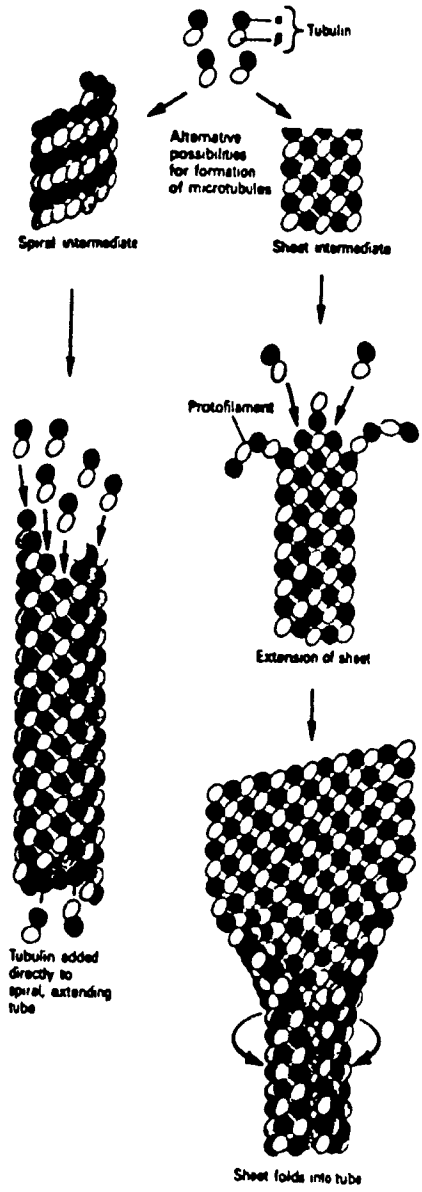


Fig. 1.3. Structure and assembly of microtubules. Microtubules can be generated in the laboratory from a concentrated solution of αβ tubulin dimers. The initial step involves formation of intermediates such as spirals of tubulin. These intermediates are unstable, and their slow formation accounts for the lag period before long microtubules are formed. Certain basic proteins can accelerate the polymerization of tubulin, possibly by acting as foci for assembly of tubulin into spirals. These intermediates act as primers for polymerization of αβ dimers, but the steps involved are not well established Tubulin may add directly to the spiral, forming a tubular microtubule Alternatively, sheets of protofilaments may be the intermediate structures. When the protofilaments become wide enough, they fold into a tube

(Darnell *et al.*, 1986)

(Krauhs *et al.*, 1981; Gunning & Hardham, 1982). However, a third minor component migrating between the two major tubulin bands has been found in porcine brain extracts (Little, 1979). Using peptide mapping, this band was identified as a form of β-tubulin. The major β-tubulin band is referred to as β_1- and the minor band, β_2-tubulin. β_1- and β_2- constitute 75% and 25% respectively, of the total β tubulin in bovine brain (Little, 1979). Little & Luduena (1985) demonstrated the marked structural differences between brain β_1- and β_2-tubulins, which suggests that β_1- and β_2- may have different functions in brain tissue. β_1- appears to be ubiquitous in all chordates, however, β_2- has so far only been found in the brains of cow, goat, pig, deer, rat, chick and dogfish (Mukhopadhyay *et al.*, 1990). In non-chordates, both β_1- and β_2- have been found only in the free living nematode *Caenorhabditis elegans* (Enos & Coles, 1990).

A head-to-tail arrangement of α and β tubulin form a hollow cylinder called protofilament (p), and usually 13 protofilaments are found per microtubule (Tilney *et al.*, 1973). MTs with different numbers of protofilaments have also been identified in crayfish sperm (15p) (Burton *et al.*, 1975), lobster neurons (12-p) (Burton & Hinkley, 1974), and cockroach epidermis (15-p). Chalfie & Thomson (1982) in a similar study, demonstrated that most cells in the free living nematode *C. elegans* have MTs with 11-p but six touch receptor neurons have MTs with 15-p; no 13-p MTs have been observed in this nematode The 11-p and 15-p MTs differ not only in protofilament number, cellular distribution, and perhaps, function but also in a number of physical and chemical properties. This suggests that for many cellular functions either 11-p or 15-p will suffice, but certain functions require MTs with specific protofilament numbers (Chalfie *et al.*, 1986).

MTs are found in cytoplasm as well as in the cell membranes The membrane tubulin is in part accessible to non-penetrating reagents and when extracted, is capable of assembling into MTs (Dustin, 1984). Membrane tubulin may provide links between membrane and cytoskeleton, links perhaps used during MT-mediated propulsion of membranous surfaces or organelles. MTs of different tissues and cells (Murphy & Wallis, 1983; Sullivan & Wilson, 1984) and within single cells (Gozes &

Sweadner, 1981), are distinguishable on the basis of the electrophoretic mobility (Feit *et al.*, 1971); proteolytic cleavage patterns (Little, 1979); protein sequencing (Postingyl *et al.*, 1981; Krauhs *et al.*, 1981); pharmacological properties (Lacey & Prichard, 1986) and immunological reactions (Draber *et al.*, 1985; Kilmartin *et al.*, 1982; Kempheus *et al.*, 1982; Sherwin & Gull, 1989).

4.2 HETEROGENEITY OF TUBULIN

Since MTs are employed in a number of diverse functions in eukaryotic cells (Sullivan, 1988), there has been speculation that different MT-mediated functions may require different forms of tubulin (Fulton & Simpson, 1976; Banerjee *et al.*, 1990) (Table 1.1). Benhke & Forer (1967) reported evidence for four classes of MTs in individual cells which differed in their drug sensitivities or thermal stability. Thompson *et al.* (1984) and Cumming *et al.* (1984) independently demonstrated, using anti-α tubulin MAbs, that α tubulin related epitopes are apparently segregated into distinguishable subsets of MTs within single cells or subsets of cells. Thus, it seems clear that although MTs appear morphologically identical, they represent a heterogeneous class of structures.

Several studies have demonstrated that both α- and β-tubulin exist as multiple isoforms encoded, in mammals or birds, by a set of 6 α- and 7 β-tubulin genes (Monteiro & Cleveland, 1987; Sullivan, 1988). Both α- and β-tubulins are regulated independently, the changes in α tubulin isoforms occur mainly before birth, while the increase of β-tubulin isoforms continues throughout development. In rats, the β-tubulin isoforms increase from 3 isoforms in embryos to 14 isoforms in adults; α-tubulin isoforms on the other hand have been resolved into 7 isoforms that are present throughout development. In the mouse brain, 5-6 α- and 10 or more β-tubulin isoforms have been identified (Wolff *et al.*, 1982), which change during maturation, α6- and β2- disappears while β6- and β10-isoforms appear; however, there are no other changes related to aging (Van Hungen *et al.*, 1981).

Havercroft & Cleveland (1984) demonstrated that chicken brain tubulin contains a number of closely related β-tubulin isoforms, some of which are either

Table 1.1. Number of α- and β-tubulin isoforms in various species.

Species	α-Tubulin isoforms	β-Tubulin isoforms	Reference
Sea urchin	10-15	10-15	Dustin, 1984
Aspergillus	4	4	Sheir-Neiss *et al.*, 1978.
Nippostrongylus brasiliensis	8	3	Tang & Prichard, 1989
B. pahangi	4-5	3	Tang & Prichard,1989
B. malayi	5	4	Tang & Prichard, 1989
Haemonchus contortus	2	2	Lubega & Prichard, 1990
Mammals	14-17	8	Tang & Prichard, 1989

brain-enriched (β2) or brain-specific (β4). In contrast, 90% of chicken erythrocyte β-tubulin is composed of β6-, an isoform which is only expressed in chicken erythrocytes and thrombocytes (Murphy *et al.*, 1984). Relative to brain β-tubulin, chicken β6-tubulin isoform from erythrocytes is more hydrophobic, less acidic by 0.4 pH units, has a different number of reactive methionines, and differs in its amino acid sequence from chicken β2-tubulin (Murphy *et al.*, 1987). The assembly properties of these tubulins are also distinct, and may reflect important functional differences in the properties.

Protein sequence analysis of porcine brain tubulin has demonstrated the presence of 2 β-tubulin isoforms (Krauhs *et al.*, 1981) and 4 α-tubulin isoforms (Postingyl *et al.*, 1981) that differ subtly in primary sequence. Sequence analysis of 2 different human β-tubulin genes has demonstrated the presence of at least 2 β-tubulin isoforms that differ significantly in sequence at the C-terminal of the polypeptide (Hall *et al.*, 1983).

Tang & Prichard (1988; 1989) examined the isoform patterns of one intestinal and two tissue nematodes and demonstrated that *Nippostrongylus brasiliensis* has 8 α- and 3 β-, *B. malayi* 4-5 α- and 4-5 β-, *B. pahangi* 4 α- and 5 β-tubulin isoforms. In a similar study, Lubega & Prichard (1991) reported the existance of at least 3 β-tubulin isoforms in BZ-susceptible *Haemonchus contortus* and the authors observed that the BZ resistant isolates of *H. contortus* shared some β-tubulin isoforms with the susceptible strain but another β-tubulin isoform present in susceptable populations was lost or diminished in the resistant population.

Micro-heterogeneity is not restricted to brain and nematode tubulins. Multiple isoforms have also been detected in *Drosophila* (Kemphues *et al.*, 1979; Raff *et al.*, 1982), *Physarum* (Burland *et al.*, 1983), the unicellular algae *Chlamydomonas* and *Polytomella* (Cleveland & Sullivan, 1985; McKeithan *et al.*, 1983), the trypanosomatid *Crithidia fasciculata* (Cumming & Williamson, 1984), the fungus *Aspergillus* (Sheir-Niess *et al.*, 1978), and essentially all vertebrate tubulin sources examined (Diez *et al.*, 1984; Little & Luduena, 1985; Murphy & Wallis, 1983).

4.3 POST-TRANSLATIONAL MODIFICATIONS OF TUBULIN

Three types of post-translational modifications of tubulin occur: (1) detyrosination/tyrosination of α-tubulin; (2) acetylation of a- and β-tubulin and (3) phosphorylation of α- and β-tubulin (Matten *et al*, 1990; Gard & Kirschner, 1985; Edde *et al.*, 1989). The impact of any of these post-translational modifications on MT function *in vivo* is unknown However, results of several studies indicate that a link exists between tubulin modification, MT assembly dynamics and morphogenesis (MacRae & Langdon, 1989; Sullivan, 1988; Schulze *et al.*, 1987).

4.3.1 Detyrosination/tyrosination of tubulin

α-tubulin undergoes a unique post-translational modification involving the enzymatic removal and addition of tyrosine at the C-terminus end of α-tubulin by an ATP-dependent enzyme, tubulin-tyrosine ligase (Kreis, 1987; Wehland & Weber 1987a,b). Tubulin-tyrosine ligase (TTL) catalyzes the ATP-dependent formation of a peptide linkage of tyrosine to the C-terminal glutamic acid residue of α tubulin (Raybin & Flavin, 1977). TTL detyrosinate prefers the soluble α-, β-dimer as substrate whereas tyrosination favors polymeric tubulin as substrate (Kumar & Flavin, 1981). The binding site of the enzyme involved in tyrosination is on β- rather than α-tubulin The incorporation and turnover of C-terminal tyrosine *in vivo* is rapid (Thompson *et al.*, 1979) and occurs on the majority of available α tubulin (Gundersen *et al.*, 1987) The demonstration that turnover of C-terminal tyrosine is dependent on MTs suggested a correlation between the dynamic properties of MTs and the α-tubulin tyrosination cycle (Thompson, 1982) Electron microscopic immunolocalization studies have revealed that individual tyrosinated or detyrosinated MTs are in fact copolymers of the post-translationally modified tubulins, being rich in tyrosinated or detyrosinated α tubulin and all interphase and mitotic MTs contain a uniform distribution of tyrosinated and detyrosinated tubulin along their lengths (Geuens *et al.*, 1986). MTs composed of the tyrosinated α tubulin exhibit different stabilities (Kreis, 1987) detyrosinated MTs exchange their tubulin subunits more slowly and are less sensitive to depolymerizing agents than tyrosinated MTs (Skoufias *et al.*, 1990)

Stable arrays of MTs in several differentiated cell types including erythrocytes (marginal band), cilia, basal bodies, centrioles, flagella, sperm (axonemes) and neurites (Gundersen & Bulinski, 1986) contain more detyrosinated tubulin than other MTs in the same cells. The enrichment of detyrosinated MTs in relatively long-lived cellular structures, such as flagella, primary cilia and centrioles (Gundersen & Bulinsli, 1986; Sherwin *et al.*, 1987) and their resistance to MT-depolymerizing drugs, but not to cold, demonstrate that such MTs are more stable than tyrosinated MTs (Wehland & Weber, 1987a; Kreis, 1987; Schulze *et al.*, 1987). Cellular morphogeneis depends on the production of stable MT assemblages within the highly dynamic intracellular MT population, However, detyrosination is not the primary cause of the stabilization (Gundersen & Bulinski, 1986). Wehland & Weber (1987b) suggested that this is an indicator of old MTs, an idea supported by the study of *Trypanosoma brucei*, a parasitic hemoflagellate (Sherwin *et al.*, 1987; Sasse & Gull, 1988).

The functional significance of this unique post-translational modification remains obscure. Skoufias *et al.* (1990) suggests that MTs rich in detyrosinated tubulin may play an active role in coalescence of the Golgi fragments and/or in the positioning of the Golgi apparatus near the MTOC and the nucleus. Since detyrosinated MTs are widely distributed in the cytoplasm of a number of cells (Wehland & Weber, 1987b), it is assumed that the functions of detyrosinated MTs are not restricted only to the Golgi apparatus.

4.3.2 Acetylation of tubulin

During regeneration of artificially removed flagella in *Chlamydomonas* and *Polytomella* (McDeithan & Rosenbaum, 1981; McKeithan *et al.*, 1983) or upon transformation of *Physarum* myxamoebae to the flagellate form (Green & Dove, 1984), a reversible post-translational modification, acetylation of α-tubulin occurs. α tubulin is acetylated on the Π-amino group of a lysine residue (L'Hernault & Rosenbaum, 1983; 1985), subsequently identified as amino acid residue 40 (LeDizet & Piperno, 1986). The enzyme responsible for acetylation termed as α-tubulin acetylase, is located in the flagellum with high specificity for α tubulin, and is preferentially reactive with

polymeric tubulin (Maruta *et al.*, 1986). Acetylated MTs tend to be resistant to drug-induced depolymerization and perhaps to cold, although there are contradictory data regarding this point (Piperno *et al.*, 1987; Sale *et al.*, 1988). Acetylated tubulin is found in mitotic spindles, primary cilia, cytoplasmic MTs, centrioles, and midbodies of HeLa and 3T3 cells in culture, but not in PtK2 cells (Piperno *et al*, 1987). Acetylated and detyrosinated tubulin often occur on the same MTs or subsets of MTs, but their rates and patterns of appearance are not the same (Bulinski *et al.*, 1988; Schulze *et al.*, 1987; De Pennart *et al.*, 1988).

4.3.3 Phosphorylation of tubulin

Tubulin is phosphorylated *in vivo* (Gard & Kirschner, 1985) and *in vitro* (Yoshikawa *et al.*, 1985). Tubulin is known to be an *in vitro* substrate of several protein kinases in brain. Casein kinase II, cAMP-dependent and Ca2+/calmodulin-dependent protein kinases have been demonstrated previously to phosphorylate on serine and threonine, residues of a basic β-tubulin isoform (Eipper, 1974a,b) identified as β2- (type III) by Luduena *et al.* (1988), at its C-terminal (Eipper, 1974b), in mouse neuroblastoma cells (Serrano *et al.*, 1987; Katz *et al.*, 1985). The phosphorylation of neuroblastoma tubulin, even in the absence of development, is stimulated by taxol and reduced by exposure to the MT depolymerizers, nocodazole, and colcemid, indicating that phosphorylation depends on the amount of polymerized tubulin and does not regulate tubulin assembly

A single β-tubulin isoform similar to the one identified in differentiated neuroblastoma cells is phosphorylated in mouse primary neurons (Edde *et al*, 1989) The phosphorylated brain β-tubulin has a unique assembly properties, but the significance of phosphorylation to assembly dynamics *in vivo* is still unclear (Banerjee *et al.*, 1990). Tubulin phosphorylated by Casein kinase II is mainly found in the assemble MT fraction (Serrano *et al.*, 1987) and could result in a stable modification of proteins (Hatawhey & Traugh, 1982). *In vivo* phosphorylation of β-tubulin may play a role in MT assembly and stabilization during neurite outgrowth in differentiating neuroblastoma cells (Gard & Kirschner, 1985). On the other hand, phosphorylation

of tubulin in Ca/calmodulin-dependent kinase II might have a regulatory role on the association of tubulin with certain cellular membranes, such as coated vesicle, synaptic vesicles, or postsynaptic densities (Hargreaves *et al.*, 1986). Phosphorylation by the Ca/calmodulin-dependent kinase was reported to block *in vitro* polymerization of tubulin (Wandosell *et al.*, 1986). Tubulin is also phosphorylated at tyrosine *in vitro* by high amounts of insulin receptor tyrosine kinase inhibiting polymerization (Wandosell *et al.*, 1987) and by the epidermal growth factor receptor kinase (Akiyama *et al.*, 1986). Recently, Matten *et al.* (1990) demonstrated that subpopulations of both α and β tubulin were phosphorylated at tyrosine residues in nerve growth cone preparations *in vitro* and *in vivo*, with tyrosine kinase $pp60^{c\text{-}src}$.

The demonstration of at least 12 isoforms of β tubulin in vertebrate brain, which expresses at most six different genetic isoforms, suggests that post-translational modification may be an important property of β-tubulin as well as of α-tubulin (Field *et al.*, 1984). Post-translational modification of tubulin may influence its assembly, its interaction with other cellular organelles, the ability to bind MAPs, and its participation in events such as cellular differentiation and morphogenesis.

4.4 POSSIBLE FUNCTIONS OF α- AND β-TUBULIN ISOFORMS

MTs perform several divergent functional roles within cells; they are the primary components through which accurate chromosome segregation is achieved during meiosis and mitosis; they serve as major structural components for ciliary and flagellar dependent cell motility; for establishment of the asymmetric morphology of neurons; and as a substrate for the transport of vesicles and organelles within the cytoplasm (Hayden & Allen, 1984; Vale *et al.*, 1985). In addition, together with actin filaments and intermediate filaments, MTs of the cytoskeleton play a major role in establishing and maintaining the dynamic and spatial organization of the cytoplasm (Kirschner & Mitchison, 1986). The function of MTs depends largely on the regulation of their assembly and their interaction with other cellular components like, mitochondria, golgi bodies, ribosomes, lysosomes, cell membranes and the nucleus (Dustin, 1984). The disruption of the tubulin-MT equilibrium directly affects the

assembly and binding properties of MTs which leads to a cascade of direct and indirect biochemical/physiological changes resulting in the loss of cellular homeostasis Conditions of cellular "disequilibrium", if maintained, are lethal (Azhar & Reaven, 1983; Durrieu *et al.*, 1987, Lacey, 1988).

There are several possibilities by which the MTs of a cell can be differentiated. (1) in many organisms, there is evidence for the existence of multi-gene families which encodes both α- and β-tubulin. The organization and number of these genes vary between organisms and it is clear that in many cases there is a complex pattern of differential expression that leads to the appearance of different tubulins in particular cell types or tissues (George *et al.*, 1981; Gozes & Sweadner, 1981; MacRae *et al* , 1989; Sullivan, 1988). The genetically encoded differences among tubulin isoforms could contribute to MT function (Lewis *et al.*, 1985; Sullivan & Cleveland, 1986); (2) the functions of MTs could also depend on the regulation of their assembly and their interaction with other cellular components such as MT-associated proteins (MAPs), a class of polypeptides the members of which are known to vary in number and composition among functionally distinct MTs (Huber & Matus, 1986; Bloom *et al.*, 1984; Binder *et al.*, 1985); (3) distinct populations of MTs have been identified in which α- and β-tubulin undergoes one or more specific post-translational modifications (Banerjee *et al.*, 1990; Edde *et al.*, 1989).

Vertebrates express approximately 7 α- and 7 β-tubulin isoforms in a nonrandom pattern reflecting both tissue specificity and developmental temporal specificity (Sullivan & Cleveland, 1986; Lewis *et al.*, 1985). These isoforms differ from each other by small protein domains, mostly at the C-terminal of each tubulin protein. The highly acidic, isoform-defining domains interact with associated proteins (Serrano *et al.*, 1984a,b). If these interactions were sequence-specific, then the expression of a particular tubulin isotype could define MT function by dictating the kinds of effector molecules that are able to bind to the resulting MTs. The presence of certain isoforms in specialized cells and tissues suggests that each isoform may be functionally significant. The use of genetic and biochemical approaches by a number of investigators has produced several lines of evidence that support the functional

equivalence of β-tubulin isoforms (Table 1.2).

Evidence for functional differentiation of tubulin isoforms is begining to emerge. Murphy *et al.* (1985; 1986) demonstrated that the assembly of chicken erythrocyte β-tubulin is comprised of 90% β6-isoform, and found it to assemble more efficiently than brain tubulin. Subsequently, Joshi *et al.* (1987) demonstrated that the β4- and β6-tubulin isoforms of chicken erythrocytes are differentially susceptible to cold-induced depolymerization. *in vivo*. Joshi & Cleveland (1989) demonstrated that rat cells express 5 isoforms, three of these isoforms were used preferentially for assembly of neurite MTs. The finding demonstrates *in vivo* biochemical differences among the β tubulin isoforms and supports the hypothesis that different isoforms are preferred substrate for different MT-based processes in neuronal cells.

Luduena *et al.* (1985) have found that assembly of brain, but not of erythrocyte tubulin, is inhibitable by a low concentration of a non-physiological sufhydryl alkylating agent, indicating that different isoforms of tubulin can behave differently *in vitro*. There is evidence that different α-tubulin isoforms occur in different regions within the same neuron, suggesting that they may perform different functions (Hajos & Gallatz, 1985) Joshi & Cleveland (1989) have shown that during neurite outgrowth in neuroblastoma cells, the β1- and β2-tubulin isoforms are incorporated into MTs in preference to the β3-isoforms. In a similar study, Gard & Kirschner (1985) and Luduena *et al.* (1988) have found that only one β-tubulin isoform β3, becomes phosphorylated at a serine residue near the C-terminus upon differentiation of neuroblastoma cells; the other β-isoforms do not appear to become phosphorylated under these conditions. It is probably significant that the C-terminus tryptic peptide of β3- contains two serines that are not present in the other neural β isoforms. The presence of cellular mechanism that use only a specific isoform mandates that such isoforms are in fact used for unique functions in cells.

Differences in the ability of tubulins to bind small ligands suggest that their biochemical properties are distinct (Little & Luduena, 1985; Banerjee & Luduena, 1987). BZ resistant strains of *C. elegans*, *A. nidulans*, *H. contortus* (Chalfie *et al.*. 1986; Driscoll *et al* , 1988; Lubega & Prichard, 1990) and *Physarum polycephalum*

Table 1.2. Properties of β-tubulin isoform classes*.

β-tubulin Isoforms	Expression
Class 1	Constitutive; many tissues.
Class 2	Major neuronal; many tissues.
Class 3	Minor neuronal; neuron specific.
Class 4a	Major neuronal; brain specific.
Class 4b	Major testis; many tissues.
Class 5	Minor constitutive; absent from neurons.
Class 6	Major erythrocytes/ platelets; hematopoeisis specific.

*Taken from Sullivan 1988; Banerjee *et al.*, 1990.

(Foster *et al.*, 1987), showed an alteration in β-tubulin isoforms compared with the susceptible wild types. The authors concluded that alterations in β tubulin caused resistance in the resistant strain

Comparison of all of the available vertebrate β-tubulin sequences reveals the existance of a highly conserved polypeptide framework in which individual sequences diverge from each other both within and between species in 2-8% of 450 residue positions. However, the C-terminal 15 residues constitute a major variable region domain for β-tubulin and to a lesser extent, for α-tubulin as well (Pratt *et al.* 1987). The stringent interspecies conservation of β-tubulin isoforms implies that each isotypic sequence has been positively selected during evolution. This observation supports the hypothesis of a functional role for the structural differentiation of β-tubulin isoforms.

4.5 IN VITRO ASSEMBLY OF TUBULIN

The multiple cellular functions of MTs such as, cytoskeleton elements, as a framework for cellular shape, cell motility, mitosis and intracellular movements of cell organelles, rely on the modulated assembly and disassembly of the microtubular system and the interaction between MT components and other components of the cytoplasm. The process of tubulin self-assembly to form MTs is one of the striking systems of self-association in eukaryotic cells (Kumar & Flavin, 1982).

MT assembly requires GTP and the nucleotide is hydrolyzed during the assembly reaction (Jacobs, 1979). MT associated proteins (MAPs) are also implicated in MT assembly and can promote tubulin assembly in the absence of co-solvents but in the presence of GTP, EGTA and Mg^{2+} ions, in addition of some other assembly promoting compounds such as sucrose, dimethylsulfoxide or glycerol at high concentrations. A temperature around 37°C is necessary for the maximal extent of assembly, while exposure of preassembled tubules to temperatures around 4°C causes their depolymerization (Roberts & Hyams, 1979). Although the tubulin molecules contain the necessary information to interact with each other and assemble into MTs, the presence of MAPs appears to be important in stimulating the assembly process

and these proteins are likely to play a physiological role in modulating MT assembly according to the cell's need for MT structures (Hill & Kirschner, 1983; Maccioni *et al.*, 1984; Maccioni *et al.*, 1981).

4.6 PURIFICATION OF TUBULIN

Since the initial isolation of the protein component of MTs (Shelanski & Taylor, 1968) and discovery of the condition to reassembly MTs (Weisenberg, 1972; Borisy & Olmsted, 1972) from microtubular protein components using GTP and EGTA in appropriate MT buffers (MES, PIPES, pH 6.6-6 8), it has been possible to purify tubulin from brain tissue and other sources, by different procedures (Kobayashi & Mohri, 1977; Lee, 1982).

Purification of microtubular proteins (tubulin and the MAPs components) is accomplished by successive cycles of assembly and disassembly (Shelanski *et al*, 1973), followed by a chromatographic step on phosphocellulose (Weingarten *et al.*, 1975). This procedure has been used for purification of tubulin from brain of a variety of sources (Farrel, 1982; Hill & Kirschner, 1983; Maccioni & Mwllado, 1981; Morejohn & Fosket, 1982;, Weatherbee *et al* , 1980; Wilson, 1982) Three temperature dependent cycles of assembly and disassembly are usually required prior to the chromatographic step. Tubulin can be purified by ionic exchange chromatography The procedure originally developed by Weisenberg (1968) contains an ammonium sulfate precipitation of tubulin in brain extracts and a chromatographic step in DEAE Sephadex in the presence of MgCl, GTP in a buffer pH 6 4 This procedure has several variants resulting from modifications introduced by several authors on the basis of the DEAE chromatographic step (Eipper, 1975; Lee *et al.*, 1978; Lee, 1982)

Tubulins have also been purified by affinity chromatography using desacetyl colchicine bound to a Sepharose affinity matrix (Morgan & Seeds, 1975b) and by poly-l-lysine sepharose chromatography (Lacey & Prichard, 1986). Both α- and β-tubulin have also been separated by hydroxylapatite chromatography and electroelution followed by SDS-polyacrylamide gel electrophoresis (Luduena, 1979; Blose *et al* , 1984; Lacey, 1988). Some of these methods are not successful for purification of tubulin from

helminths (Friedman *et al.*, 1980), due to the following reasons: (a) the amount of tubulin as percentage of total protein is usually very low (< 1%) in helminths (Friedman & Platzer, 1981; Kohler & Bachmann, 1981), and (b) endogenous inhibitors of tubulin polymerization such as, proteolytic activity and GTP-ase activity may play a role in degrading the already limited protein concentration (Roobol *et al.*, 1980).

4.7 TUBULIN DOMAINS GENERATED BY LIMITED PROTEOLYSIS

The domain structure of tubulin and the interactions between domains have been investigated by several approaches, such as limited proteolysis, the binding of subunit- and domain-specific antibodies, chemical cross-linking and sequence analysis (Mandelkow *et al.*, 1985; Kirchner & Mandelkow, 1986; Luduena *et al.*, 1985; Sullivan, 1988).

Limited proteolysis is a powerful tool in the study of the structures and functions of proteins as well as of regulatory mechanisms of proteins *in vivo* (Jacobson, 1964; Luduena & Roach, 1991). This classical technique has been applied to tubulin for the identification of domains for the binding of colchicine (Serrano *et al.*, 1984b; Avila *et al.*, 1987), nucleotides (Maccioni & Seeds, 1983), calcium (Serrano *et al.*, 1986) and MT associated proteins (Serrano *et al.*, 1984a; 1985).

Tubulin is a heterodimer containing two α- and β- subunits, each of which has been sequenced and is composed of about 450 amino acids (Krauhs *et al.*, 1981; Postingl *et al.*, 1981). Both α- and β-subunits are acidic, with the β subunit slightly more acidic than α- (pI = 5.3 - 5.4), respectively (Berkowitz *et al.*, 1977). The C-terminus end of each is remarkably rich in acidic residues, especially in glutamic acid (Krauhs *et al.*, 1981). These regions contain about 40% of all the glutamates and about 20% of all the aspartates in tubulin. At physiological pH, the sequences are highly charged and stay in an extended conformation (Sackett & Wolff, 1986). Proteolysis of tubulin with subtilisin has revealed that small regions at the C-terminal ends of both subunits are exposed to the solvent and are readily cleaved (Serrano, 1984c; Sackett *et al.*, 1985).

Limited proteolysis of tubulin consist of the enzymatic treatment of the protein

under analysis with substoichiometric amounts of proteolytic enzymes for short incubation times followed by further biochemical analysis of the cleavage products. Occasionally proteins can be cleaved under appropriate conditions in only one or two sites in the sensitive regions of the protein molecule. In contrast to exhaustive proteolysis resulting in extensive cleavage, limited proteolysis is dependent upon both protein conformation and location of specific amino acid residues in the protein sequence. Since conformation is an important factor for the nature of cleavage, the experimental conditions for enzymatic proteolysis have to be well controlled Alterations of the digestion conditions result in changes in cleavage patterns. This approach has provided useful information for the conformational analysis of tubulin upon the interaction of several functionally important ligands. Studies on tubulin subdomains displaying specific functions have yeilded novel information on the localization of the major structural sites of tubulin.

Digestion of native tubulin with trypsin or chymotrypsin shows that both α- and β-monomers split into two major domains comprising roughly the N-terminal 3/5 and the C-terminal 2/5 of the protein (Mandelkow *et al.*, 1986) The susceptibility to cleavage depends on the protease used Trypsin cleaves primarily α-tubulin (main fragments of molecular weight 36 and 14 kDa, and a minor band of 17 kDa), chymotrypsin cleaves mainly β-tubulin (main fragments at molecular weight 31 and 20 kDa), and a minor band at 21 kDa). Sequence analysis of the fragments shows that the main cleavage sites are at Arg 339 (trypsin, α) and Tyr 281 (chymotrypsin, β) Thus the large fragments are N-terminus, and the smaller ones are C-terminus (Mandelkow *et al.*, 1985). A second site of trypsin appears to be close to residue 300 of α-tubulin, and a second site of chymotrypsin is within 1-2 kDa of the C-terminus of β-tubulin, as shown by immunoblots with antibodies specific for the C-terminus peptide. In general, the domain boundary and the C-terminus region are susceptible to cleavage by a variety of proteases. The region near the C-terminus which carries a high negative charge is one of the most antigenic parts of both α- and β-tubulin so that a fraction of the antibodies raised against α tubulin recognizes the C-terminus stretch of the chain and cross-reacts with the C-terminus of β-tubulin

4.7.1 Differential stability of tubulin domains

Tubulin is a labile protein. This can be demonstrated by the tendency for spontaneous degradation of purified tubulin. This process becomes noticable especially at high temperature (Zale & Klibanov, 1984). The C-terminus domain of α-tubulin and the N-terminus domain of β-tubulin are more labile than their complementary domains. For example, autolysis of β-tubulin leads to a range of fragments between 49-30 kDa, all of which carry the C-terminus domain. The differential stability correlates well with the differential antigenic response since the antibodies are directed against the stabler domains. The domains most susceptible to autolysis are also most easily degraded by progressive enzymatic digestion of the native protein. Several proteases e.g. V8 protease, papain, clostripain, thermolysin, proteinase K, yeild comparable patterns of fragments and immunoblots. The most sensitive areas are the boundary between the domains, the C-terminus region, and the region around the glycine cluster; they roughly agree with areas of high predicted flexibility (Sullivan, 1988).

4.8 ANTIGENICITY OF TUBULIN

4.8.1 Hydrophilicity

The domains defined by the enzymes have different antigenic activities. This means that the specific antibodies do not identify tubulin chains as a whole, but those fragments that contain the active domain, α-large or β-small fragments. Although the complementary domains are nearly silent they contain antigenic sites that are recognized by the cross-reacting antibodies. If a process similar to spontaneous degradation took place in the animal after immunization, the labile domains would largely disappear before antibodies are produced. Thus the antigen would be represented mainly by the stabler domains. Antigenic sites are on surfaces and therefore tend to be composed of hydrophilic residues. Mandelkow *et al.* (1986) used hydrophilicity to estimate hydrophilic regions along the tubulin α and β chains. The authors reported that there are eight major peaks of hydrophilicity in each tubulin subunit. The sites occur in three main regions around 100 to 200 (in the vicinity of the

presumptive GTP binding site), 300 to 325 (near the domain boundaries), and 410 to 450 amino acid (close to the C-terminus). Those occurring in homologous regions could correspond to antibodies cross-reacting between the two tubulin monomers, while non-homologous regions would represent subunit specific antibodies (subject to the constraint of differential antibody response caused by tubulin's lability or other factors.

4.8.2 Flexibility

There is a debate on whether flexibility rather than hydrophilicity is the key property of an antigenic determinant (Westhof *et al.*, 1984). Mandelkow *et al.* (1986) have demonstrated the flexibility along the tubulin chains In general, the peaks of flexibility do not coincide with those of hydrophilicity, the most prominant exception being the 10-15 C-terminus residues Interesting regions of high flexibility occur between residues 140-150 (the loop containing the glycine cluster) and around the domain boundary. In fact, both proteolytic cleavage sites are preceded by a flexible stretch of about 5 residues Moreover, there is an extended flexible region in α tubulin around position 40 which would be compatible with one of the observed binding regions of the α specific antibody fraction These data support the view that flexibility is a useful indicator for predicting epitopes, cleavage sites, or hinges between domains (Fig. 1.4).

4.9 MICROTUBULE INHIBITORS

A number of drugs such as, colchicine and derivatives, vinca alkaloids, benzimidazole carbamates and derivatives etc. interact with the tubulin-microtubule system and block tubulin assembly although by different mechanisms or induce tubulin association into structures different than MTs and are considered as MT poisons because of their inhibitory effect on cell growth. These compounds have played a fundamental role in MT research and the study of their effects on MT assembly has provided valuable information for clinical and pharmacological investigations

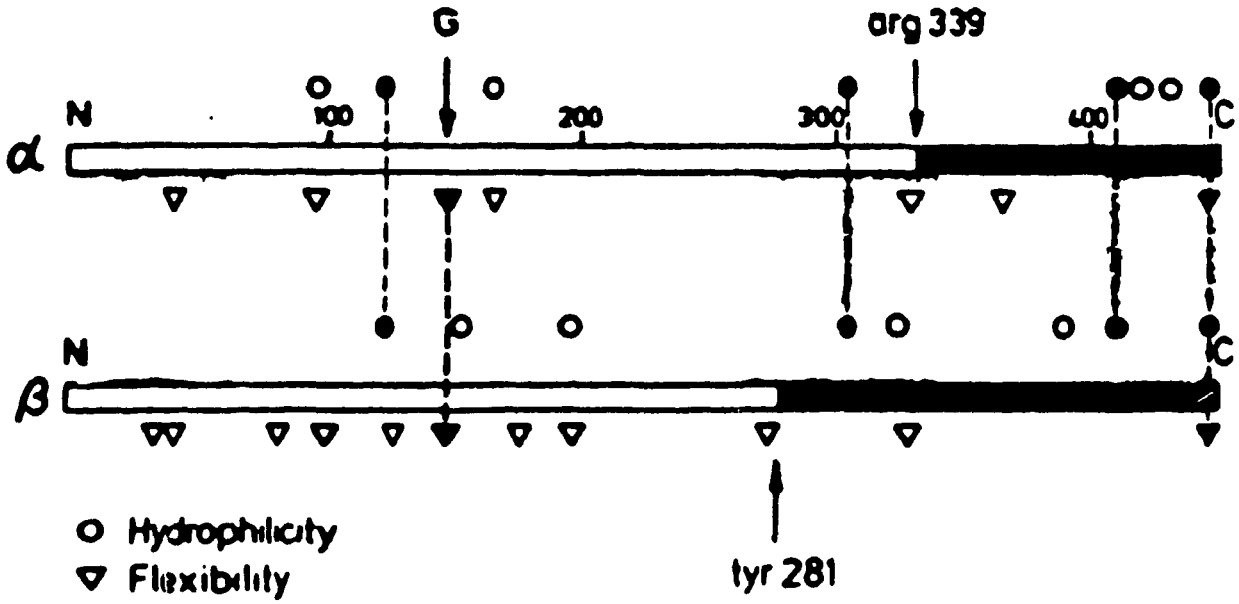


Fig. 1.4. Protease sensitivity, antigenicity and predicted hydrophilicity and flexibility of tubulin The N-terminal domains are represented by empty bars, the C-terminus ones are filled Shaded regions indicate areas of preferred interaction with the polyclonal antibodies Predicted peaks of hydrophilicity are indicated by circles above the bars; those in homolgous regions are filled (possible determinants for cross-reacting antibodies Peaks of flexibility are shown as triangles below the bars (homologous reions, filled). A high flexibility is predicted around the glycine cluster G (142-146), at the cleavage sites, and near the C-terminus. This roughly agrees with the observed protease sensitive areas. The antigenicity in the N-terminal region agrees with predicted flexibility, but not with hydrophilicity.

(Mandelkow *et al.*, 1986)

4.9.1 COLCHICINE

Colchicine, a substance isolated from the plant *Colchicum autumnale* binds specifically and with high affinity to tubulin (Garland & Teller, 1975; Wilson, 1975; 1982) and has been shown to effect cell division (Dustin, 1984) Tubulin dimer has two sites for the binding of colchicine (Floyd *et al.*, 1989). One of these sites, the high affinity site, has been extensively studied (Luduena & Roach, 1991) The specificity and the high affinity of colchicine for tubulin have provided the basis for the determination of the levels of tubulin in different tissue extracts (Wilson, 1975) The process of colchicine binding to tubulin is slow, highly dependent on the temperature, and associated with successive conformational changes in the tubulin molecule induced by the drug prior to the formation of a stable complex (Garland & Teller, 1975).

Colchicine and some BZs are believed to share the same binding site on the tubulin dimer because they competitively inhibit each others binding to nematode tubulin (Friedman & Platzer, 1980) and mam alian brain tubulin (Laclette *et al.*, 1980). The affinity of colchicine is lower for helminth tubulin than mammalian tubulin (Kohler & Bachmann, 1981) but it inhibits the binding of mebendazole, FBZ and OFZ, but not of TBZ, OBZ or PBZ (Friedmen & Platzer, 1978). Colchicine and TBZ occupy different binding sites on tubulin. TBZ is thought to bind to the β-tubulin subunit of fungal tubulin (Sheir-Neiss *et al.*, 1978) whereas the colchicine site is on the α-tubulin (Schmitt & Atlas,1976). It is possible that in mammalian tubulin the binding sites are closely associated on the tubulin dimer and side chains of either colchicine or the BZs obstruct the BZs binding sites on the other subunit. Since the α-tubulin of *A gallı* and sheep brain have different mobilities on electrophoresis (Ireland *et al* , 1982), the structure of these tubulins may differ. Structural differences could account for the lower binding of colchicine with helminth tubulin and differences in the relationships between the BZs and the colchicine binding sites in helminths and mammalian tubulin. For example, the side chains of MBZ, FBZ and OFZ but not TBZ, OBZ, or PBZ may encroach on the binding site for colchicine on helminth tubulin

Serrano *et al.* (1984a,b,c) using limited proteolysis reported that colchicine

binding site is located in a 16 kd carboxy-terminal fragment of α-tubulin subunit. MAPs also bind the C-terminus of the tubulin subunits. These proteins compete with colchicine for the tubulin binding site (Nunez *et al.*, 1979). However, Avila *et al.* (1987) found that cleavage of the 16 kd α-tubulin trypsin fragment with subtilisin produces a 4 kd and 12 kd fragment, the former originating from the extreme C-terminus and associated with MAPs binding and the latter associated with colchicine binding.
The BZs may bind to regions of the tubulin molecule which are involved in Mt assembly. Alternatively, BZs binding to tubulin may disrupt Mt assembly as a result of a conformational change in the tubulin dimer. Colchicine binding to tubulin produces a change in the tubulin dimer conformation (Serrano *et al.*, 1984c).

Mukhopadhyay *et al.* (1990) reported that α tubulin C-terminus regulates the colchicine tubulin interaction A possible structural contribution of β subunit to the tubulin interaction with colchicine is suggested by the observation that chinese hamster ovary mutants resistant to colchicine present an altered β tubulin (Cabral *et al.*, 1980). Another approach using sulfhydryl modifications with cross-linking reagents suggests that the site could be located in the interface between both tubulin subunits (Maccioni *et al.*, 1984a,b).

4.9.2 VINBLASTINE

Vinblastine is an alkaloid, from *Vinca rosea* which has been widely used in cancer chemotherapy. Vinblastine bind to tubulin at site distinct from the colchicine and podophylotoxin binding sites (Bryan, 1974). The vinblastine reaction with tubulin is reversible and has different characteristics than the tubulin interaction with colchicine (Luduena, 1979; Wilson, 1975). The binding is relatively temperature independent and rapid and it has been shown to be insensitive to GTP, Ca2+ and colchicine. Two high affinity vinblastine binding sites per tubulin heterodimer have been described which appear to have similar affinities (Roberts & Hyams, 1979; Soifer, 1975). Vinblastine binding induces changes in ultra-violet characteristics of tubulin and promotes fluorescence but does not change significantly its conformation and tubulin precipitation with the drug results in the release of the total nucleotide

content of tubulin (Luduena, 1979). A striking characteristic of vinblastine interaction with tubulin is the formation of paracrystalline structures containing stoichiometric ratios of the drug to tubulin, and formation of rings and other spiral structures, which are produced at high concentrations of the drug. However, low concentrations of vinblastine causes dimerization of tubulin (Roberts & Hyams, 1979, Soifer, 1975) Preassembled MTs are sensitive to vinblastine, which produce a separation of protofilament pairs associated to coiling of the protofilament into spirals (Erickson, 1974; Fujiwara & Tilney, 1975).

4.9.3 BENZIMIDAZOLE CARBAMATES (BZs)

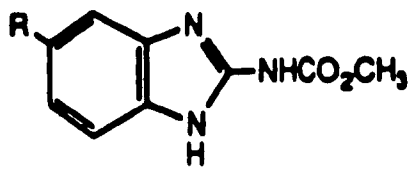
4.9.3.1 Chemistry and mode of action

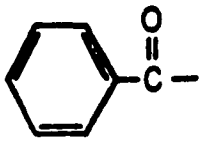
Benzimidazole carbamates (BZs) represent the only class of truly broad-spectrum anthelmintics, however, they also show activity against fungi and mammalian cells (Lacey, 1990). With the discovery of thiabendazole (TBZ) as a class of low-dose broad-spectrum drugs with a high therapeutic index was established (Brown *et al.*, 1961). The discovery of TBZ opened the doors for the development of a further 15 BZs and BZ prodrugs (Townsend & Wise, 1990). BZs compounds are of similar chemical structure and mode of action, they vary in structure (R & R') around the central benzene and imidazole rings (Fig. 1.5). Various substitutions of the BZ ring lead to substances which are used as fungicides, anthelmintic drugs and in cancer chemotherapy (Dustin, 1984). Since the mid-1960s the mode of action of BZs has been extensively investigated All the BZs probably share common mechanisms of action against helminths as they are structurally similar and the parasites resistant to one drug share side-resistance to the others (Lacey, 1988).

4.9.3.2 Effects of BZs on the metabolism of eukaryotes

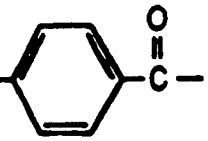
Initial studies of the mode of action of BZs focused on their role in carbohydrate metabolism. Allen & Gottlieb (1970) demonstrated that TBZ inhibited respiration in fungi and concluded that inhibition of terminal electron transport was probably the primary site of action. In helminths, BZs also inhibit the enzymes of

Benzimidazole Carbamates

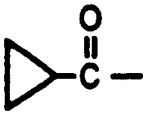


R = 

Mebendazole[10]
crystals, acetic acid, mp 288°C
insoluble in water

R = F 

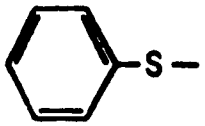
Flubendazole[10]
crystals, mp 260°C
insoluble in water

R = 

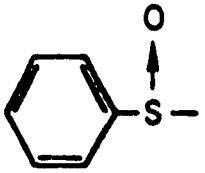
Ciclobendazole[10]
crystals, mp 250°C

R = $CH_3CH_2CH_2S$—
Albendazole[10]
crystals, mp 208°C

R = $CH_3CH_2CH_2O$—
Oxibendazole[12]
crystals, mp 230°C
practically insoluble in water

R = 

Fenbendazole[13]
powder, mp 233°C
insoluble in water

R = 

Oxfendazole[14]
crystals, mp 253°C

R = $CH_3CH_2CH_2CH_2$—
Parbendazole[9] crystals,
mp 225°C, insoluble in water

Fig. 1.5. Chemical structures of some benzimidazole anthelmintics.

(Lacey, 1990)

metabolic pathways, including some associated with electron transport.

Prichard (1970) demonstrated complete inhibition of NADH oxidation in the presence of the anaerobic enzyme fumarate reductase by TBZ in *H contortus* extracts. Inhibition of fumarate reductase is an important hypothesis as the site of action because the enzyme is unique to the parasite in the host/parasite system and it is important in the energy metabolism of the parasite. In comparative studies of BZ-susceptible and resistant *H. contortus*, differential inhibition of the fumarate reductase by TBZ was observed (Prichard, 1973) Cambendazole (CBZ), fenbendazole (FBZ) and oxfendazole (OFZ) but not mebendazole (MBZ) also inhibit the fumarate reductase of BZ-susceptible and resistant isolates of *H contortus* (Prichard, 1973; Romanowski *et al.*, 1975; Rahman *et al.*, 1977; Prichard *et al* , 1978).

Several studies have demonstrated that BZ can significantly interfere with the transport of low molecular weight nutrients by helminths. MBZ reduces the uptake of glucose, fructose, proline, glycine, methionine and palmitate by *A. suum in vitro* (Van Den Bossche, 1972; Van Den Bossche & De Nollin, 1973). BZs have also been shown to inhibit glucose uptake both *in vitro* and *in vivo*, in *Trichinella spiralis*, *Schistosoma mansoni*, *Moniezia expansa* and *Hymenolepis diminuta*, which in some cases is associated with a compensatory depletion of glycogen reserves (Lacey, 1988) In addition, there have been few other reports on the effects of BZs on enzymes from disrupted mitochondria. The succinate decarboxylase system from submitochondrial particles of *Fasciola hepatica* was inhibited by MBZ and CBZ (Kohler & Bachmann, 1978). The enzymatic activity of phosphoenolpyruvate reductase and malate dehydrogenase from different helminths was inhibited by various BZs (Rahman *et al.*, 1977).

BZs have also been shown to inhibit the *in vitro* secretion of acetylcholinesterase (AChE) in *Nippostrongylus brasiliensis* by a process which is thought to be MT dependent (Watt *et al.*, 1982) and important for allowing the worm to maintain itself in host intestine (Rapson *et al.*, 1981). In a similar study, Sangster *et al.* (1985) showed that BZ treatment produced greater effects on acetylcholinesterase secretion, the presence of MTs in intestinal cells and colchicine

binding, in BZ-susceptible compared with resistant *Trichostrongylus colubriformis*. Other effects of BZs on the helminths, such as inhibition of serotonin metabolism, membrane-bound monoamine oxidase activity and NA^+ uptake have also been reported (Moreno & Barret, 1979, Beames *et al.*, 1976).

4.9.3.3 Effects of benzimidazoles on microtubules

MTs are proteinacious organelles which are found in all eukaryotes from yeast to man. The major structural component of MT, tubulin is a dimeric protein comprised of α and β subunits, which undergoes a reversible polymerization to form MTs. MTs play a major role in cell shape, intracellular transport, absorption, secretion and cell division (Sullivan, 1988). To perform these functions MTs exist in dynamic equilibrium with tubulin The equilibrium can be altered both *in vivo* and *in vitro*, by exogenous substances such as low temperature, pH, drugs, known as MT inhibitors. Most, but not all, such inhibitors exert their action by binding to tubulin to prevent the self-association of subunits onto the growing MTs. This results in a "capping" of the MTs at the associating end while the MT continues to dissociate from the opposing end, with a net loss of MT length One implication of this phenomenon is that it is not necessary for MT inhibitors to bind all tubulin dimers to inhibit polymerization, it is sufficient for them simply to "cap" the MT (Robert & Hyams, 1979; MacRae & Langdon, 1989).

BZs have been shown to exert their biochemical action as anthelmintics, fungicides and anti-cancer agents by binding to tubulin and inhibiting the formation of polymeric MTs (Davidse & Flach, 1978; Lacey & Watson, 1985; Lacey & Prichard, 1986; Lacey, 1990). The hypothesis that tubulin is the target for BZs was first based on studies of the mode of action of the prodrug benomyl and its active principle carbenazim in fungi (Clemon & Sisler, 1971) and ultrastructural studies (Rubino *et al.*, 1983; Verheyen *et al.*, 1975). Borgers & De Nollins (1975) demonstrated that MBZ *in vivo* causes disappearance of MTs from the intestinal cells of the nematodes *A. suum* and *Syngamus trachea*. Loss of MTs apparently causes the accumulation of secretory granules, the formation of autophagic vacoules and death of these parasites.

In contrast, the MT of host cells appear normal. Several other investigators observed similar degenerative changes in the absorptive cells of some other nematodes and cestodes (Verheyen *et al.*, 1976; Atkinson *et al.*, 1980; Sangster *et al* , 1985; Franz *et al.*, 1990a,b; Enos & Coles, 1990)

Eilers *et al.* (1989) demonstrated that the treatment of polarized human intestinal adenocarcinoma cell with the nocodazle, a BZ derivative, blocks the transport of newly synthesized proteins to the apical cell surfaces, while delivery to the basolateral surface is not affected. These studies support the assumption that tubulin is the primary target of the BZs and in nematodes, the intestinal tubulin/MT system appears to be of particular importance in this respect, since the morphological changes are observed in the intestine first after the MBZ treatment (Atkinson *et al.*, 1980; Borgers *et al.*, 1975).

4.9.3.4 Efficacy of benzimidazoles in vitro and in vivo

In vitro studies demonstrated that BZs such as MBZ, OBZ, PBZ, FBZ, ABZ and CBZ are capable of binding to purified mammalian brain tubulin and inhibiting MT assembly (Friedman & Platzer, 1978; DeBrabander *et al.* (1976; 1982) and OBZ, FBZ and CBZ compete for the colchicine binding site on tubulin (Ireland *et al* , 1979; Laclette *et al.*, 1980). TBZ and MBZ inhibit mitosis in many lower eukaryotes such as yeast, fungi and slime moulds (Burland & Gull, 1984; Davidse & Flach, 1978; DeBrabander *et al.*, 1976). Dawson *et al* (1984) demonstrated that BZs inhibited DMSO-induced polymerization of *A. galli* tubulin to MT Lacey & Prichard (1986) examined the total binding of ABZ, FBZ, MBZ, OBZ, OFZ and PBZ to supernatant preparations from BZ susceptible and resistant adult *H contortus* and found lower binding to the resistant isolates

Lubega & Prichard (1990) resolved the total BZ binding into high and low affinity binding to the extracts from BZ resistant and susceptible strains of *H contortus*. The authors concluded that BZ-resistant strain had a significant decrease in high affinity receptors for both MBZ and OBZ compared with the susceptible strain. However, the binding affinity values for the high affinity receptor were shown to be

similar between strains. In a subsequent study, Lubega & Prichard (1991) compared the tubulin content and high affinity BZ binding to unembryonated eggs with that to larvae and adult worms for BZ-resistant and susceptible strains of *H. contortus*. The authors demonstrated that high affinity binding was lost from the resistant strain at all three stages of development, and that the egg contained a greater concentration of tubulin per milligram of protein and bound more drug per milligram of protein than the larvae or adult worms

Friedman & Platzer (1980) demonstrated that BZs and colchicine bind to tubulin from *Ascaris* embryos and intestine (Kohler & Bachmann, 1981) and they demonstrated that FBZ was 250-fold and MBZ was 400-fold more potent as inhibitors of [^{3}H]colchicine-binding to the tubulin of embryonic *A. suum* than to mammalian tubulin; thus exhibiting an apparently higher affinity for helminth tubulin. However, using direct binding studies with [^{3}H]MBZ and *A. suum* intestinal tubulin, Kohler & Bachmann (1981) failed to find any change in BZ affinity. They concluded that selectivity was related to the differing pharmacokinetics of BZs within the host and the parasite.

Inhibition of MT polymerization, or accelerated depolymerization, is a plausable hypothesis for the primary mode of action of BZs because MTs are ubiquitous organelles. Considering the variety of functions performed by MTs , it is likely that the metabolic and biochemical changes induced by BZs are dependent upon an initial disruption of the polymerization of tubulin to form MTs. There is now enough evidence to indicate that the primary mode of action of BZs is to bind to tubulin and disrupt the tubulin-MT equilibrium.

The potency of BZs (mg/kg dose) based on *in vivo* efficacy studies demonstrates a rank order efficacy of TBZ < CBZ < PBZ < OBZ < MBZ < FBZ ~ ABZ ~ OFZ over a 10 fold range. The doses required to achieve efficacy against nematodes are lower than those used for cestode and trematode control. In the latter classes, usually control is only obtained at higher doses or following multiple treatment (Van Den Bossche *et al.*, 1982). It has been observed that extra-intestinal parasites, particularly intravascular and interstitial dwelling parasites, are less sensitive than gastrointestinal parasites

(Van Den Bossche *et al.*, 1982). BZs activity against developing stages is greater than that against arrested or adult stages in comparable habitats For examples, (a) gastrointestinal parasite egg development (*in vivo* and *in vitro*), hatching and larval development are inhibited at doses which are sub-efficacious against adults *in vivo* (Kirsch & Schleich, 1982) and (b) interstitial filarial nematodes, such as *Onchocerca gibsoni*, display microfilarial control in the absence of adult efficacy (Forsyth *et al* , 1985).

Important host-related pharmacological principles affecting *in vivo* efficacy of BZs are:

1. Host toxicity is low at efficacious anthelmintic doses, however, transient adverse reactions at higher or chronic doses are observed (Seiler, 1975);
2. Efficacious BZ doses are host-dependent due to differing dynamics and clearance rates of drugs *in vivo* (Van den Bossche *et al.*, 1985);
3. BZs are extensively metabolized in host species usually to less potent anthelmintics. The pathways of metabolism for each BZ are similar for all hosts (Hennessy, 1985);
4. Despite earlier reports of poor systemic absorption of BZs after oral administration *in vivo* (MBZ), subsequent data suggest >50:% absorption (Van den Bossche *et al.*, 1982). Reduced bioavailablity was initially assumed in the absence of biliary excretion data. Within the BZ class, biliary excretion is favoured by MBZ, FBZ and OFZ while predominantly urinary excretion is observed for TBZ, CBZ, PBZ, OBZ and ABZ (Hennessy, 1985),
5. BZs are hydrophobic and water insoluble and therefore bioavailability and pharmacodynamics and thus efficacy can be altered by formulation and presentation (Hennessey, 1985).

There are three fundamental aspects of BZs pharmacology *in vivo*: the role of host pharmacodynamics, host-parasite interactions and the biochemical pharmacology of BZs which must be addressed to account for the efficacy variations observed against helminths.

4.10 TUBULIN AND ANTIBODIES

Elevated levels of serum anti-tubulin antibodies in humans have been reported to be associated with Graves's disease and Hashimoto's thyroiditis (Rousset *et al.*, 1983), demylinating disease (Newcombe *et al.*, 1985), infectious mononuculeosis (Mead *et al.*, 1980) and alcoholic liver disease (Kurki & Virtanen, 1984). High quantities of IgM anti-tubulin antibody were detected in all these diseases. IgG antibodies were also detected in all groups studied, but levels were not as high as those of the IgM antibody. These antibodies were assessed using immunofluoresence assay, radioimmunoassay and affinity chromatography.

Howard *et al.* (1987) detected elevated levels of anti-tubulin antibodies in sera from patients with leishmaniasis, african trypanosomiasis, onchocerciasis, schistosomiasis, leprosy and to some extent in Chagas disease and chronic hepatitis. The anti-tubulin antibodies detected, were of IgG isotype. Levels of serum anti-tubulin antibodies were significantly elevated in 67% of patients with visceral leishmaniasis, in 89% of patients of onchocerciasis, in 100% of patients of schistosomiasis and in 94% patients with leprosy

There are several mechanisms by which infectious agents may induce anti-tubulin antibodies. Tissue damage during infection may release a host antigen that is not normally exposed to the immune response, but is, upon release immunogenic and capable of by-passing tolerance mechanisms. Alternatively the host antigen, in this case tubulin, may be altered during the course of the infection rendering it immunogenic. Tubulin occurs in all cell types, the highest levels have been found in neurons. MTs are present in oligodendrocytes and cytoplasmic loops of myelin (Peters *et al.*, 1976), and tubulin is an integral component of rodent brain myelin (Gozes & Richter-landsberg, 1978). It is therefore possible that in demyelinating conditions tubulin is released from degenerating oligodendrocytes and myelin or even axons. For some of the other infections such as leishmaniasis, trypanosomiasis, tubulin is a major content of the parasite and this tubulin may be immunogenic in mammalian hosts. Even with infectious agents that do not contain tubulin, such as *M. leprae* and hepatitis B virus, antigens may be present that cross-react with components of the

mammalian host, tubulin. The mechanisms and significance of the response is unclear and require cautious interpretation.

4.10.1 Poly- and monoclonal antibodies

Polyclonal antibodies have been raised against electrophoretically purified tubulin monomers, injected into the lymph nodes of rabbits and separated into subunit-specific and cross-reacting fractions by affinity chromatography (Mandelkow *et al.*, 1985). These antibodies had high affinities and proved to be domain-specific; the antibodies raised against α-tubulin were directed mainly against the large N-terminus domains of α-tubulin in the case of the α-specific antibodies, and towards both α- and β-tubulin in the case of the α-cross-reacting antibodies, while the antibodies raised against β-tubulin recognized the small C-terminus domain. Thus immunization with purified monomers elicited complementary antigenic responses (Herrmann, 1981). The highly specific interaction between an antibody and its antigenic determinant has proven to be an extremely useful tool in studying the structure and functions of many different proteins (Atassi, 1977a,b; Attasi, 1979). Antisera raised against tubulin are no exception.

MTs have been localized in cells using anti-tubulin antisera and the techniques of indirect immunoflouresence and immunogold electron microscopy (Sherwin & Gull, 1989; Joshi & Cleveland, 1989). Anti-tubulin antisera have provided an alternative to the colchicine binding assay for the quantitation of tubulin in cell extracts through the technique of radio-immunoassay and ELISA (Tang & Prichard, 1989). Anti-tubulin polyclonal sera have also been used to assess structural differences and similarities between tubulins isolated from different sources (Banerjee *et al.*, 1990; Lubega & Prichard, 1991; Sullivan, 1988)

The use of monoclonal anti-α-tubulin antibodies in the ELISA, Western blot, and immunofluorescence (IFA) unequivocally demonstrated that α-tubulin from different sources and even from different tissues of the same species, are non-identical (Asai *et al.*, 1982a,b). One MAb A.1, stained MTs of chick embryo fibroblast cells immunofluorescently but did not react with MTs of rat kangaroo fibroblasts; another

MAb A.2, stained both cell types. This shows that A.1 and A.2 recognize discrete and different epitopes on α-tubulin. Furthermore, these MAbs inhibited the bend amplitude of reactivated sea urchin spermatozoa without affecting beat frequencies, showing that they can have a differential effect on microtubule functions. IFA with anti-tubulin MAbs has also demonstrated that, in addition to their role in the mitotic spindle, MTs are a basic component of the cytoskeleton of cells in interphase and are long structures extending from the centrosphere just outside the nucleus to the plasma membrane (Osborn & Weber, 1976).

In order to determine the heterogeneity among MTs of the cytoplasmic microtubule complex, three MAbs were tested by Thompson *et al.* (1984). They found that two of these MAbs bound to all of the cytoplasmic microtubules but one MAb bound only a subset of cytoplasmic microtubule. This indicates that the cytoplasmic microtubule complex contains antigenically different types of microtubules. MAbs specific for mammalian β-tubulin have been shown to recognize the microtubule cytoskeleton of the flagellated protozoan *Trichomonas vaginalis*. Two out of seven MAbs demonstrated axostyle, costa, recurrent flagellum and anterior flagella by IFA (Schwartzman *et al.*, 1986).

MAbs have also been used to localize the exchangeable GTP-binding site on tubulin and it provided the direct evidence that only β and not α-tubulin contains the exchangeable GTP-binding site (Hesse *et al.*, 1984). Gianni & Margeret (1985) showed that MAbs raised against tubulin from the axonemes of sea urchin sperm flagella recognize an acetylated form of α-tubulin present in the axoneme of a variety of organisms. Birkett *et al.* (1985) used MAbs to analyse the expression of a multiple tubulin family in *Physarum polycephalum*. They found that their MAbs reacted with the β-tubulin isotypes expressed in both *Myxamoebae* and *Plasmodia*. These antibodies showed a spectrum of reduced reactivity with the Plasmodial β-tubulin and revealed a new level of complexity amongst the tubulin isotypes expressed in *Physarum*.

Since many of the biological activities of MTs are associated with their assembly in the cell, at the appropriate time and place, it is also important to

investigate if anti-tubulin antibodies can be used to inhibit assembly of MTs The domain-specific antibodies have been shown to prevent the assembly of tubulin and cause the disassembly of preformed MTs *in vitro* (Mandelkow *et al* , 1985), as well as in cell models or after microinjection *in vivo* (Fuchtbauer *et al* , 1985)

Morgan *et al* (1978a,b,c) using anti-chick brain polyclonal antibodies, demnostrated that these antibodies inhibited *in vitro* assembly of tubulin into MTs, in a concentration-dependent manner The authors suggested that the antibody binding site(s) on the tubulin molecule are at or near the site(s) of tubulin-tubulin interactions. However, it is also possible that the sites are spacially separate and that the presence of antibody blocks sufficiently close contact between tubulin molecules or will not allow a necessary conformational change to take place in the tubulin molecule so that it can polymerize

Mandelkow *et al* (1985) studied the substructure of the tubulin molecule by limited proteolysis and high affinity polyclonal antibodies specific for α-and β-tubulin The epitope of one α-tubulin antibody was very close to the C-terminus, the other is close to the N-terminus. The epitope of the β-cross-reacting antibody is also located near the C- terminus. These antibodies prevented MT assembly and cause disassembly of preformed MTs The authors suggested that the disassembly caused by their antibodies must be due to their interference with some bonding surfaces. Conversely, since the C-terminus is exposed on the outside of the MTs (Wehland *et al* 1983), it is probably not involved in bonds between subunits, and this would explain why some antibodies do not depolymerize MTs This is being investigated in more detail. Draber *et al* (1990) also reported the differential inhibitory effect of MAbs against N-terminal domains of tubulin subunits on *in vitro* assembly of MTs Some MAbs strongly inhibited the MT assembly The authors suggested that surface regions of N-terminal domains of both tubulin subunits are involved in the formation of MT structures. Some of the target epitopes recognized by the inhibitory antibodies could be essential for tublin polymerization The polymerization process is not compatible with simple endwise shortening of MTs, and the microinjection studies suggest internal breaks along the MTs. The inhibitory effect of the antibodies is probably due

to their tight binding to some epitope that plays a role in the dimer-polymer equilibrium (Mandelkow *et al.*, 1986).

All the above mentioned studies suggests that anti-tubulin specific MAbs can be used as an immunological tool for the study of tubulin isoforms, molecular mapping of its functional domains and for study of orientation of subunits in MTs.

RESEARCH OBJECTIVES

5.1 Preamble

The literature cited above suggests that nematodes occupying the tissues or the intestine of human present many unusual structural and developmental features, they also possess an as yet undefined array of chemicals or antigens, some of which are secreted/excreted into their environment To develop new approaches to combat effectively the potentially debilitating disease caused by infection with these organisms one should·

(a) define parasite products chemically;

(b) determine the ability of these products to induce protective immunity or to counter a protective immune response; and

(c) establish their potential for the diagnosis of infection

MTs are proteinacious organelles which are localized in the cytoplasm and cell membranes of all eukaryotic cells, where they function in cell division, cell shape, intracellular transport, nutrient absorption and excretion. Tubulin, the main structural component of MTs, is a heterodimer composed of two subunits, α and β. Both α and β subunits exist in multiple isoforms in different organisms, tissues and even single cells. It has been suggested that α- and β-isoforms may be unique to or selectively enriched in different parts of the cell and this may have some functional importance. Tubulin is considered to be a highly conserved protein but differences have been found in the tubulin between nematodes and mammals, among nematodes and even within the various tissues of the same nematode In addition to differences in the structure, tubulin also differs in drug binding abilities Nematode tubulin is more sensitive to BZ attack than mammalian tubulin. The selective action of BZs on microtubules (MTs) is likely to be a complex mixture of. (1) Differential sensitivity of the tubulin; (2) The dynamics of MTs assembly; (3) Pharmacokinetics; and (4) The unusual structure of MTs.

Tubulin has particular importance in nematodes as microtubules are targets for anthelmintic chemotherapy and also because nematodes present many unusual

structural and biochemical features It is important to determine the pattern of expression of the different tubulin family members because different tissues have different functions and behaviours. If a protein is expressed differentially one might gain insight into its function through knowledge of tissue function or behaviour. It is also necessary to determine the localization of the protein within the cell, as sorting of different isoforms of a single protein type within the cell implies that the different isoforms have different functions

Previous ultrastructural and drug binding studies have focused exclusively on intestinal MTs, and suggested that nematode intestinal tubulin is the most sensitive target of BZs. However, the mechanism or the basis of this selection has not been explained precisely in terms of heterogeneity or content of tubulin in this particular tissue No data are available on the interactions of BZs with tubulin from body wall muscle or reproductive tract of nematodes. To understand this phenomenon, characterization of tubulin from various tissues of intestinal and tissue dwelling nematodes was chosen as the basis of this study. Detailed knowledge of the heterogeneity in α- and β-subunits, isoforms and distribution of tubulins, in different species and various tissues of the same species, may help us to define more precisely, the basis of differential sensitivity of various tissues and interactions of different proteins and drugs with tubulins

The advent of hybridoma technology has added a new dimension to efforts directed towards identification, isolation and characterization of antigen and developing a specific, sensitive immuno-diagnostic test to detect filarial infections. The use of monoclonal antibodies (MAbs) rather than polyclonal serum has the advantage that a single antigen can be studied with MAbs. Similarly, the action of a single antibody against an organism can be examined using MAbs. Antibodies specific for tubulin have been employed as specific probes for study of tubulin isoforms, post-translation modifications of tubulin, molecular mapping of its functional domains and for study of the orientation of α and β subunits in microtubules in a number of species. However, there are no immunological or biochemical data on the tubulin from various tissues of *A suum*. In order to understand better the heterogeneity and

sensitivity of tubulin in this nematode, my study has focused on the production of monoclonal antibodies (MAbs) to nematode tubulin and their use in the characterization of the tubulins from intestinal and tissue dwelling nematodes *Ascaris suum* and *Brugia pahangi*.

The research objectives of this thesis were.

1) **Purification of nematode tubulin.**

To use this purified tubulin as an antigen, for the production of anti-tubulin monoclonal antibodies

2) **Production of anti-tubulin monoclonal antibodies.**

To study the heterogeneity and distribution of α and β tubulin in intestinal and tissue nematodes

3) **Identification and comparison of tubulin subunits in different nematodes and mammalian cells.**

To determine the specificity of anti-tubulin MAbs; to investigate the differences (if any) in the tubulin contents of different nematodes; and to get an indication of relative concentrations of tubulin subunits.

4) **Identification and compar son of tubulin isoforms in *A. suum* and *B. pahangi*.**

To gain some insight into the importance of α and β tubulin diversity; and to determine whether they are expressed indiscriminately in all tissues or they show differential tissue specificity.

5) **Localization and comparison of the domains of *A. suum* and *B. pahangi* tubulin to which the MAbs bind.**

To localize any functional domains that could be involved in the interaction of tubulin with other proteins and drugs or in any other specific function

6) **Compare the specific binding of mebendazole to the tubulins from body wall muscle, intestine and reproductive tract of *A. suum*.**

To determine if different tissues of *A. suum* are equally sensitive to mebendazole and to determine binding constants K_a (affinity constant) and B_{max} (maximum binding at infinite drug concentration)

7) **Effects of anti-*B. pahangi* tubulin MAbs on the viability of adult worms *in vitro*.**

To determine the biological activities of anti-tubulin MAbs.

8) **Immunohistochemical localization of tubulin in *B. pahangi*.**

To study the distribution of tubulin in the different tissues of *B. pahangi*; which may indicate some of the functional roles of tubulin in filarial worms.

9) **Detection of tubulin in the sera and peritoneal fluid of infected gerbils and sera from infected cats, dogs and humans using Dot-ELISA.**

To determine if anti-tubulin MAbs could be used as an immuno-diagnostic tool.

CHAPTER 2

CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF MONOCLONAL ANTIBODIES SPECIFIC TO *BRUGIA PAHANGI* TUBULIN

INTRODUCTION

Microtubules (MTs) are proteinaceous organelles that are implicated in a variety of cellular functions including mitosis, intracellular transport, the maintenance of cell shape and the formation of cilia, flagella and sensory organelles (Dustin, 1984) The major structural component of MTs is tubulin, which is composed of α- and β-subunits, the dimer having a molecular weight of 110 kDa Both α- and β-tubulins are expressed as heterogeneous but closely related families of multiple isoforms, in different organisms, tissues and even within single cells of the same organism (Fulton & Simpson, 1976; Gozes & Littauer, 1978, Sullivan, 1988; Birkett *et al*, 1985) The heterogeneous population of tubulin isoforms may result from both the differential expression of distinct tubulin genes and post-translational modifications (Cleveland *et al.*, 1980; Gard & Kirschner, 1985, Luduena *et al.*, 1988) It has been suggested that the diversity in tubulin isoforms may have implications for specific MT functions (Lewis & Cowan, 1988). The precise nature or role of α- and β-tubulin isoforms have not yet been elucidated, although several groups have demonstrated that many *in vivo* functions of tubulin are to some extent, isoform specific (Gundersen *et al*, 1984) Benzimidazoles (BZs), anti-mitotic and anti-fungal agents are widely used in the chemotherapy of parasitic diseases Several chemicals such as colchicine, vinblastine and BZs have been shown to bind to tubulin. BZs exert toxic effects on nematodes by

binding to tubulin and inhibiting polymerization of the heterodimer into MTs (Ireland *et al.*, 1979; Laclette *et al.*, 1980; Lacey & Prichard, 1986). BZs induce paralysis and slow growth in the free-living nematode *Caenorhabditis elegans* (Driscoll *et al.*, 1989). These drugs are potent filaricides for *B. pahangi* (Denham *et al.*, 1978) and have been shown to bind to tubulin-enriched extracts of adult *B. pahangi* and *B malayi* (Tang & Prichard, 1989). However, the precise BZs binding site has not been determined

MAbs have made it possible to recognize different domains of tubulin in different species in order to study the structure, distribution and functions of tubulin (Hesse *et al*, 1984; Warn *et al*, 1987; Vina *et al.*, 1988; Banerjee *et al.*, 1990). Tang and Prichard (1989) reported the presence of 4–5 β-tubulin isoforms in the tubulin-enriched extracts of adult *B pahangi*. In addition, immunogold studies with *B. malayi* adult and microfilariae (Mf) using anti-tubulin MAbs have revealed the presence of β-tubulin in the somatic muscle blocks beneath the cuticle, intestinal brush border and intra-uterine Mf of the adult worms (Helm *et al.*, 1989).

In this chapter, the production of MAbs raised against *B. pahangi* tubulin that react with a nematode-specific β-tubulin epitope(s), is reported. Anti-*B. pahangi* tubulin MAbs P3D and 1B6, were used: (1) to investigate the heterogeneity of tubulins from nematodes and mammals and (2) to determine their effects on the viability of adult *B pahangi* These results were compared to those obtained with anti-chick brain β-tubulin MAb 357

MATERIALS AND METHODS

Preparation of antigens

Gerbils (*Meriones unguiculatus*), 9–10 months old and previously infected intraperitoneally with 400 *B pahangi* infective larvae, were obtained from Dr. J. McCall (University of Georgia, USA). The adult worms were isolated from the infected gerbils as described by Ash & Riley (1970). *B. pahangi* (0.7 g) were harvested from the peritoneal cavities of gerbils in warm physiological saline (0.85% NaCl), washed with 0.025 M MES buffer containing 1 mM EGTA, 0.5 mM $MgSO_4$ and 1 mM GTP, and were homogenized (with a glass homogenizer with a Teflon pestle) in 7 ml of MES

buffer. The homogenate was centrifuged at 100,000 g for 1 h at 4°C The supernatant after ultracentrifugation was retained and used for further purification The same procedure was used to prepare tubulin from other filarial (*B. malayi* and *D immitis*) and non-filarial nematodes (*A suum*, BZ-susceptible and resistant strains of *H contortus*). Tubulin from pig brain was prepared by 2 cycles of polymerization-depolymerization, according to the method of Shelanski *et al* (1973) *Giardia muris* antigen was prepared as a sonicate as described by Butscher & Faubert (1988) A peptide corresponding to amino acid residues 430–448 of *B pahangi* β-tubulin, synthesized using an Applied Biosystems Peptide Synthesizer, HPLC purified, sequenced and coupled to the carrier protein, keyhole Limpet Hemocyanin (KLH) (The Alberta Peptide Institute), was also used as an antigen in ELISA

B. pahangi, B. malayi, D immitis, A. suum and *H. contortus* tubulins were partially purified using polylysine affinity chromatography (Lacey & Prichard, 1986) Five ml of supernatant after centrifugation were applied to the column and eluted sequentially with 0 025 M MES buffer, 1% $(NH_4)_2SO_4$ in MES buffer, and 5% $(NH_4)_2SO_4$ in MES buffer Fractions were collected for protein determination The protein concentration was determined by the method of Bradford (1976) using BSA as the standard. The elution profile consisted of 3 distinct peaks The first protein peak was eluted with MES buffer, the second with 1% aqueous $(NH_4)_2SO_4$, and the third with 5% $(NH_4)_2SO_4$. Fractions for each peak were pooled and concentrated separately in centriflo (Amicon) at 400 *g*. Polylysine-purified proteins were separated on SDS–PAGE, protein bands of the molecular weight corresponding to tubulin were excised, and the protein was electro-eluted (Electro-eluter, Bio-Rad) (Blose, 1984) The eluted protein was precipitated three times with 80% acetone at -20°C for 5 h and then dissolved in 0.125 M Tris-HCl (pH 6 8), 0.1% SDS and 1 mM EDTA, dialysed overnight against this buffer at 4°C and stored at -70°C until used.

Immunization and preparation of monoclonal antibodies

Six week old female BALB/c mice (Charles River Canada Inc., St. Constant, Québec) were injected subcutaneously at three week intervals with purified eluted *B.*

pahangi tubulin (100 µg/injection) using equal volumes of complete Freund's adjuvant for the first injection and incomplete adjuvant for the second injection. The third immunization of 100 µg of tubulin in PBS was administered intraperitoneally (i.p.). At this stage, mice were bled and serum was tested for anti-tubulin antibodies by Enzyme-linked immunosorbent assay (ELISA) and Western blotting. The spleen cells from the mouse giving the highest titer were fused with the non-secreting myeloma cell line, P3-x63-Ag8 653 (ATCC, Rockville, MD), as described by Hurrell (1982). Positive cultures as determined by ELISA and Western blotting, were cloned twice by limiting dilution

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described by Voller *et al.* (1976). Microtiter plates (Falcon) were coated with the polylysine-purified tubulin or an 18 amino acid peptide corresponding to the extreme C-terminal residues 430-448 of *B. pahangi* tubulin (Guénette *et al*, 1991) at a concentration of 10 µg/ml in PBS. Plates were incubated with 200 µl of 1% BSA in PBS Horseradish peroxidase-labeled anti-mouse IgG or IgM (Bio-Can, Mississauga, Ontario) at dilutions of 1:5000 and 1:20,000, respectively, was added to each well and incubated for 1 h at 37°C The substrate was (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)(Sigma) The plates were read on a Titertek multiskan plate (Flow Laboratories, Irvine, Ayrshire, UK) at 414 nm. Normal mouse serum or culture medium used to grow hybridoma cells (Iscoves modified Dulbecco's medium (IMDM) with 20% FCS, 10% NCTC 135 and HT) was used as a negative control.

Monoclonal antibodies (MAbs)

Three MAbs, all specific for tubulin, were investigated in this study. Anti-chick brain MAb 357, which cross-reacts with β-tubulins from a spectrum of eukaryotic cell types, was purchased from the Radiochemical Centre (Amersham, England) and MAbs P3D and 1B6 were raised in our laboratory against the tubulin of adult *B. pahangi*. All anti-tubulin MAbs used in this study were of IgG isotype.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were run in a Mini Protean II dual slab cell (Bio-rad, Richmond, CA) using the discontinuous system of Laemmli (1970) consisting of 4% polyacrylamide as stacking and 12% polyacrylamide as separating gels.

Isoelectric focusing and two-dimensional electrophoresis (IEF-2D SDS-PAGE)

IEF gels were prepared and run according to the method of O'Farrell (1975) as modified by Chung (1987) Briefly, IEF was performed in tube gels (1 5 x 8 cms) containing 9 5M urea (LKB) and 2% (w/v) ampholines (LKB) (1 6% pH 4–6 and 0 4% pH 3.5–10) IEF was conducted at 400 V for a period of 16 h and then at 800 V for 3 h. Electrophoresis was performed in 4% polyacrylamide stacking and 12% polyacrylamide separating gels, running at 50 V for 30 min and at 150 V for 60 min, in the Mini Protean II slab cell After 2-dimensional (2D) SDS-PAGE, gels were either stained with silver stain (Bio-Rad), or the proteins were transferred onto nitrocellulose (NC) sheets for Western blot analysis

Western blotting

After 1 and 2D SDS-PAGE, tubulin subunits, individual tubulin isoforms and peptides were electrophoretically transferred (Towbin *et al* , 1979) onto NC sheets for 2 h at 4°C. The NC sheets were cut into several strips containing an identical pattern of separated proteins To visualize protein bands, two NC strips were stained with amido black. The remaining strips were washed in PBS and incubated for 2 h at room temperature in 10% newborn calf serum (Gibco) in Tris-buffer saline (140 mM $NaCl_2$, 50 mM Tris-HCl, pH 7 4, with 0 1% (v/v) Tween 20 (TBS-T)) to saturate the unoccupied protein binding sites of NC After washings, the strips were incubated overnight at 4°C with anti-tubulin MAbs or IMDM (negative control) The NC strips were then washed 6 x 5 min with TBS-T, immersed in peroxidase-conjugated goat anti–mouse IgM or IgG (Bio-Can) diluted at 1:500 with high salt buffer (1 M $NaCl_2$, 10 mM Tris-HCl, pH 7.4; 0.5% (v/v) Tween 20 (HSB-T) with 10% NBCS), and incubated for 2 h at room temperature. After washing the NC strips with TBS-T for

30 min, the bound peroxidase was detected with the substrate 4-chloro-1-naphthol (Sigma) at 3 mg/ml in methanol/PBS, 1:5 (vol/vol), containing 0.075% of 30% hydrogen peroxide (Birkett *et al*, 1985)

Limited proteolysis of tubulin

Limited proteolysis of tubulin in gel slices was performed as described by Cleveland *et al.* (1977) and modified by Chung (1987). Gel pieces corresponding to the tubulin were cut out of the polyacrylamide gels and placed directly into the sample well of a second 15% SDS-polyacrylamide gel. Gel pieces were overlaid with one of the following proteases: α-chymotrypsin from bovine pancreas (Sigma) or *S. aureus* V8 protease (Boehringer Mannheim) The SDS-PAGE was performed at 50 V until bromophenol blue dye reached the bottom of the stacking gel and then increased to 150 V for the remainder of the electrophoresis. After SDS-PAGE, the digested peptides were either stained with silver stain or transferred onto NC sheets, in the same way as described for the Western blot analysis, and reacted either with anti-*B. pahangi* tubulin MAbs or anti-chick tubulin MAb 357.

Inhibitors

Anti *B. pahangi* β-tubulin MAbs P3D, 1B6, 1A5 (in culture medium), anti-chick β-tubulin MAb 357 (in ascites fluid) and mebendazole (MBZ) (in DMSO), a BZ anthelmintic drug, were used as inhibitors in the *in vitro* assays. Anti-*B. pahangi* MAbs P3D and 1B6 were in culture medium Iscove's Modified Dulbeco's Medium/NCTC-135 supplemented with 20% fetal calf serum (IMDM/FCS) and used undilute. However, anti-chick brain MAb 357 was in ascites fluid and was diluted to the appropriate concentration with culture medium IMDM/FCS.

Culture in vitro

B. pahangi were isolated from peritoneal cavities of gerbils, as described earlier in a sterile hood. Following isolation, *B. pahangi* were washed 5 times with sterile IMDM/FCS medium, for surface sterilization. Three wells in 24-well plates (Nunc)

were set up for each test MAb, drug and for the control cultures. To each well was added 2 ml of the appropriate test medium containing pure MAb P3D, 1B6, 1A5, 357 alone or MAb + MBZ and two adult worms. The plates were incubated at 37°C in a humidified incubator in the presence of 95% air and 5% CO_2. Worm activity was observed every two hours, and motility was assessed subjectively by observation with a naked eye. The experiment was terminated after 48 h. During the 48 h incubation the culture medium was not changed. Control medium contained an identical volume of the IMDM/FCS without MAbs or drug.

Optimization of MTT reduction assay

Previous studies (Alley *et al.*, 1988; Finley *et al.*, 1986) have demonstrated the utility of MTT-formazan colorimetry in proliferation and cytotoxicity assays in anti-cancer chemotherapy. Subsequently, Comley *et al* (1989a) demonstrated the successful application of this assay to the determination of filarial viability and its use in *in vitro* anti-filarial drug screening. MTT is pale-yellow in solution but when incubated with living cells is reduced by active mitochondria to yield a dark blue crystalline deposit (formazan) within cells, which once solubilized can be quantified colorimetrically.

Live female *B. pahangi* worms were placed in 0.5 ml of IMDM containing 0.5 mg/ml MTT and incubated at 37°C for various time intervals ranging from 0–90 min (MTT-reduction). Female worms that had previously been heat-killed were also incubated with MTT for selected time intervals over this range For each time point three replicate worms were used Following MTT incubations, worms were carefully removed and transferred to individual wells of a microtitre plate, containing 200 µl of DMSO, and allowed to stand at room temperature for 1 h (formazan-solubilization) An ELISA reader was used to measure absorbance of the formazan solution at 550 nm, using DMSO as a blank (Comley *et al.*, 1989a).

Quantification of B. pahangi viability

A three-step colorimetric assay based on MTT [3-(4,5 dimethyl(thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma), was used to assess viability of adult *B.*

pahangi (Comley *et al.*, 1989b). MTT was dissolved in PBS at a concentration of 5.0 mg/ml and subsequently diluted to 0.5 mg/ml with PBS. Female worms were incubated for 30 min at 37°C (MTT reduction). After incubation, worms were transferred to 96 well plates containing 200 µl of DMSO. The plates were allowed to stand for 1 h at room temperature (formazan solubilization). The absorbance was determined at 550 nm in the presence and absence of worm and compared with a DMSO blank. Worms were killed for control purposes by heating in PBS at 100°C for 10 min.

Statistical analysis

The data for any statistical significance, were analysed using the Dunnett's procedure or Student's t-test. P values less than 0.01 were declared significant.

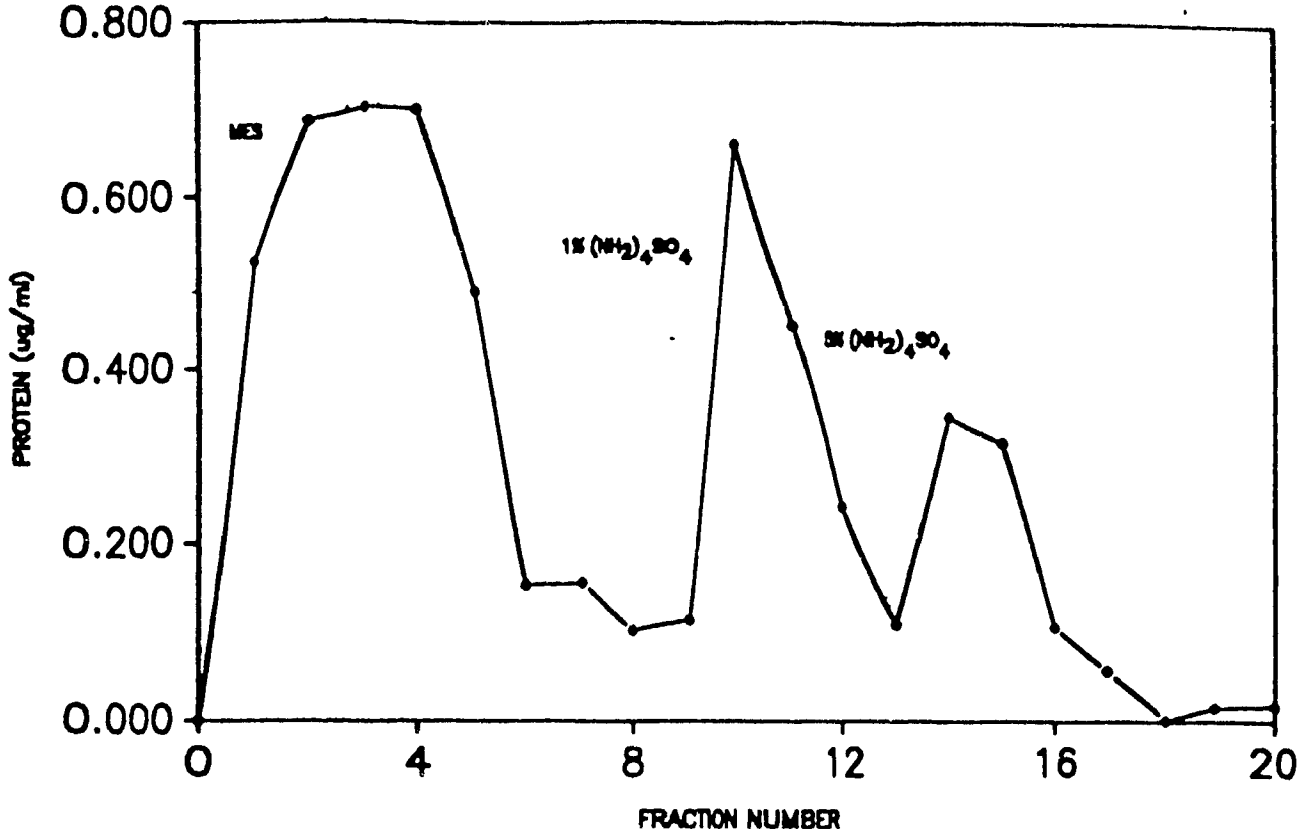
RESULTS

Preparation of antigen

After centrifugation of the adult *B. pahangi* homogenate, the supernatant of adult *B. pahangi* was subjected to partial purification on a polylysine agarose column. Several fractions were obtained from polylysine chromatography. The protein content of each fraction was determined and it was observed that the elution profile consisted of 3 distinct protein peaks. The first peak includes the elution fractions 1-8, the second peak includes the fractions 9-12 and the third peak includes the fractions 13-18 (Fig. 2.1).

The pooled protein fractions from each peak were further fractionated by SDS-PAGE, transferred onto NC and probed with commercial anti-tubulin MAbs. The results show that the third peak was enriched with tubulin, however, tubulin could not be detected in the first and second peak (Fig. 2.2). This is consistent with the previous report by Tang & Prichard (1989). Third peak proteins were concentrated and then subjected to SDS-PAGE, respectively. The tubulin band was cut out of the SDS-gels and subjected to electro-elution for further purification.

Fig. 2.1. Partial purification of *B. pahangi* tubulin by polylysine agarose chromatography. Five ml of *B. pahangi* crude homogenate in 0.025 M MES buffer was applied to a column packed with 25 ml of polylysine agarose at 4°C and eluted with a concentration gradient of $(NH_4)_2SO_4$ in MES buffer. Each fraction contained five ml eluate. The details of the assay are given in Materials and Methods.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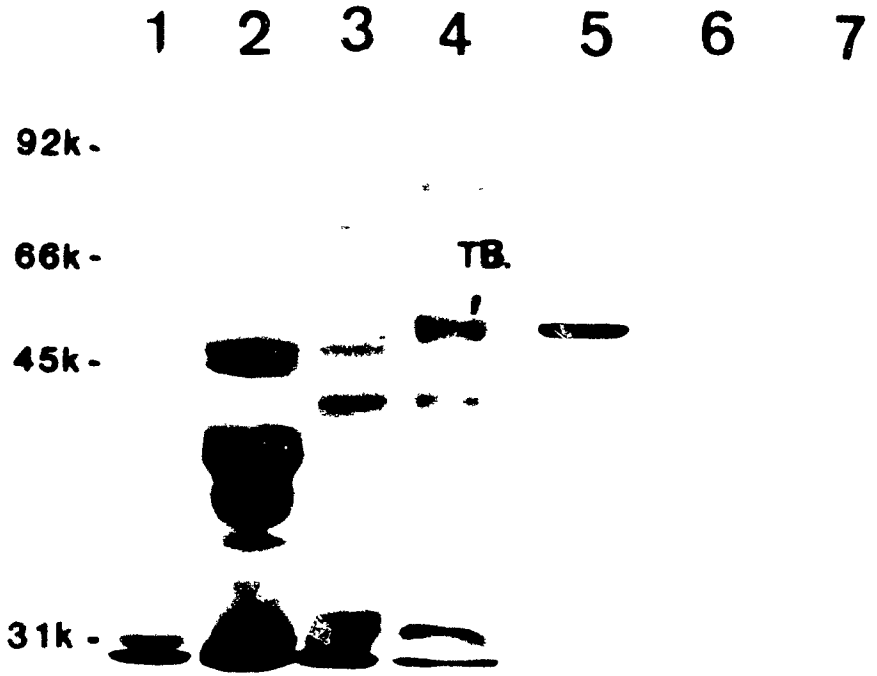
0
4
8
12
16
20
FRACTION NUMBER

Fig. 2.2. SDS-PAGE and Western blotting of *B. pahangi* total proteins fractionated on polylysine column. Lane 1, molecular weights; lane 2, first protein peak; lane 3, second protein peak; lane 4, third protein peak stained with Coomassie blue; lane 5, immunoblot of third protein peak; lane 6, second protein peak; lane 7, first protein peak; immunostained with anti-α and β tubulin MAbs. The details of the procedure are given in the Materials and Methods.


1 2 3 4 5 6 7
92k -
66k -
45k -
31k -
TB.

Monoclonal antibodies

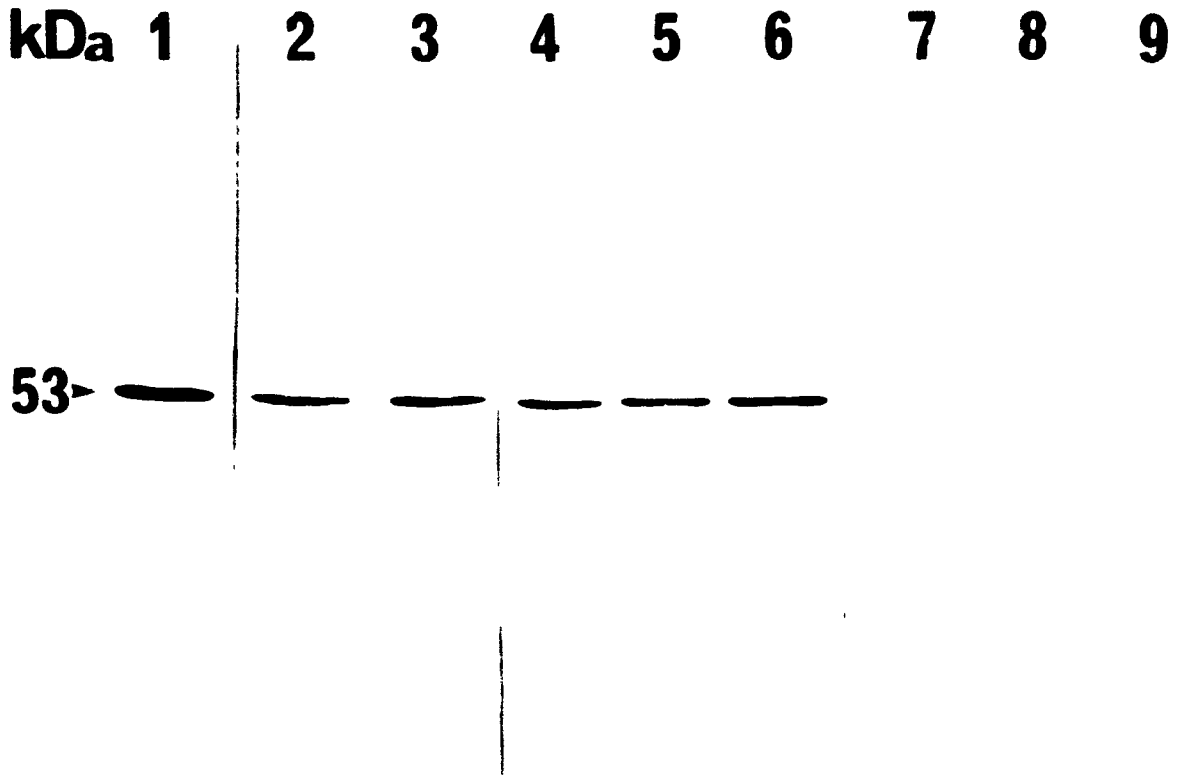
Two different isotypes of anti-*B. pahangi* MAbs were obtained. Seven out of fifty-four MAbs were polyreactive IgM, recognizing tubulin as well as other high and low molecular weight proteins, whereas the remaining MAbs represented two populations of the IgG isotype Of these, four out of fifty-four reacted with tubulin and other low molecular weight proteins; however, forty-three MAbs were specific for tubulin. MAbs P3D and 1B6 specific to nematode tubulin, were chosen for further characterization. All MAbs used in this study were of IgG isotype.

Specificity of MAbs P3D, 1B6 and 357

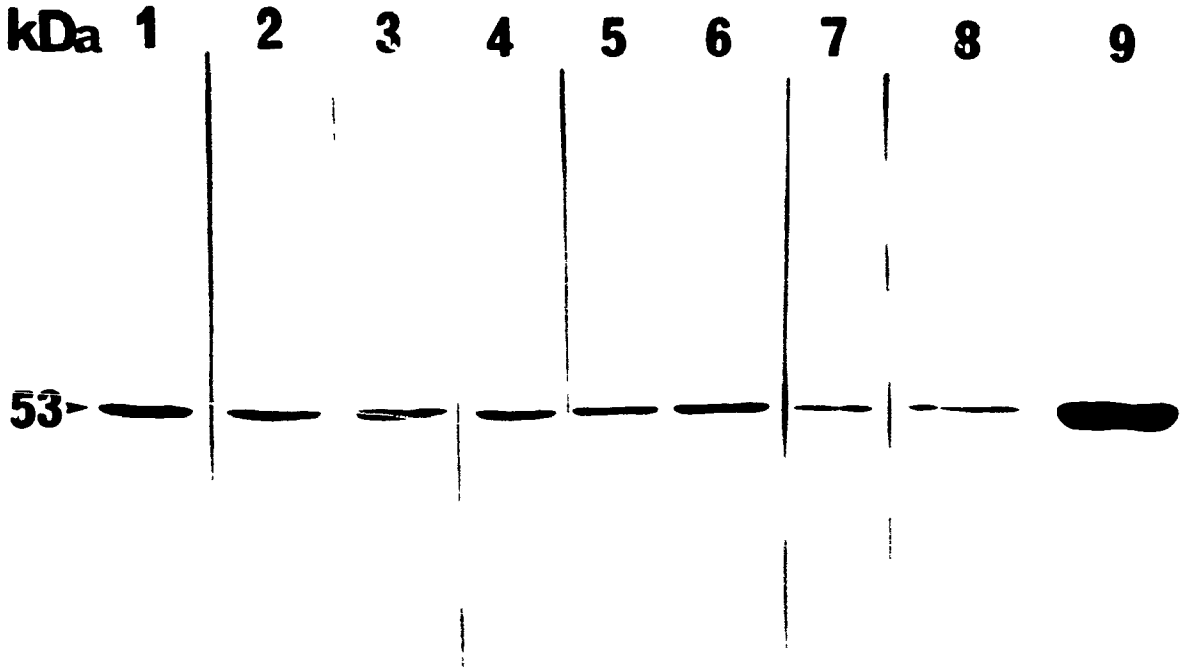
The specificity of these MAbs was investigated by determining their reactivity to proteins from a variety of filarial and non-filarial nematodes, protozoa and mammalian cells using ELISA and Western blot analysis. Using ELISA, the anti-*B. pahangi* MAbs P3D and 1B6 did not react with *G. muris* tubulin, which is recognized by anti-chick brain tubulin MAb 357. Crude and partially purified extracts of adults and microfilariae of *B pahangi*, adult *B. malayi* and *D. immitis*, eggs of *H. contortus*, adult *A. suum*, pig brain and 3T3 mouse fibroblast cell tubulins were separated on SDS-PAGE and electrophoretically transferred onto NC sheets. The blots were treated with: (1) MAb 1B6; (2) MAb P3D; and (3) MAb 357 Analysis of Amido black stained blots revealed that crude extracts of adults and microfilariae of *B. pahangi*, adult *B. malayi* and *D. immitis*, eggs of susceptible and resistant strains of *H. contortus*, adult *A. suum*, pig brain and 3T3 mouse fibroblast cell contained many bands in the tubulin region (not shown) Tubulin from the various nematodes and mammalian extracts were separated into two bands designated α and β. Anti-*B. pahangi* MAb P3D recognized specifically β-tubulin from adult and microfilariae of the filarial worms *B. pahangi, B. malayi* and *D. immitis* (Fig 2.3A, lane 1–4). It also reacted with equal intensity to tubulin from the intestinal nematode *H. contortus* (BZ-susceptible and BZ-resistant strains) (Fig. 2.3A, lane 5–6). Tubulin from *A. suum* did not show very strong reactivity with this MAb (Fig. 2.3A, lane 7), due to the weak reactivity tubulin band from *A suum* can not be seen in fig. 2.3A, lane 7. No cross-reactivity to 3T3 mouse

Fig. 2.3. SDS-PAGE and Western blot analysis of the total protein extract of adult *B. pahangi* (lane 1); *B. pahangi* microfilariae (lane 2); adult *B malayi* (lane 3); *D immitis* (lane 4); *H. contortus* BZ-susceptible strain (lane 5); *H contortus* BZ-resistant strain (lane 6); *A. suum* (lane 7); mouse fibroblast cells (lane 8); and pig brain (lane 9). Extracts were electrophoresed on a 12% SDS-polyacrylamide gel, transblotted and immunostained with anti-*B. pahangi* MAb P3D (A) and anti-chick brain tubulin MAb 357 (B). See Materials and Methods for details. Twenty-five µg of each antigen was loaded per lane.

A



B



fibroblast cells or pig brain tubulins was detected (Fig. 2.3A, lane 8–9). Similar results were obtained using MAb 1B6 (not shown), whereas, cross-reactive anti-chick β-tubulin MAb 357 recognized β–tubulin from all nematodes and mammalian cells (Fig. 2.3B, lane 1–9).

Identification of tubulin isoforms

Anti-*B. pahangi* β-tubulin MAbs P3D and 1B6, and anti–chick β–tubulin MAb 357, were used to characterize β-tubulin isoforms in *B. pahangi* tubulin. MAb P3D (Fig. 2.4A) and MAb 357 (not shown) recognized the same isoform pattern, reacting with two β-tubulin isoforms in the crude as well as partially purified extracts of *B. pahangi*, whereas, MAb 1B6 specifically recognized only one β-tubulin isoform in the extract of *B. pahangi* (Fig. 2.4B). The β-tubulin isoforms were in the pH range of 5.1–5.3.

MAb 357 probed blots were re-probed with MAb P3D and 1B6 respectively, to demonstrate that the same spots were recognized by this MAb. Furthermore, to show the full complement of β-tubulin isoforms, P3D and 1B6 probed blots were re-probed with MAb 357. The results indicated that all these MAbs recognized the same isoforms in tubulin-enriched extracts of adult *B. pahangi*. However, MAb 1B6 was specific to one isoform

Interaction of anti-tubulin MAbs with tubulin proteolytic fragments

In order to localize the antibody epitope on tubulin fragments, Western blotting experiments using anti-*B. pahangi* MAbs, following limited proteolysis of tubulin with chymotrypsin and V8 protease, were performed Western blots of peptides digested with chymotrypsin showed that MAb P3D reacted with a 21 kDa chymotrypsin fragment (Fig 2.5A, lane 2) and a 21 kDa V8 protease β-tubulin fragment (Fig. 2 5A, lane 3). In contrast, MAb 1B6 reacted with the two chymotrypsin-digested fragments of 42 and 34 kDa (Fig 2.5B, lane 2). It reacted strongly with the 42 kDa and weakly with the 34 kDa fragment However, the same MAb did not recognize any β-tubulin fragment resulting from digestion with V8 protease (Fig. 2.5B, lane 3). These results

Fig. 2.4. Western blot analysis of anti-*B pahangi* tubulin MAbs to the total protein extract (20 µg) of adult *B. pahangi* after 2D electrophoresis The gels were transblotted onto nitrocellulose paper and immunostained with MAb P3D (A) and MAb 1B6 (B).

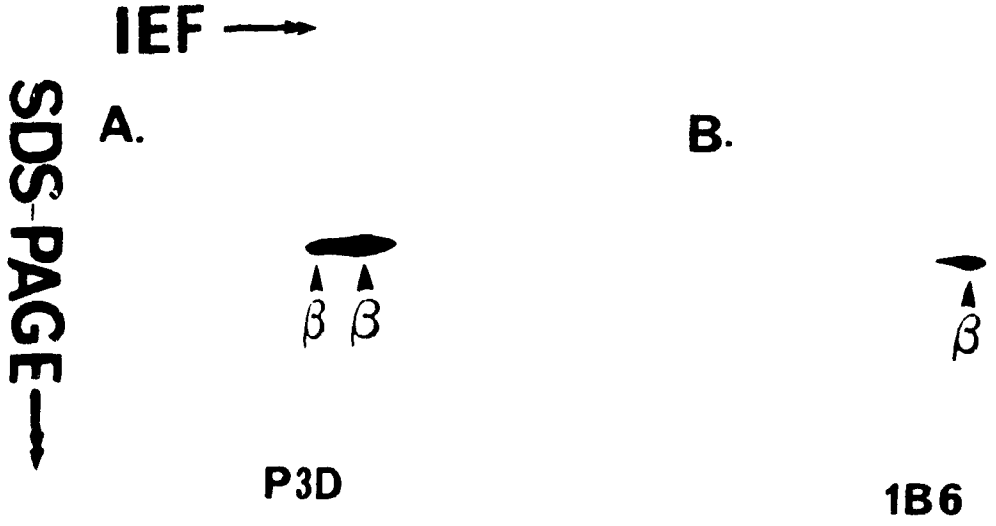
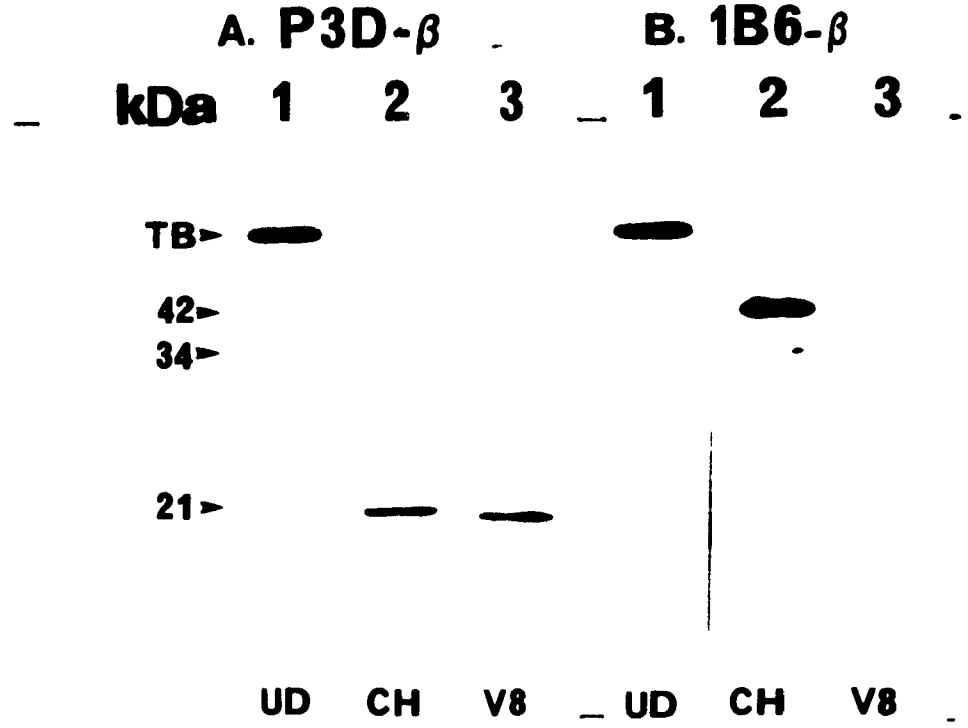

IEF
SDS-PAGE
A.
B.
β β
β
P3D
1B6

Fig. 2.5. Limited proteolysis and Western blots of *B pahangi* tubulin The proteins of adult *B. pahangi* were separated by 12% SDS-polyacrylamide gel electrophoresis After staining, the tubulin band was excised and submitted to partial proteolysis in the gel with 2 µg chymotrypsin or V8 protease/well. The peptides generated were analyzed by electrophoresis on a 15% gel. The electrophoretically separated peptides were then transferred to nitrocellulose sheets and immunostained with anti-*B. pahangi* MAb P3D (A) or MAb 1B6 (B).

UN = undigested tubulin; CH = tubulin digested with chymotrypsin; V8 = tubulin digested with V8 protease.


A. P3D-β
B. 1B6-β
kDa 1 2 3 1 2 3
TB►
42►
34►
21►
UD CH V8 UD CH V8

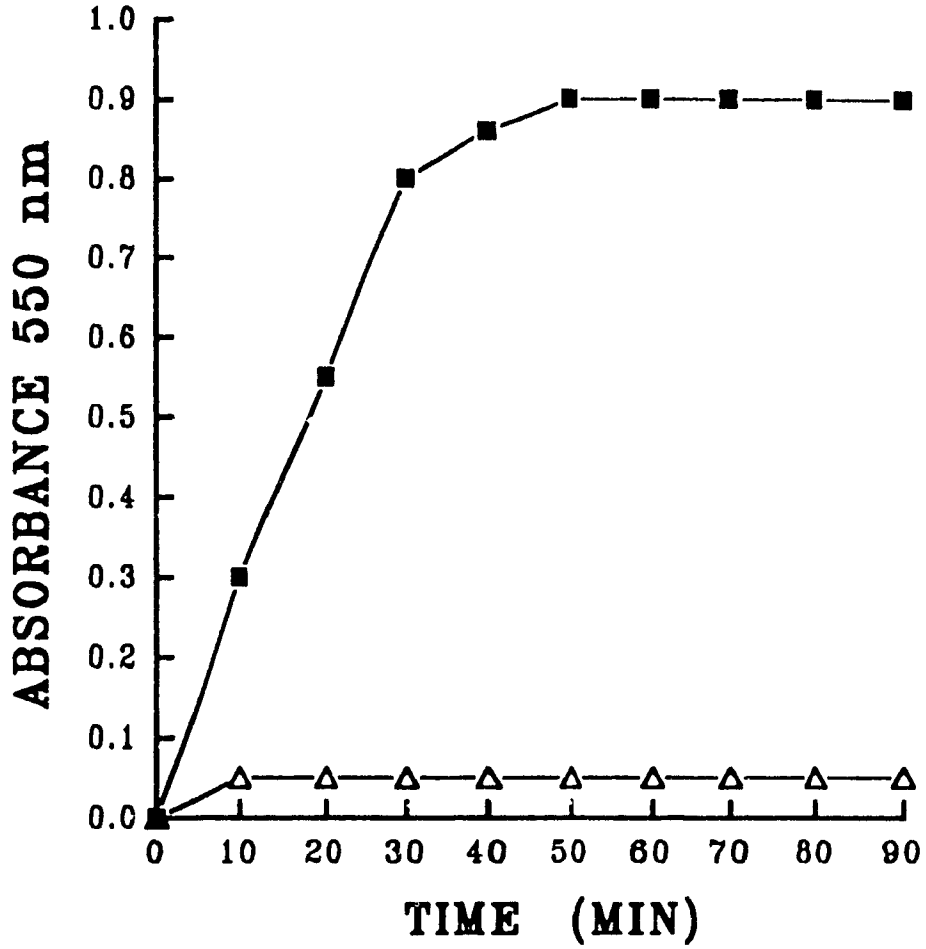
of the limited proteolysis analysis indicate that the antigenic site recognized by MAb P3D differs from that recognized by MAb 1B6. Although MAb 357 reacts strongly to intact β-tubulin from *B. pahangi*, no interaction was seen with β-tubulin fragments digested with chymotrypsin or V8 protease. Protease digestion appears to destroy the reactivity of *B. pahangi* tubulin towards MAb 357.

The silver staining showed that chymotrypsin preferentially cleaves β-tubulin into three fragments of apparent mass 42, 34 and 21 kDa but other minor fragments are also seen and the α-tubulin remained intact (not shown). This pattern of cleavage is similar for pig brain tubulin (Mandelkow *et al.*, 1985; Serrano *et al.*, 1986; Cleveland *et al.*, 1977; Vina *et al.*, 1988; Payelle-Brogard *et al.*, 1989). Small differences in the molecular weights of proteolytic fragments are explained by different conditions of proteolysis and/or gel electrophoresis. Limited proteolysis of *B. pahangi* tubulin with V8 protease also resulted in the appearance of proteolytic β-tubulin fragments of 42, 30 and 21 kDa which is in agreement with the results of pig brain proteolysis with V8 protease (Vina *et al.*, 1988). Both for chymotrypsin and V8 protease, large proteolytic fragments are N-terminal, and the small ones represent C-terminal (Mandelkow *et al.*, 1985)

Optimization of MTT reduction assay

The effects of anti–tubulin MAbs on the viability of adult *B. pahangi* were determined using the MTT assay. The selection of an optimum MTT reduction time was derived from data shown in fig. 2.6. Viable control female *B. pahangi* showed rates of formazan formation that were maximal and linear during the first 30 min of the incubation with MTT By one hour formazan formation had begun to plateaue between 60–90 min. Heat-killed worms showed only background levels of formazan formation (Fig. 2.6). Based on these results I chose 30 min as an optimum incubation time for the three-step MTT (reduction, formazan solubilization and formazan quantitation) method To determine if the presence of female worms had any effect on the absorbance values, after DMSO solubilization for 1 h, the absorbance of the resulting formazan solution was determined in the presence or absence of the worms.

Fig. 2.6. Time course of formazan formation by single female *B. pahangi* (■) after incubation for 2 h with MTT (0.05 mg/ml) in IMDM/FCS at 37°C relative to heat killed worms (100°C for 10 min) (△). (n = 3 replicates). The details of the assay are given in the Materials and Methods.


ABSORBANCE 550 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
90
TIME (MIN)

It was found that in the presence of worm there was a slight increase in the absorbance values, obtained.

Quantification of B. pahangi viability

Analysis of MTT assays demonstrated that female *B. pahangi* incubated with MAb P3D showed significant decline in their ability to reduce MTT to formazan (Fig. 2.7A) In comparison to untreated live control worms, MAb P3D alone caused a highly significant 80% reduction in worm viability, 48 h post-treatment. Worms exposed concurently to MBZ and MAb P3D caused a significant, 70% reduction in the viability of worms, compared to untreated live control worms. The high reduction in the viability of worms seems to be due to the presence of MAb P3D and not MBZ. As MBZ alone induced a minimal decrease (10%) in the viability of worms. The dose of MBZ used is sufficient to induce biochemical changes that are associated with a change in parasite viability. Exposure of worms to MAb 1B6 resulted in 40% decrease in the ability of worms to reduce MTT (Fig. 2.7B). Similar result were obtained when male worms were incubated in the presence of MAb P3D and 1B6. No significant effect on the viability of worms was obtained after incubation with anti-*B. pahangi* MAb 1A5 (Fig. 2.7C) or anti-chick β-tubulin MAb 357 (Fig. 2.7D). Direct visual observations on adult worms treated with anti-*B. pahangi* MAbs P3D alone and together with MBZ showed a detectable decrease in motility 12 hours post-treatment. Anti-*B. pahangi* MAb 1B6 alone, or in the presence of MBZ, also exhibited an apparent decline in the motility of worms. However, no mortalities were observed using these MAbs during the experiment. No noticeable reduction was observed in the motility of the worms treated with either anti-*B. pahangi* MAb 1A5 or anti-chick brain MAb 357 alone, or when they were incubated in the presence of MBZ. From these observations, it is suggested that the reduction in the worm motility is caused mainly by the anti-*B. pahangi* MAbs alone, since MBZ alone did not have any effect on the motility of the worms, nor did it enhance the effect of MAbs.

Fig. 2.7A. The effects of anti-*B. pahangi* tubulin MAb P3D on the viability of adult female *B. pahangi in vitro*. Worms were incubated for 48 hours at 37°C in IMDM/FCS in the presence of anti-*B. pahangi* MAb in the presence or absence of MBZ. The untreated controls were incubated with the culture medium IMDM/FCS alone. Worm viability was assessed by MTT assay as described in the Materials and Methods. Six worms were used in each determination and the experiment was performed three times. Data shown in this figure are mean absorbance values per worm and S.E.M. ($P < 0.01$).

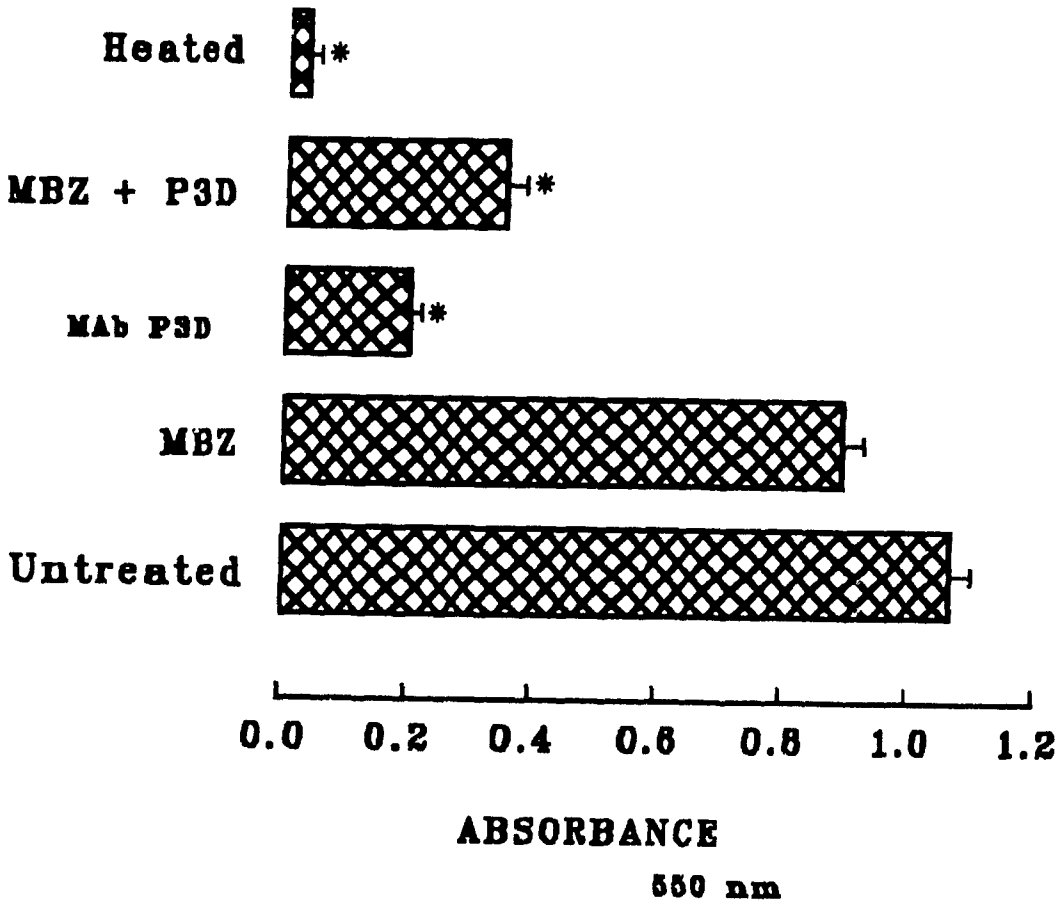

Heated
*
MBZ + P3D
*
MAb P3D
*
MBZ
Untreated
0.0
0.2
0.4
0.6
0.8
1.0
1.2
ABSORBANCE
550 nm

Fig. 2.7B. The effects of anti-*B. pahangi* MAb 1B6 on the viablity of female *B. pahangi in vitro*. Experimental protocol and statistical analysis are identical to those detailed in the legend to Fig. 2.7A.

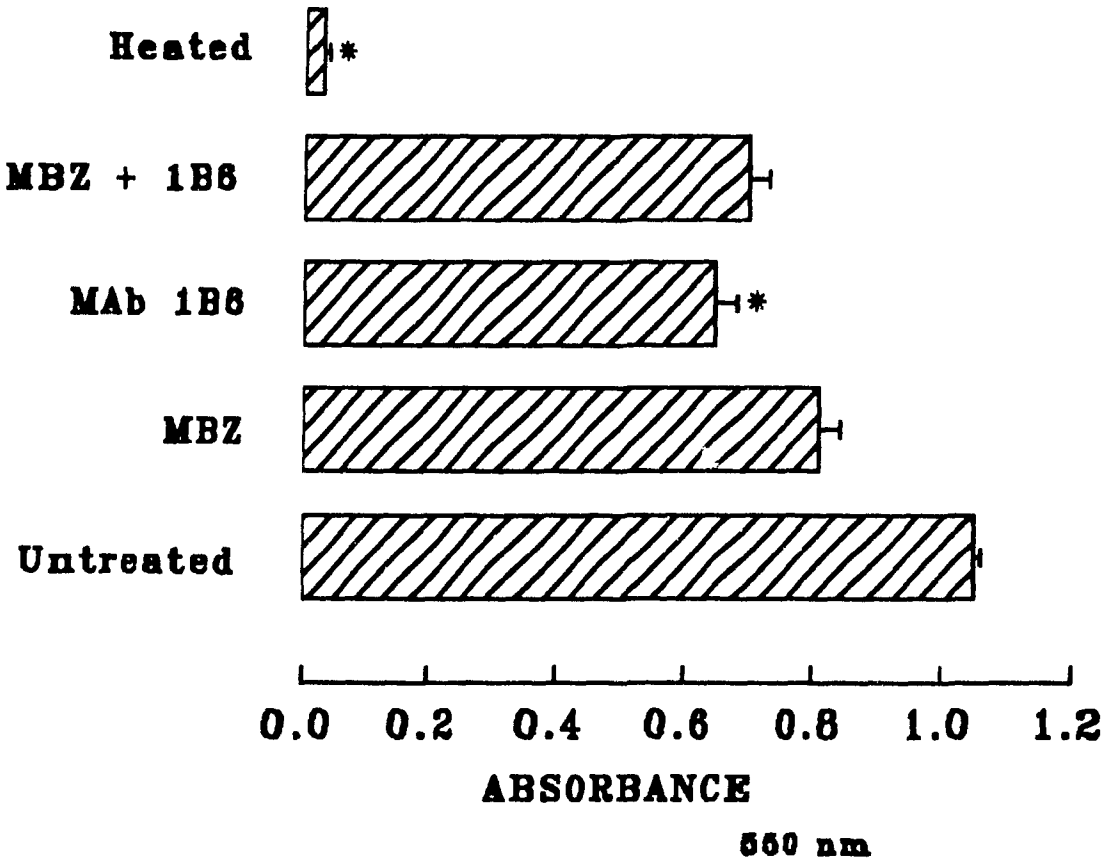

Heated
*
MBZ + 1B6
MAb 1B6
*
MBZ
Untreated
0.0
0.2
0.4
0.6
0.8
1.0
1.2
ABSORBANCE
550 nm

Fig. 2.7C. The effect of anti-*B. pahangi* tubulin MAb 1A5 on the viability of female *B. pahangi in vitro*. The details of the assay are given in the Materials and Methods section and statistical analysis are identical to those detailed in the legend to fig 2.7A.

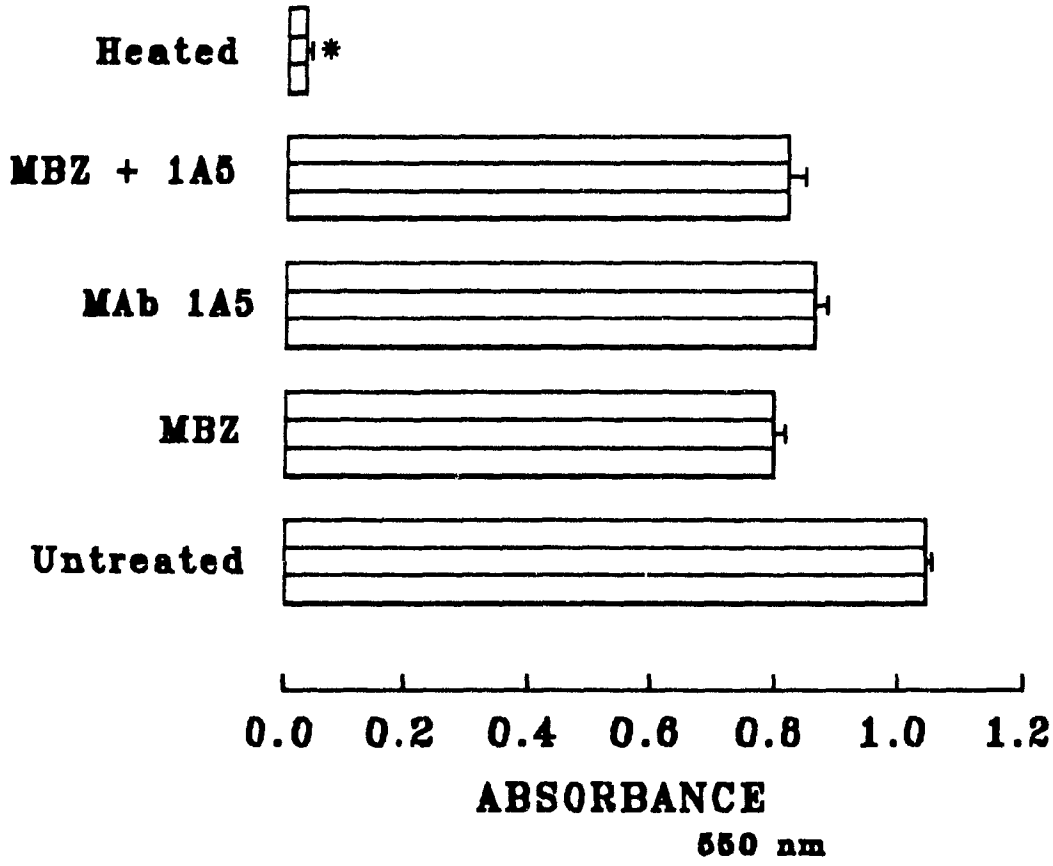
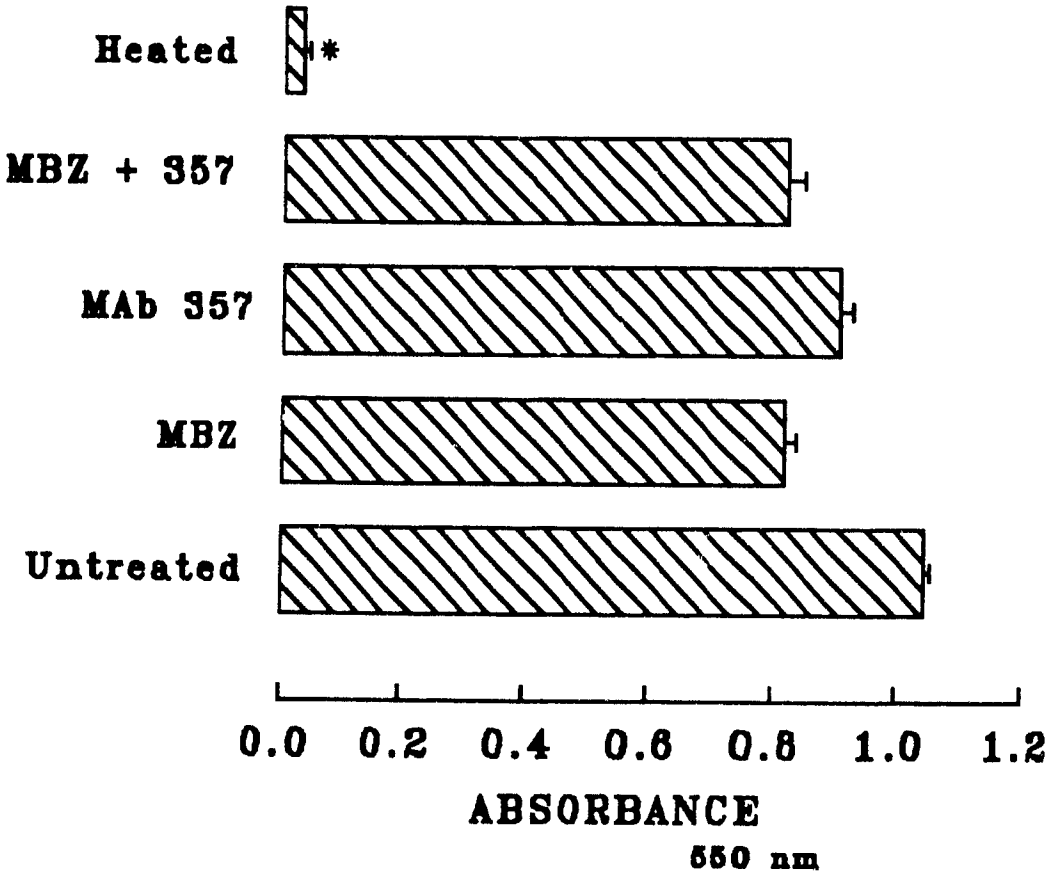

Heated
*
MBZ + 1A5
MAb 1A5
MBZ
Untreated
0.0
0.2
0.4
0.6
0.8
1.0
1.2
ABSORBANCE
550 nm

Fig. 2.7D. The effect of anti-chick brain tubulin MAb 357 on the viability of female *B. pahangi in vitro*. The details of the assay are given in the Materials and Methods section and statistical analysis are identical to those detailed in the legend to fig. 2.7A.


Heated
*
MBZ + 357
MAb 357
MBZ
Untreated
0.0
0.2
0.4
0.6
0.8
1.0
1.2
ABSORBANCE
550 nm

DISCUSSION

In this chapter, is reported the production and characterization of MAbs raised against *B. pahangi* tubulin. In total, fifty-four anti-*B pahangi* tubulin MAbs were obtained after immunization of mice with purified *B pahangi* tubulin Because of their remakable specificity for tubulin, MAbs P3D and 1B6 have been selected for more extensive characterization. Western blot analysis of one-dimensional SDS-PAGE showed that anti-*B. pahangi* MAbs P3D and 1B6 recognized tubulin from a number of filarial nematodes (*B. pahangi, B malayi* and *D. immitis*) and an intestinal nematode (*H contortus*). However, the same MAbs did not cross-react with tubulin from pig brain, 3T3 mouse fibroblast cells or the parasitic protozoan *G. muris* On the other hand, anti-chick MAb 357 reacted with pig brain, 3T3 mouse fibroblast and *G muris* tubulins as strongly as it did with filarial and other nematode β-tubulins These results indicate that anti-*B pahangi* tubulin MAbs recognize an epitope(s) that is conserved between filarial and intestinal nematode β-tubulin but not in protozoan and mammalian β-tubulin, whereas cross-reactive anti-chick MAb 357 recognizes an epitope that is conserved among filarial and intestinal nematodes as well as protozoan and mammalian β-tubulin The epitope of MAb 357 has been localized to a region of β-tubulin between amino acid 339-417 in the proteolytic fragments of pig brain tubulin (Serrano *et al.*, 1986). These data suggest that anti-*B. pahangi* tubulin MAbs are highly specific to nematode tubulin

In previous studies, several anti-tubulin MAbs raised against parasitic protozoa and nematodes have been characterized, but all of them have been found to cross-react with tubulin from other species. For example, Draber *et al* (1985) reported a MAb raised against pig brain tubulin which reacted with microtubules from mammals, bird, amphibian, fungi, echinoderm, platyhelminth, slime moulds, but not protozoan tubulin. Similarly, Birkett *et al.* (1985) generated an anti-β-tubulin MAb against *Physarum myxamoebae* which reacts with β-tubulin from various fungi, algae, higher plants, avian, insect and several mammalian sources In addition, Helm *et al.* (1989) have raised MAbs against microfilariae of *Brugia* species. Contrary to my anti-*B. pahangi* β-tubulin MAbs, their MAbs cross-reacted with mammalian tubulin.

The 2D gel analysis of crude and partially purified *B. pahangi* extracts revealed that anti-*B. pahangi* tubulin MAbs interacted only with the isoforms of the β-subunit of tubulin, and not with the α-subunit or microtubule-associated proteins. MAb 1B6 appears to be specific to one β-tubulin isoform whereas MAb P3D shows an isoform pattern similar to the one observed with the anti-chick β-tubulin MAb 357. Moreover, MAb P3D recognized a similar isoform pattern as anti-peptide antibody raised against the 18 amino acid sequence of the extreme C-terminus region of *B. pahangi* β-tubulin (Guénette *et al.*, 1991). MAb 357 and the anti-peptide antibody recognize two β-tubulin isoforms in *B. pahangi* extract.

Limited proteolysis of *B. pahangi* tubulin followed by Western blot analysis showed that the anti-*B. pahangi* MAb P3D reacted with the C-terminal fragment of the chymotryptic and V8 protease digests. It has been suggested by Mandelkow *et al.*, (1985) that the strong reactivity with the C-terminal fragments could be due to the fact that this region is exposed in native tubulin. The *B. pahangi* β-tubulin contains the chymotrypsin recognition site at amino acid 281 (Guénette *et al.*, 1991), thus confirming the conserved chymotryptic digestion pattern. MAb 1B6 reacted with the N-terminal fragments of the chymotrypsin-digested β-tubulin. However, with V8 protease proteolytic fragments, no reaction was seen and it reacted only with the undigested tubulin. Using MAb 357 in a similar study, no proteolytic fragments were found to react. Yet MAb 357 recognized undigested β-tubulin, suggesting that the epitope is destroyed by proteolytic cleavage. The results observed with MAb 357 are in agreement with those obtained by Mizuno *et al.* (1985), using V8 protease-digested fragments of sheep brain and mung bean β-tubulin.

Measurement of the *in vitro* activity of anti-*B. pahangi* tubulin MAbs P3D, 1B6, 1A5 and anti-chick brain tubulin MAb 357 against female *B. pahangi* was carried out to determine whether anti-*B. pahangi* or anti-chick tubulin MAbs could independently cause any damage to the intact adult worms. MBZ was included in the study to determine whether the presence of MBZ drug alone or in conjunction with MAbs had any differential effect. The results in this study demonstrated qualitatively a decline in the motility, when the worms were cultured with anti-tubulin MAbs P3D

and 1B6. However, no noticeable reduction in the motility was observed, when the worms were treated with anti-*B. pahangi* MAb 1A5, anti-chick MAb 357, MBZ or IMDM/FCS culture medium without antibodies. The viability of the worms was determined by MTT assay. Anti-*B. pahangi* MAb P3D and 1B6, significantly reduced the viability of adult *B. pahangi in vitro*. However, no significant reduction in the viability was observed when adult *B. pahangi* were exposed to anti-*B. pahangi* MAb 1A5, anti-chick MAb 357 and /or MBZ.

The validity of assays based on the metabolism of MTT for the quantitative staining of living cells is well established (Finley *et al.*, 1986; Franz, 1988). Observations with *B. pahangi* and *D. viteae* have shown that MTT uptake and metabolism occurs in all developmental stages of these worms (Comley *et al.*, 1989a). In contrast, the developmental stages of *H. contortus* and *T. colubriformis* failed to show any formazan formation (Comley *et al.*, 1989b) These data strongly suggest that filarial species may be unique amongst nematodes in their ability to take up MTT. This observation is consistent with the different morphology of the filarial cuticle (Franz, 1988) and the increased permeability relative to the cuticle of other parasitic nematodes. Comparison of an adult filarial and an adult intestinal nematode shows that filarial worms have a much smaller intestine and less musculature in the body wall, whilst the lateral cords of hypodermal tissues are expanded to subtent a large proportion of the sub-cuticular area (Howells, 1987). The outer plasma membrane of the hypodermis, which forms a basal membrane to the cuticle, is much folded in filarial worms and possesses characteristics of an absorptive surface (Howells, 1980). Correlated with the absorptive function of the cuticle, acid phosphatase activity is present in the hypodermal tissues of adult *B. pahangi* but not in the intestine. Adult *B. pahangi* has been shown to be able to take up glucose, amino acid and certain filaricidal compounds through the cuticle (Howells & Chen 1981). Damage to the cuticle could render the worm more permeable, but this might upset the ionic balance of the tissues, render the parasite more vulnarable to the host's immune response and increase the penetration of certain anthelmintic drugs and chemicals (Ottesen, 1987). Dick & Wright (1973) demonstrated microtubule-like structures in the cuticle of a non-

filarial pin worm, *Syphacia obvelata*. Subsequently, Rogers *et al.* (1974) reported the presence of tubulin between the hypodermis and the basal stratum of the cuticle, where it may serve a morphological function or may function in intracellular transport or nutrition.

It is suggested that the mechanisms by which the anti-*B. pahangi* β-tubulin MAbs reduce the viability of adult worms, may be due to their interference with microtubules in the body wall muscle of *B. pahangi*. For example, (a) the antibody may interfere with certain microtubule functions, but not with cellular distribution. This has been found for a MAb raised against tyrosinated α-tubulin of rat (Kilmartin *et al.*, 1982). When complexed with colloidal gold particles and injected into PtK2 cells, the antibody blocked the saltatory movement of such particles along individual particles (Wehland *et al.*, 1983), (b) antibodies may block several MT-dependent motility processes, by aggregating MTs. Saltatory movement of endogenous organelles and chromosomal separation were inhibited by anti-tubulin MAbs in the PtK2 cells, (c) the antibody may interfere with cellular architecture by disrupting links between MTs and other cytoplasmic structures; the intermediate filament network collapsed into perinuclear caps, and the Golgi apparatus disintegrated into dispersed vesicles (Blose *et al.*, 1984), (d) the antibody may interfere with microtubular integrity by binding to epitopes on the tubulin molecule which are essential for microtubule polymerisation or maintenance of structure. In a recent study, Draber *et al.* (1990) reported that five MAbs against N-terminal domains of tubulin, inhibited the assembly of tubulin into microtubules *in vitro* from porcine brain. Similarly, Mandelkow *et al.* (1985) reported that some polyclonal anti-tubulin antibodies were able to inhibit tubulin assembly *in vitro*.

Reduction in the viability of adult *B. pahangi* worms, by antibodies raised against an internal or non-surface protein is an interesting finding. These data of an internal protein being able to have a significant inhibitory effect, are in agreement with the study of Pearce *et al.* (1988). The authors have demonstrated that non-surface schistosome antigen, paramyosin, when administered intra-dermally, conferred significant resistance against *Schisotoma mansoni* challenge infection in mice. Their

study shows that non-surface antigen can be protective.

In previous studies, *in vitro* assays have been used to evaluate the importance of antibodies in the resolution of filariasis Tan *et al.* (1989) demonstrated that two MAbs against *B. malayi* L3 antigens decreased larvae motility and caused surface damage in vitro independently of buffy coat cells. Parab *et al.* (1988) reported the development of a MAb against L3 of *B. malayi* that promoted destruction of the larvae by mouse macrophages. Antigens with molecular weight of 80, 67, 52 and 36 kDs were recognized by this MAb and have been identified in the somatic extracts of *B malayi* L3.

MAbs specific for the α- or β-subunit of tubulin would allow the subcellular localization and the function of each subunit to be studied. Proteins of the size of tubulin are generally built of several structural domains that have distinct functions. In the case of tubulin, such functions include binding of anti-microtubule drugs, GTP or microtubule-associated proteins and the association between monomers, dimers or protofilaments. Nematode-specific anti-tubulin MAbs may serve to characterize the structure and distribution of the *B. pahangi* tubulin molecule, and to define microtubule stability and functional domains.

CONNECTING STATEMENT

In chapter 2, the production and characterization of MAbs raised against *B. pahangi* tubulin was reported. It was demonstrated that anti-*B. pahangi* MAbs recognized β-tubulin from a number of filarial and intestinal nematodes however, they did not cross-react with mammalian tubulin. Two MAbs P3D and 1B6 were selected for more extensive characterization. MAb P3D recognized two and MAb 1B6 recognize one β-tubulin isoform in *B. pahangi* whole worm extract. Limited proteolysis analysis revealed that MAb P3D reacted with the C-terminal, whereas, MAb 1B6 reacted with the N-terminal fragment of β-tubulin. Measurement of viability of adult *B. pahangi* revealed that anti-*B. pahangi* MAbs significantly reduced the viability of worms *in vitro*.

After obtaining these interesting results, anti-*B. pahangi* MAbs were assessed for their ability to detect β-tubulin (antigen) in the sera and peritoneal fluid from infected gerbils, and sera from infected cats, dogs and human. These MAbs were also used to localize the distribution of β-tubulin isoforms in adult *B. pahangi*. The data of this study are reported in the following chapter 3.

CHAPTER 3

DETECTION OF β-TUBULIN IN ANIMAL AND HUMAN FILARIASIS: USE OF ANTI-β-TUBULIN SPECIFIC MONOCLONAL ANTIBODIES FOR IMMUNO-DIAGNOSIS

INTRODUCTION

Lymphatic filariasis caused by *B. malayi* and *W. bancrofti* is a major health problem in tropical countries manifesting a broad spectrum of acute and chronic clinical features (Partono, 1987) Improved methods for diagnosis of active filarial infections are needed to identify pre-clinical infections, monitor control efforts and to evaluate new drugs (WHO, 1984). Diagnostic methods based on microfilarae (Mf) detection in peripheral blood have some practical and biological limitations. For example, Mf detection is inconvenient because of nocturnal periodicity of Mf; and it is not useful for detection of amicrofilaremic infections.

Although host antibodies to filarial adult and microfilaremic antigens of molecular weight in the range of 15-110 kDa are detected in most patients (Weil *et al.*, 1984; Dissanayake *et al.*, 1984), the serodiagnosis of filariasis based on the detection of antibodies is not always accurate and valid because (1) the presence of antibodies indicates exposure to the parasite but does not reflect the presence or extent of active infections; and (2) the problems of specificity due to the extensive antigenic cross-reactivity among nematode parasites (Ambroise-Thomas, 1984).

The difficulties encountered with parasitological and antibody-based serological methods for diagnosis of active filarial infections have led to the development of immunodiagnostic tests based on the detection of circulating parasite antigens in the

sera or other body fluid. Studies of human onchocerciasis and lymphatic filariasis, as well as animal lymphatic filariasis, have reported the presence of circulating antigens and immune complexes during the patent stages of infection (Des Moutis *et al.*, 1983; Weil *et al.*, 1984; Dissanayake *et al.*, 1982). Several studies have demonstrated the use of a parasite antigen detection for diagnosis and quantitation of filarial infections (Santhanam *et al* , 1989; Weil & Liftis, 1987; Forsyth *et al.*, 1985; Weil *et al.*, 1990; Wenger *et al.*, 1988); and for monitoring the efficacy of therapy (Forsyth & Mitchell, 1984; Weil *et al.*, 1986; Weil, 1988; More & Copeman, 1991a,b; Chandrashekar *et al.*, 1991).

Howard *et al.* (1987) reported the presence of high levels of anti-tubulin antibodies in sera from patients with leprosy, chronic hepatitis, leishmaniasis, african trypanosomiasis, schistosomiasis and onchocerciasis. In a similar study, Helm *et al.* (1989) reported that antibodies to tubulin can be detected in sera taken from patients with lymphatic filariasis (asymptomatic microfilaraemic, microfilaraemic with symptoms, amicrofilaraemic with chronic disease, as well as in sera from endemic residents with neither microfilaraemia nor disease manifestations). Highest levels of anti-tubulin antibodies were found in the amicrofilaraemic cases. The presence of anti-tubulin antibodies in the sera of infected animals, have raised the question of whether filarial tubulin can be detected as a circulating antigen in the sera of infected animals and as a non circulating antigen in the peritoneal fluid of infected animals.

In this study, two MAbs P3D and 1B6 raised against *B. pahangi* tubulin and one MAb 357 against chick brain, specific to β-tubulin were selected as a probe for detecting tubulin in the sera of infected animals. These MAbs were selected for this study because Western blot analysis of one dimensional SDS-PAGE showed that MAbs P3D and 1B6 recognized tubulin from a number of filarial (*B. pahangi*, *B. malayi* and *D. immitis*) and non-filarial nematodes (*H. contortus*). The same MAbs did not react with tubulin from pig brain, mouse fibroblast cells or the parasitic protozoan *Giardia muris*. Sequence analysis of *B. pahangi* β-tubulin has indicated some divergence from mammalian β-tubulins (Guénette *et al.*, 1991). The anti-chick MAb 357 recognize a conserved epitope on β-tubulin and cross-reacted with β-tubulin from all nematodes,

mammals and protozoa. It seems evident that anti-*B. pahangi* MAbs recognized epitope(s) that are conserved between filarial and an intestinal nematode β-tubulin but not in protozoan or mammalian tubulin (Chapter 2).

Chapter 3 reports on the further characterization of these MAbs. Anti-*B. pahangi* and anti-chick brain β-tubulin MAbs were assessed for their ability to: (1) detect β-tubulin in the sera and peritoneal fluid of infected gerbils and sera from infected cats and dogs using Dot-ELISA, and (2) to localize the distribution of β-tubulin in *B. pahangi*.

MATERIALS AND METHODS

Preparation of antigens

Gerbils (*Meriones unguiculatus*) previously infected intraperitoneally with 400 *B. pahangi* infective larvae, were obtained from Dr. J. McCall (University of Georgia, U.S.A.). Individual serum were collected by bleeding from the retro-orbital sinus from the gerbils at the time of the necropsy. The adult worms were isolated from the infected gerbils as described by Ash & Riley (1970). Adult and Mf of *B. pahangi* were harvested from the peritoneal cavities of gerbils in warm physiological saline (0.85% NaCl). Mf were separated from peritoneal cells by density gradient centrifugation (Gusmao *et al.*, 1981). Soluble antigens of adult *B. pahangi* were extracted as previously described (Chapter 2). The protein concentration was determined using the method of Bradford (1976), using BSA as the standard.

Peritoneal fluid

Peritoneal lavage was prepared from fluid recovered after intraperitoneal injection of 10 ml IMDM into the peritoneal cavity of CO_2-killed infected and uninfected gerbils. Peritoneal fluid was centrifuged at 3000 x g for 10 min. The supernatant was retained and used in Dot-ELISA.

Monoclonal antibodies (MAbs)

Three MAbs, all specific for tubulin, were used in this study. Anti-chick brain

MAb 357, which cross-reacts with β-tubulins from a spectrum of eukaryotic cell types, was used as a positive control in this study. This MAb 3~7 was purchased from the Radiochemical Centre (Amersham, England) and MAbs P3D and 1B6 were raised by me against the tubulin of adult *B pahangi* as described previously (Chapter 2).

Sera

Three groups of sera were analysed: (1) four sera from (microfilaraemic) gerbils infected with *B. pahangi*, (3) three sera from cats (amicrofilaraemic) infected with *B. malayi*, and (4) four sera from dogs (microfilaraemic) infected with *B. pahangi*. Control All sera were stored at -70°C until used. Serum from infected and un-infected cats and dogs were provided by Dr. J. McCall (University of Georgia, U.S.A.).

Infected and uninfected animal sera were tested using Dot-ELISA with and without heat treatment. Heat treatment was performed to release antigens from immune complexes and to inactivate interfering proteins before performing the Dot-ELISA. For heat treatment, one part serum and one part of 0.1M Na_2-EDTA (disodium (ethylene-dinitrilo)-tetraacetic acid) pH 7.5 were mixed and boiled for 7 min. The samples were centrifuged at 16 000 x g for 5 min. After centrifugation, the supernatant was retained and used in Dot-ELISA assays (Weil, 1988).

Dot-ELISA

Dot-ELISA was performed to detect tubulin (antigen) in the sera and peritoneal fluid of gerbils and sera from cats and dogs. All infected and uninfected sera and peritoneal fluid were diluted with PBS in the range of 1:10-1:640. Nitrocellulose paper (NC) (Bio-Rad) was cut into strips. 10 µl of sera, peritoneal fluids were placed on the NC strips, dried for 20 min and then incubated overnight at 4°C in a solution of 10% newborn calf serum in Tris-buffered Saline (140 mM Tris-HCl, pH 7.4, with 0.1% (v/v) Tween (NBCS-TBS-T)), to saturate the unoccupied protein sites. After washing, the strips were incubated overnight at 4°C with anti-tubulin MAbs or controls. The strips were washed 5 x 5 min with TBS-T, then immersed in horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Can), diluted 1:500 with NBCS-TBS and

incubated for 3 h at 4°C. After washing, the NC strips with TBS-T for 30 min, the bound peroxidase was detected by adding the substrate 4-chloro-1-naphthol (Sigma) at concentration of 3 mg/ml of methanol/PBS 1:5 (vol/vol) containing 0.075% of hydrogen peroxide (Sigma) (Birkett *et al.*, 1985) The development of a well-defined purple dot on the NC strips was considered positive. In order to demonstrate the specificity of the Dot-ELISA, the following negative and positive controls were used. (a) normal sera + MAb; (b) IMDM + MAb; (c) tubulin + MAb; and (d) conjugate + substrate.

Electron microscopy: Fixation and embedding

In order to localize β-tubulin in adult *B. pahangi* at the ultrastructural level, female *B. pahangi* were cut into small pieces. These fragments were fixed in 0.5% gluteraldehyde-4% formaldehyde in 0.1 M sodium phosphate buffer pH 7.2 for 2 h at room temperature. Samples were washed for 30 min with sodium phosphate buffer (3 x 10 min), dehydrated in a graded series of ethanol (25%, 50%, 75%, 95% and 100%) (10 min each) at 4°C and then infiltrated with L R White resin (medium grade) (J.B EM., Montreal, Canada). The L R White resin was polymerized at 55–60°C for 36 h in gelatin capsules (J.B. EM., Montreal, Canada) that were completely filled and covered, to exclude air. Thin sections approximately 80 nm were cut with a glass knife on a Reichert ultramicrotome (Hayat, 1972; Armbruster *et al.*, 1983)(E.M. Centre, McGill University).

Immunogold labelling

The sections were mounted onto nickel grids and blocked with 0.1M phosphate buffer, pH 7.4 containing 10% NBCS and 0.25% (v/v) Tween-20 for 1 h at room temperature to saturate the non-specific protein binding sites. Sections were incubated with anti-tubulin MAb P3D, 1B6 or 357, for 8-10 h at room temperature followed by 2 h incubation with 1:10 dilution of 20-nm colloidal gold labelled Protein-A at room temperature (Sigma). All sections were stained with 5% uranyl acetate for 10 min, washed with distilled water, examined and photographed with an electron microscope

(Philipps 410 TEM, E.M. Centre, McGill University). Sections were washed 5 x 5 min with TBS-T, between each incubation. Negative controls consisted of sections treated with IMDM culture medium, or an irrelevant anti-*Giardia muris*, IgG isotype MAb, substituted for anti-B. pahangi MAb followed by an incubation with Protein-A colloidal gold, or the sections were incubated with Protein-A colloial gold alone. Anti-chick brain MAb 357 was used as a positive control.

RESULTS

Dot-ELISA

In this study, Dot-ELISA with anti-tubulin MAbs P3D, 1B6 and 357 was used to detect β-tubulin (antigen) in sera and peritoneal fluid from *B. pahangi* infected gerbils, sera from *B. malayi* and *B. pahangi* infected cats and dogs, respectively. All positive gerbil sera and peritoneal fluid samples reacted at the dilution of 1:80. All positive cat sera gave reactions at 1:10 and positive dog sera reacted at 1:20.

Anti-tubulin MAbs P3D, 1B6 and 357 recognized tubulin from the sera and peritoneal fluid of *B. pahangi* infected gerbils. All MAbs reacted with equal intensity to the tubulin from the infected sera and peritoneal fluid from four different gerbils as they did with the tubulin from partially purified *B. pahangi* extracts (Table 3.1A), similar results were obtained with the sera and peritoneal fluid from all four gerbils. Therefore, in this chapter the results obtained with sera and peritoneal fluid from one gerbil is shown. Tubulin was also detected in the sera from infected dogs (Table 3.1B) and cats (Table 3.1C), using MAb P3D, 1B6 and 357. All sera and peritoneal fluid were used in three replicates and experiments were repeated twice. The specificity of this assay was very good. No false positive results were observed in sera and peritoneal fluids from uninfected gerbils or sera from uninfected cats or dogs (Table 3.1A-C) with any of the three anti-tubulin MAbs.

Immunogold electron microscopy

In this study, immunogold electron microscopy was performed with anti-*B. pahangi* MAb P3D, 1B6 and anti-chick MAb 357, to demonstrate the anatomic location

Table. 3.1A. Dot-ELISA analysis of anti-tubulin MAbs P3D, 1B6 and 357, to detect β-tubulin in sera and peritoneal fluid from *B. pahangi* infected and uninfected gerbils Sera and peritoneal fluid was assayed from four infected gerbils. However, in this figure reactivity of sera and peritoneal fluid taken from one gerbil is shown; since the other three gave the same results. All gerbils were infected on 15-6-1990 with 400 *B. pahangi* third stage larvae and the sera and peritoneal fluid was collected on 10-5-91. Details of the assay are given in Materials and Methods section.

A

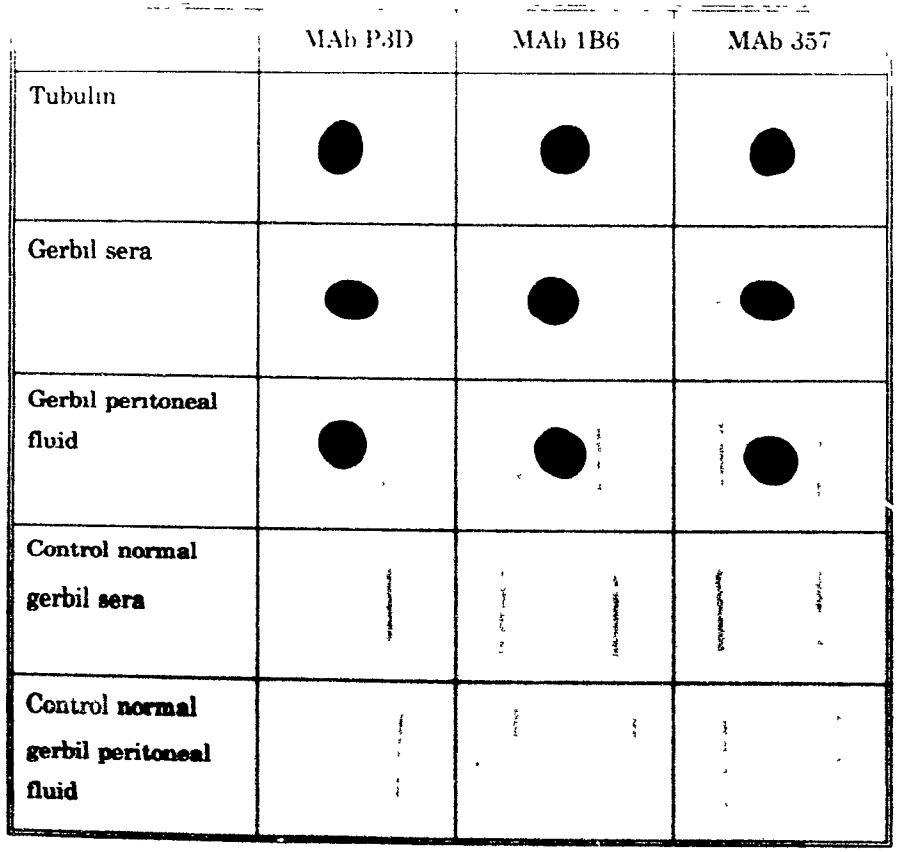

MAb P3D
MAb 1B6
MAb 357
Tubulin
Gerbil sera
Gerbil peritoneal fluid
Control normal gerbil sera
Control normal gerbil peritoneal fluid

Table. 3.1B. Dot-ELISA analysis of anti-tubulin MAbs P3D, 1B6 and 357, to detect β-tubulin in sera from *B. pahangi* infected and uninfected dogs. Sera was assayed from four infected dogs, however, the reactivity of sera from one dog is shown, as the other three gave the same results. All dogs were infected on 12-8-1989 with 200 *B. pahangi* third stage larvae and the sera was collected on 27-6-1990. Details of the assay are given in Materials and methods.

B

	MAb P3D	MAb 1B6	MAb 357
Dog sera			
Control normal dog sera			

Table. 3.1C. Dot-ELISA analysis of anti-tubulin MAbs P3D, 1B6 and 357, to detect β-tubulin in sera from *B. malayi* infected and uninfected cats. Sera was assayed from three infected cats. The reactivity of only one sera with anti-tubulin MAbs is shown. All cats were infected on 11-6-1987 with 200 *B. malayi* third stage larvae and the sera was collected on 27-6-1990. Details of the assay are given in Materials and Methods section.

C

	MAb P3D	MAb 1B6	MAb 357
Cat sera			
Control normal cat sera			

of the epitope and the possible origin of β-tubulin.

The ultrastructure of adult *B. pahangi* and *B. malayi* have been described previously, with particular reference to the body wall and somatic musculature (Rogers *et al.*, 1974). The authors demonstrated that the exterior covering of these filarial nematodes is a thick extracellular layer termed the cuticle, synthesized by the underlying hypodermal tissue (Martinez-Palomo, 1978), which is organized as an undivided syncytium. The cuticle is divided into three distinct layers, termed as basal, median and cortical, in order of distance from the hypodermal plasma membrane (Bird, 1980). The hypodermis is a thin cellular layer between the cuticle and the somatic musculature. The bulk of the hypodermis consists of longitudnal cords that separate the somatic muscles into longitudnal fields. The cords contain most of the nuclei of the hypodermis. Somatic muscle cells are spindle shaped and are arranged longitudinally with the long axis of the nematode. Each somatic muscle cell is composed of a basement membrane adjacent to the hypodermis, contractile muscle and the fibers attached to the basement membrane and a sarcoplasmic or afibrillar area enclosing the nucleus (Inglis, 1983).

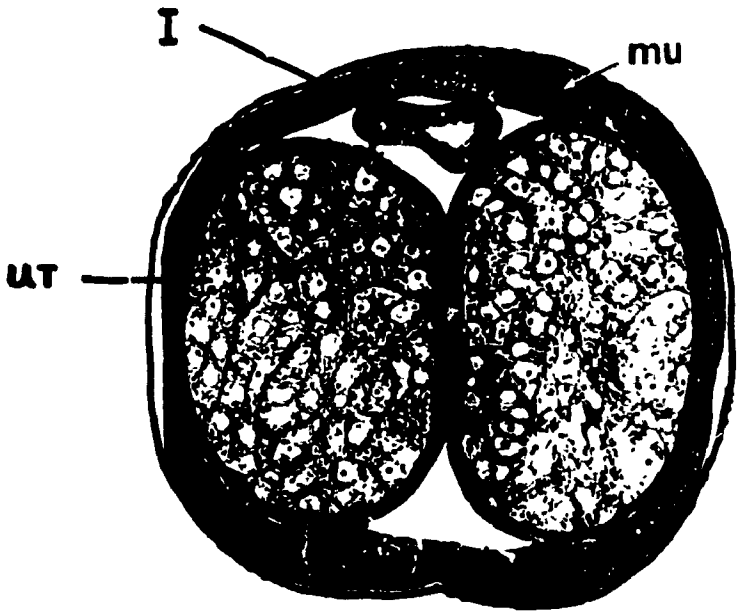
Immunogold staining of female *B. pahangi* sections with MAb P3D revealed that the β-tubulin isoforms were distributed uniformly as distinct clusters along the MTs, in various sites. Cross-sections of *B. pahangi* demonstrated that large amounts of gold particles were present in the median and basal layers of cuticle (Fig. 3.2), the gold particles represent the presence of β-tubulin isoforms recognized by MAb P3D. Tubulin was also demonstrated in the hypodermal layer, fibrillar portion of muscle cells (Fig. 3.3) and afibrillar portion of muscle cells (Fig. 3.4). Small amount of gold particles were clearly observed in the sub-cuticular upfolds and the somatic tissues of the uterus containing embryonic microfilariae (Fig. 3.5). However, no gold staining was detected in the intestine with anti-*B. pahangi* MAb P3D. The results suggest that although the epitope is present in the adult *B. pahangi*, it is strongly represented in the cuticle and somatic musculature. The specificity of the immunogold assay was confirmed by using several negative controls. No gold particles were observed when *B. pahangi* sections were incubated with IMDM or anti-*G. muris* or Protein-A

Fig. 3.2. Immunogold localization of β-tubulin (antigen) in the cuticle of female *B. pahangi*. (A) Cross-section showing the morphology of female *B. pahangi;* (B) Electron micrograph of sections of *B. pahangi* immunostained with anti-*B. pahangi* tubulin MAb P3D followed by incubation with Protein A linked with 20-nm colloidal gold. Gold particles indicate the distribution of β-tubulin in the various layers of cuticle of *B. pahangi*. Details of the assay are given in the Materials and Methods section. Fig. 3.2 A is taken from Franz *et al.* 1990.

Magnification: x 65 880.

I = intestine, UT = uterus, mu = muscle layer, T = tubulin, CL = cuticular layer, ML = median layer, BL = basal layer, H = hypodermal.

A



B

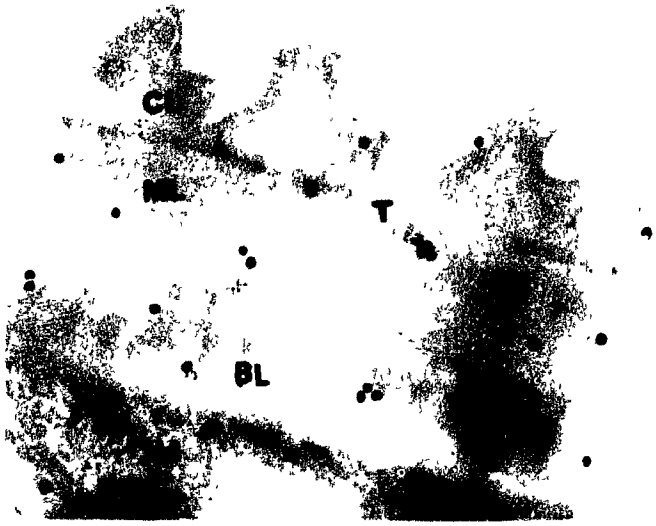


Fig. 3.3. Electron micrographs of somatic muscle cells of female B. pahangi. Sections were immunostained with anti-*B. pahangi* MAb P3D followed by incubation with Protein A linked with 20 nm colloidal gold. Gold particles indicate the distribution of β-tubulin in the fibrillar muscle cells of the body wall of *B. pahangi*. The experimental protocol is similar to that explained in the legand of fig. 3.2.

Magnification: 65 880.

BL = basal layer; H = hypodermis; FMC = fibrillar muscle cells.


BL
H

Fig. 3.4. Electron micrographs of somatic muscle cells of female *B. pahangi*. Sections were immunostained with anti-*B. pahangi* MAb P3D. Gold particles indicate the distribution of β-tubulin in the fibrillar portion and afibrillar portions of body wall of *B. pahangi*. Details of the assay are given in the Materials and Methods section. Magnification: x 56 160.

FMC = fibrillar muscle cells, AMC =afibrillar muscle cells.

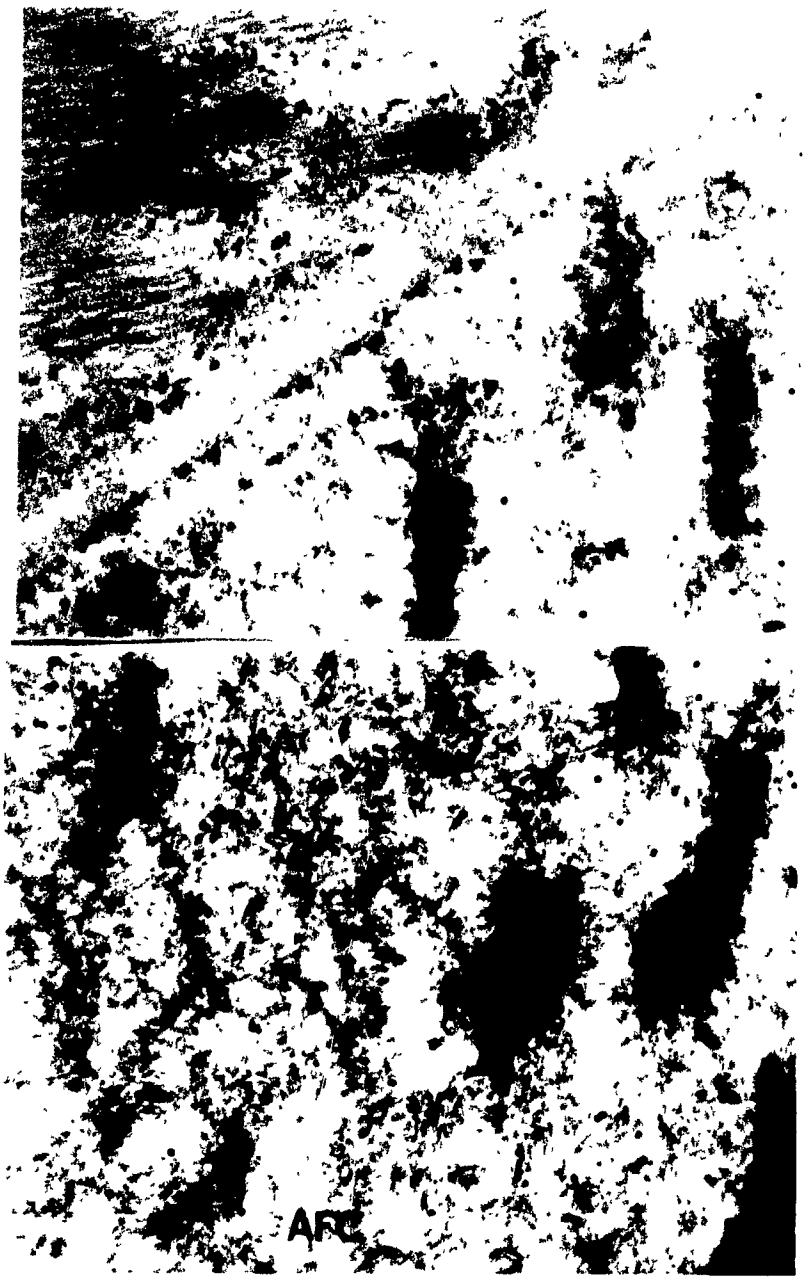

AFC

Fig. 3.5. Electron micrograph of the uterus (containing embryonic microfilaraie) of female *B. pahangi*. Sections of *B. pahangi* were immunostained with anti-*B. pahangi* tubulin MAb P3D and followed by incubation with Protein-A linked with 20-nm colloidal gold particles. Gold particles are clearly seen in the sub-cuticular upfolds, cell nuclei and the somatic tissue of the uterus.

Details of the assay are given in the Materials and Methods.

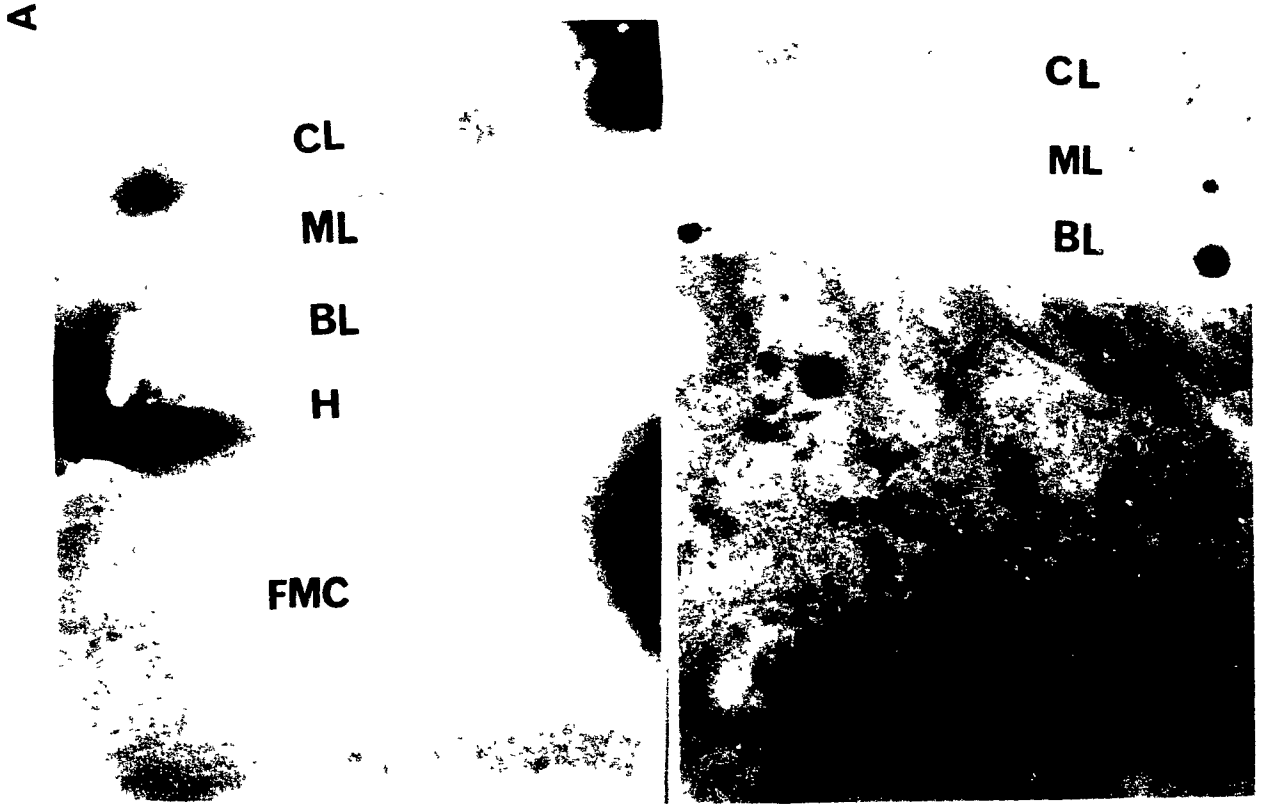
Magnification: x 65 880.

SU = subcuticular upfolds; N = nucleus, ST =somatic tissues.

Fig. 3.6. Electron micrograph of body wall (A) and somatic muscle cells (B) of female *B. pahangi* treated with either IMDM (A) or with an irrelevant MAb (B, upper) followed by colloidal gold-conjugated Protein-A or with colloidal gold-conjugated Protein-A alone (B, lower). The non-specific reaction could not be detected in the body wall and muscle cells of *B. pahangi* treated with these negative controls. Details of the assay are given in the Materials and Methods section.

Magnification: (A) upper, x 56 160, lower, x 39 420; (B) x 56 160.

CL = cortical layer; ML = median layer; BL = basal layer; FMC = fibrillar muscle cells; AMC = afibrillar muscle cells.


A
CL
ML
BL
H
FMC
CL
ML
BL

B

conjugated with colloidal gold alone (Fig. 3.6A,B). All assays performed in this study were repeated three times and were reproducible. All three anti-tubulin MAbs produced similar results, therefore the results obtained with MAb P3D are shown in this chapter.

DISCUSSION

In a previous study, Tang & Prichard (1989) reported that tubulin accounts for 2.9% of soluble protein in *B. pahangi* extracts. Payelle-Brogard *et al.* (1989) and Helm *et al.* (1989) independently reported the presence of high levels of anti-tubulin antibodies in sera from patients with onchocerciasis and lymphatic filariasis.

The present study focused on identification of β-tubulin (antigen) in the sera and peritoneal fluid of *B. pahangi* infected gerbils and in the sera of *B. pahangi* infected dogs and *B. malayi* infected cats with anti-tubulin MAbs, using Dot-ELISA. All gerbil sera, peritoneal fluid and dog sera from *B. pahangi* infected animals reacted strongly with anti-tubulin MAbs The strong reactivity of the gerbil sera, peritoneal fluid and dog sera may be due to the continuous release of tubulin from damaged or dead adults or circulating Mf in the sera. In my study, the gerbils infected with 400 third stage larvae of *B. pahangi* demonstrated higher levels of circulating and peritoneal fluid antigen than cats or dogs or humans. These observations are in agreement with the previous study on gerbils infected with *B. malayi*, in which parasite antigen levels correlated with the numbers of third stage larvae used to infect the animals (Weil, 1988)

The immunocytochemical staining method, employed in this study, using colloidal gold conjugated to Protein-A as a probe, seems to be a powerful tool for investigating intracellular antigens in such a way that in routine immuno-cytochemical studies, the antibody cannot have access to it. Although the number of antigenic moieties involved in immunoreaction on the cut surface of the sections is supposedly small, the immunogold technique was sensitive enough to detect such weak reaction.

Previously, Dick & Wright (1973) demonstrated microtubule-like structures in

the cuticle of a non-filarial pinworm, *Syphacia obvelata*. In another study, Roger *et al.* (1974) reported the presence of tubulin between the hypodermis and the basal stratum of the cuticle. In this study, immunogold staining of female *B. pahangi* revealed the presence of β-tubulin in the median and basal layers of cuticle, hypodermal layer, somatic muscle cells and developing microfilariae.

Howell & Chen (1981) demonstrated that atypical methods of nutrient uptake occur in filarial nematodes, where the nutrients are taken up transcuticularly rather than via the mouth and intestine. It was postulated that the intestine of the filarial nematodes is non-functional and that the cuticle serves as an absorptive surface. Therefore, it is suggested that the MTs found in the cuticle and body wall muscle of *B. pahangi* may play a morphological function or may function in intracellular transport or nutrition, since these functions are routinely performed by MTs in the functional intestines of *A. suum, A. galli* etc. The presence of β-tubulin as observed in this study, in the cuticle and somatic musculature of *B. pahangi* could serve as a source of circulating or peritoneal fluid filarial β-tubulin.

It is believed that a MAb P3D-based Dot-ELISA could be useful as a specific and potentially practical test for detecting active infection of *B. malayi* and *O. volvulus*. Detection of *B. malayi* tubulin with Dot-ELISA in conjuction with anti-*B. pahangi* β-tubulin MAb P3D, could be considered as a useful addition to the diagnosis of lymphatic filariasis, leading to improved understanding of the epidemiology of filariasis.

CONNECTING STATEMENT

In the following chapter 4, characterization of α and β tubulin from intestine, body wall muscle and reproductive tract of *A. suum* is reported. In this chapter, the sensitivity of tubulins from intestine, body wall muscle and reproductive tract to mebendazole anthelmintic drug have been determined. Tubulin contents in these tissues have also been compared, to determine whether differential sensitivity of the intestinal, body wall muscle and reproductive tract tubulin to mebendazole drug was associated to differential density of tubulin in these tissues.

CHAPTER 4

INTERACTION OF MEBENDAZOLE-ANTHELMINTIC DRUG WITH TUBULIN FROM BODY WALL MUSCLE, INTESTINE AND REPRODUCTIVE TRACT OF *Ascaris suum*.

INTRODUCTION

Benzimidazole carbamates (BZs) have been introduced as fungicides (Delpi & Klopping, 1968), as anthelmintics (Van Den Boosche, 1982; Lacey, 1990), and as anti-tumor drugs (Attasi & Tagnon, 1975; Lacey, 1988). Several studies have proposed that a possible mode of action of BZs is to bind to tubulin and promote the disassembly of microtubules (MTs) (Friedman & Platzer, 1978;1980; Kohler & Bachmann, 1978;1981). Although the biological activity of BZs is based on its interference with the formation and functioning of MTs, not all organisms or tissues are equally sensitive to BZ compounds. The molecular basis of selectivity of the BZs is as yet unknown. The selective action of BZs is likely to be a complex mixture of: (1) differential sensitivity of the tubulin; (2) the dynamics of MTs assembly and (3) pharmacokinetics.

The curative action of mebendazole (MBZ) in animals as well as in human helminthiases implies selectivity with respect to host and parasite. Ultrastructural studies in *A. suum*, *Trichostrongylus colubriformis* and *Ascaridia galli* have shown that upon treatment of their respective hosts with MBZ, cytoplasmic and spindle MTs in cells of these nematodes are completely destroyed. On the other hand, MTs of the host cells remain unaffected (Borgers & De Nollins, 1975; Sangster *et al.*, 1985; Atkinson *et al.*, 1980). Davis & Gull (1983) studied the protofilament number in *Ascaridia galli* and *Trichostrongylus colubriformis* and found that *A. galli* contained 11 protofilament MTs and *T. colubriformis* contained 11, 12, and 14 protofilament

MTs. These nematodes lacked 13 protofilament MTs which are common among mammalian cells. The authors postulated that the selective toxicity of the BZs could be due to the differences in protofilament number found in mammals and nematodes.

Selective toxicity of BZs to intestinal tissues of the parasite has also been reported. Several investigators in their *in vivo* studies reported that the degenerative modifications in the absorptive cells leading to the disappearance of MTs, were first observed in intestinal cells of several nematodes such as, *B malayi*, *L. carinii*, *Syngamus trachea* and *A. suum* (Borgers & De Nollin, 1975; Franz *et al.*, 1990a,b) These studies suggest that intestinal tubulin of the nematodes is the most sensitive target of the BZs attack; and the primary mechanism of action of BZs is to bind to tubulin and disrupt the tubulin-MTs equilibrium.

In vitro studies demonstrated that MBZ is capable of binding to purified mammalian brain tubulin and inhibiting MT assembly (Ireland *et al.*, 1979; MacRae & Langdon, 1989; Lacey, 1990). Friedman & Platzer (1980) demonstrated that MBZ binds to tubulin in cytosolic extracts from *A. suum* embryos and the affinity of this nematode tubulin towards MBZ appears to be more than two orders of magnitude higher than that of bovine brain tubulin. Kohler & Bachmann (1981) demonstrated that the *in vitro* incubation of the *A. suum*, in the presence of MBZ resulted in a complete loss of colchicine binding ability of intestinal extracts Biochemical evidence supported the identification of the colchicine binding receptor in *A suum* intestinal extracts as on tubulin. In a subsequent study, Barrowman *et al.* (1984) reported that all BZs and their metabolites bind to intestinal tubulin from *A. suum* The binding of BZs and their inactive metabolites, to *A. suum* tubulin was measured by the inhibition of colchicine binding by intestinal extracts of the nematode. Subsequently, Dawson *et al.* (1984) and Barrowman *et al* (1984) demonstrated the inhibition of polymerization of intestinal tubulin from *A. galli* and *A suum*. All of these studies have demonstrated that intestine is the most sensitive target of BZs However, the mechanism or the basis of this selective toxicity has not been explained precisely in terms of differential drug binding, heterogeneity or content of tubulin in different tissues No data are available on the interaction of BZs with tubulin from body wall muscle or reproductive

tract of *A. suum*.

The first aim of this study was to determine if the tubulins in the intestine, body wall muscle and reproductive tract of *A. suum* were equally sensitive to MBZ. MBZ binding has been resolved into high affinity or specific binding and low affinity or non-specific binding and the binding constant such as K_a (binding affinity) and B_{max} (maximum binding at infinite ligand concentration) determined. Due to the limited availability of labelled MBZ, only saturation assays are reported for this ligand. The second aim of this study was to compare the tubulin content in the intestine, body wall muscle and reproductive tract of *A. suum*, using electron microscopy, to determine whether differences in the sensitivity of the tubulin in intestine, body wall muscle and reproductive tract to MBZ were related to the tubulin content.

MATERIALS AND METHODS

Preparation of parasite extract

Adult *A. suum* were isolated from the intestines of experimentally infected pigs into Minimum Essential Medium (MEM) salt solution and washed with 0.025 M MES buffer containing 1 mM EGTA, 0.5 mM $MgSO_4$ and 1 mM GTP. The worms were dissected and the intestine and reproductive tracts removed. For partial purification of *A. suum* tubulin, body wall muscle, intestine and reproductive tract was washed with MES buffer and homogenized separately in 10 ml of MES buffer per gram of tissue (1 ml/100 mg tissue). The homogenate was centrifuged at 100 000 g for 1 h at 4°C (Chapter 2). The resultant supernatant was retained and used in binding studies.

Mebendazole Binding assays

The MBZ binding assays were performed as described previously (Lacey & Prichard, 1986; Tang & Prichard, 1989). To determine the binding constants K_a (binding affinity) and B_{max} (maximum binding at the infinite ligand concentration), [^{3}H]MBZ binding of *A. suum* intestine, body wall muscle or reproductive tract (total binding) was separated into high affinity binding (HAB) and low affinity binding (LAB).

Since BZ carbamates are insoluble in water, therefore the stock concentration of unlabelled MBZ was prepared in pure DMSO, and the stock concentration of [^{3}H]MBZ (Dr. Lacey, CSIRO, Australia) was prepared in 20% (v/v) DMSO in MES buffers. For the binding assay, 10 µl of pure DMSO or 10 µl of 200 µM unlabelled MBZ were pre-incubated in two replicates with 80 µl of either MES buffer (blank), intestine, body wall muscle and reproductive tract supernatants, respectively, for 30 min at 37°C in Eppendorf tubes in a shaking water bath. Then 10 µl of 0 12-5 µM stock [^{3}H]MBZ were added to each tube and incubated for a further 30 min It was assumed that the high concentration of the unlabelled MBZ will block all the high affinity receptors for MBZ. Consequently, in tubes containing the unlabelled drug, the [^{3}H]MBZ bound to the low affinity receptors while in the tubes without unlabelled drug, [^{3}H]MBZ bound to both low affinity binding (LAB) and high affinity binding (HAB) receptors. Following the incubation, the assay was stopped by adding 500 µl (10 mg/ml) of animal charcoal (Fisher) containing 1% BSA (Sigma) to make a final volume of 0 6 ml. This charcoal suspension was incubated at 37°C for 10 min to absorb the free [^{3}H]MBZ (Sherline *et al.*, 1974). The charcoal suspension was routinely prepared fresh and stirred constantly during use. The charcoal was sedimented by centrifugation at 13 000 g for 5 min at room temperature. After centrifugation, 0.45 ml of the resultant supernatant (containing bound drug) was added to 4.5 ml diluted scintillant PCS (Amersham) and toluene (Fisher) (PCS/toluene = 2/1) and counted by liquid scintillation counter (Rack Beta, LKB). Some experiments were carried out at 4°C in which depolymerization of MTs to free tubulin was promoted, experiments were performed as described above except before starting the binding assays, the tubulin samples were incubated at 4°C for 24 h.

The raw data, corrected to dpm was transformed into pmol/mg protein The HAB for MBZ was calculated by subtracting LAB from total binding. HAB is the difference between total and LAB. The bound drug (dpm) was determined and TB and LAB calculated as follows:

$TB = (dpm_{1a} - dpm_{1b}) \times 1.3$

$LAB = (dpm_{ua} - dpm_{ub}) \times 1.3$

Where dpm_{ls} is the dpm of sample (protein) and dpm_{lb} is the dpm of blank (protein free), incubated with the labelled ligand only, dpm_{us} and dpm_{ub} is the dpm of sample and blank, respectively, incubated with the unlabelled and labelled ligand; and 1.3 is the correction factor for the fraction of the assay counted (0.45 ml out of 0.6 ml final assay volume was counted) (Lubega & Prichard, 1991). The data were transformed into pmoles/mg protein as follows:

pmole (mg protein)$^{-1}$ = net dpm/[protein] x specific activity.

HAB was then calculated as follows:

HAB = TB - LAB

Saturation assay

Saturation assays were carried out as described above except that the concentrations of [^{3}H]MBZ were varied while the protein concentration (180 µg protein/assay) and the unlabelled MBZ concentration remained fixed (20 µM MBZ/assay).

Calculation of K_a and B_{max}

K_a (binding affinity) and B_{max} (maximum binding at infinite ligand concentration) were calculated by two computer programs EBDA and LIGAND (Munson & Rodbard, 1980; McPherson, 1983), as previously described by Lubega & Prichard (1990; 1991). EBDA was utilized to obtain initial estimates of K_a and B_{max} and to predict the binding sites (1 site versus 2 or more sites). Then the initial estimates and the predicted model were fed to LIGAND to calculate final estimates of K_a and B_{max}.

Statistical analysis

The binding data was analysed statistically using Student's t-test, to evaluate the significance of difference of means.

Electron microscopy

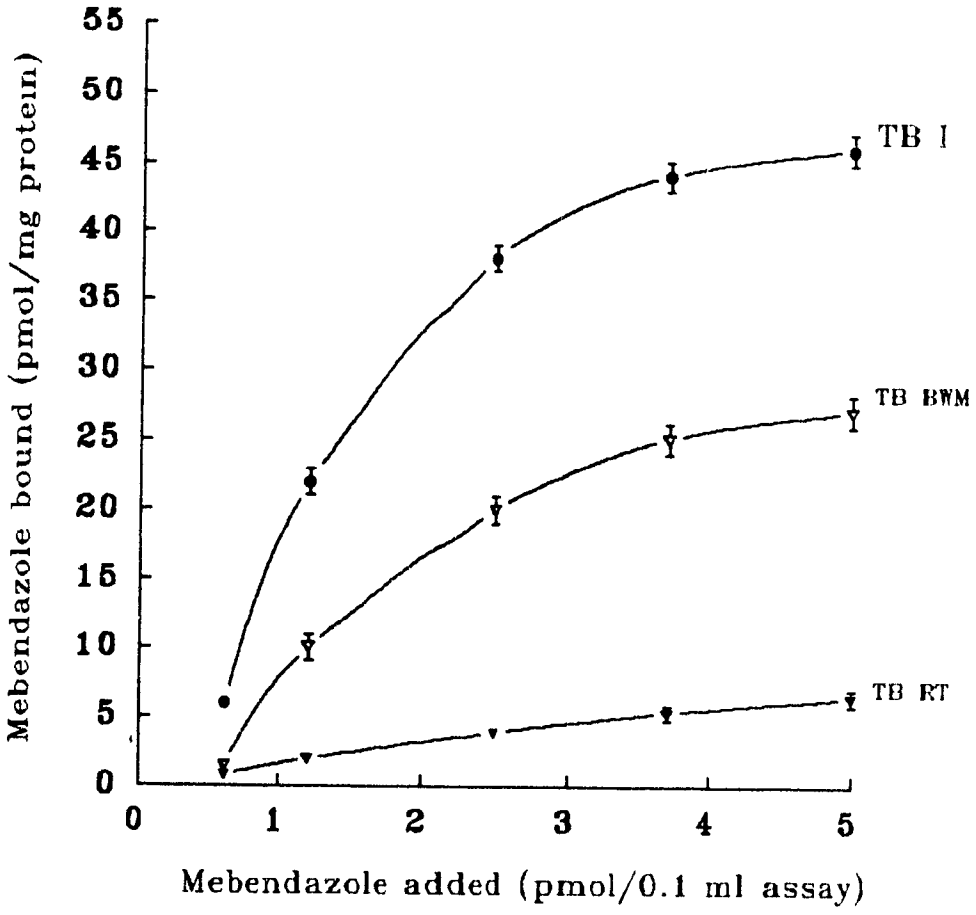
Electron microscopy was used to determine the relative density of tubulin in the intestine, body wall muscle and reproductive tract of *A. suum*. For uranyl acetate staining, worms were isolated as described in the preparation of parasite extract section. The body wall muscle, intestine and reproductive tract tissues were cut into small pieces. These fragments were immediately fixed in 3% gluteraldehyde in 0.1 M sodium phosphate buffer pH 7.2 for 3 h at room temperature. After washing with sodium phosphate buffer, the fragments were incubated with 2% tannic acid overnight. Samples were postfixed in 1% Osmium tetroxide for 1 h at 4°C, then stained with uranyl acetate for 6 h. Samples were washed and dehydrated in a graded series of ethanol for 2 h. Tissue fragments, dehydrated in ethanol, were placed in pure propylene oxide for 10 min, then in a mixture of 1:1 epon:propylene oxide for 10 h, 2:1 epon:propylene oxide for 2 h, and finally in pure epon for 2 h. Then the specimens were embedded in pure epon and polymerized at 56°C for 36 h (Sheffield, 1964). Sections were cut on a Reichert ultramicrotome, mounted on formvar coated mesh grids, examined and photographed in a Philips 410 TEM electron microscope (Hayat, 1972)(E.M. Centre, McGill University).

RESULTS

Mebendazole binding assays: Graphical analysis

Due to the difficulties in getting *A. suum* tubulin in pure form, the supernatants of intestine, body wall muscle and reproductive tract obtained after ultra-centrifugation were used in the drug binding assays. The binding was uniform for protein concentrations in the range of 100-180 µg protein/assay for intestine, body wall muscle and reproductive tract extracts. Below or above 100-180 µg per assay, the binding was affected by protein content. The binding of [^{3}H]MBZ to the extracts of intestine, body wall muscle and reproductive tract of *A. suum* was measured. Total binding for intestinal and body wall muscle tubulin was saturable, within the concentration range of [^{3}H]MBZ used. However, [^{3}H]MBZ binding to tubulin from reproductive tract of *A. suum* was not saturable (Fig. 4.1). The results obtained for

Fig. 4.1. Total binding (TB) by [^{3}H]MBZ to intestine (I), body wall muscle (BWM) and reproductive tract (RT) of *A. suum*. The procedure is described in detail in Materials and Methods. The data was transformed into picomoles per milligram of protein. Each point is the mean and S.E.M of 3 experiments.


Mebendazole bound (pmol/mg protein)
55
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Mebendazole added (pmol/0.1 ml assay)
TB I
TB BWM
TB RT

intestinal extracts, are in agreement with those reported by Kohler & Bachmann (1981).

There was a marked difference in binding of MBZ to intestine (Fig. 4.2A), body wall muscle (Fig. 4.2B) and reproductive tract (Fig. 4.2C) of *A. suum*. Total binding was highest in intestine followed by body wall muscle and in the reproductive tract it was the least. The total binding of MBZ in the intestinal tissue was significantly different from that in body wall muscle and reproductive tract ($P< 0.01$). The binding in the body wall muscle in turn was significantly higher than that in the reproductive tract ($P< 0.01$). The LAB of the intestine was not significantly different from body wall muscle, for MBZ. However, in the reproductive tract, the LAB was similar to the total binding (Fig. 4 2A-C). The extracts of intestine showed greater high affinity binding then body wall muscle (Fig. 4.2D). High affinity binding in the intestine was significantly higher than that of body wall muscle ($P< 0.01$). In the reproductive tract extract the high affinity binding of [^{3}H]MBZ could not be measured, because the binding was not saturable at the drug concentration used.

Calculation of K_a and B_{max}

After correction for the non-specific binding of MBZ, the K_a and B_{max} for intestine and body wall muscle were calculated using the computer programs EBDA and LIGAND. Mathematical analysis of MBZ binding for reproductive tract extract was not performed because the total binding in this tissue could not be resolved into HAB and LAB and was not saturable at either 37°C or 4°C, within the range of MBZ concentrations used.

K_a values

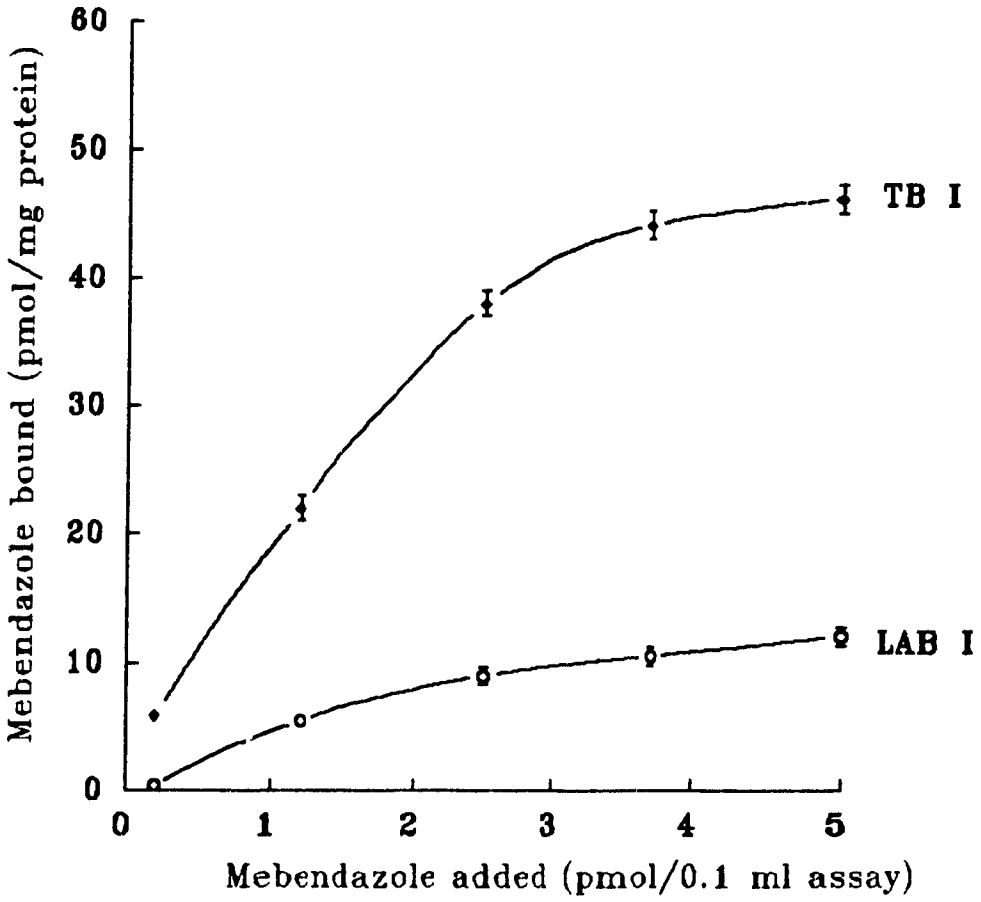
There was no significant difference in the K_a values between the intestine and body wall muscle tubulin of *A. suum* (Table 4.1).

Bmax values

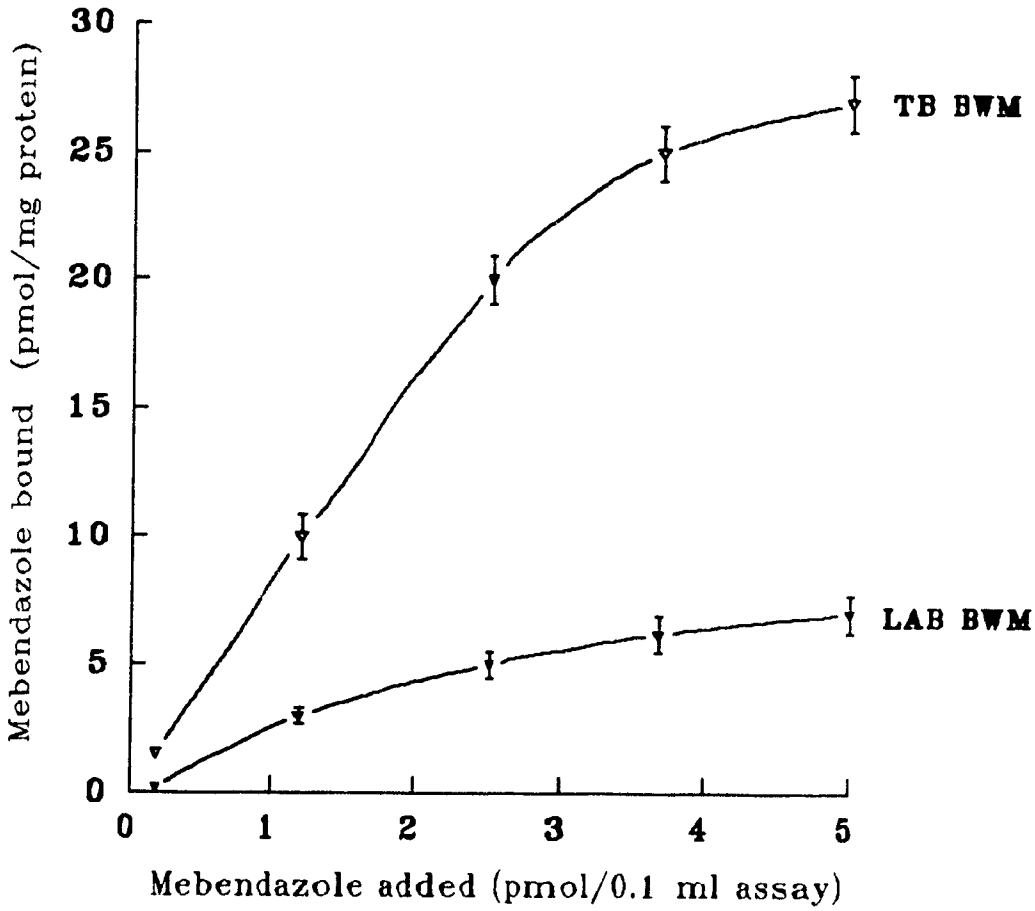
The B_{max} values of intestinal extract were significantly higher ($P< 0.001$) than

Fig. 4.2. Total binding (TB) and low affinity binding (LAB) by [^{3}H]MBZ to intestine (I) (Fig. 4.2A), body wall muscle (BWM) (Fig. 4.2B) and reproductive tract (RT)(Fig. 4.2C). High affinity binding (HAB) by [^{3}H]MBZ to intestine and body wall muscle is shown in Fig. 4.2D. The procedure is described in detail in experimental protocol section. The data was transformed into picomoles per milligram of protein. HAB was determined by subtracting LAB from TB. Each point is the mean and S.E.M of 3 separate experiments.

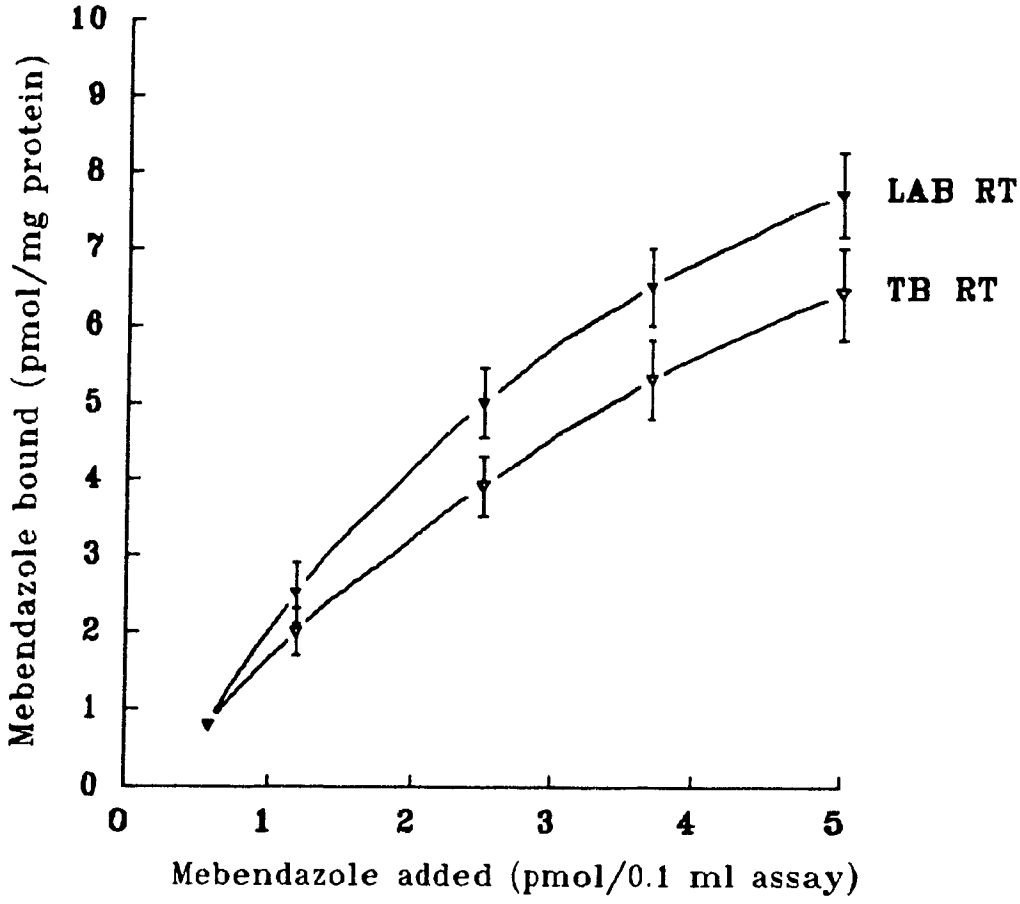
A


Mebendazole bound (pmol/mg protein)
60
50
40
30
20
10
0
0
1
2
3
4
5
Mebendazole added (pmol/0.1 ml assay)
TB I
LAB I

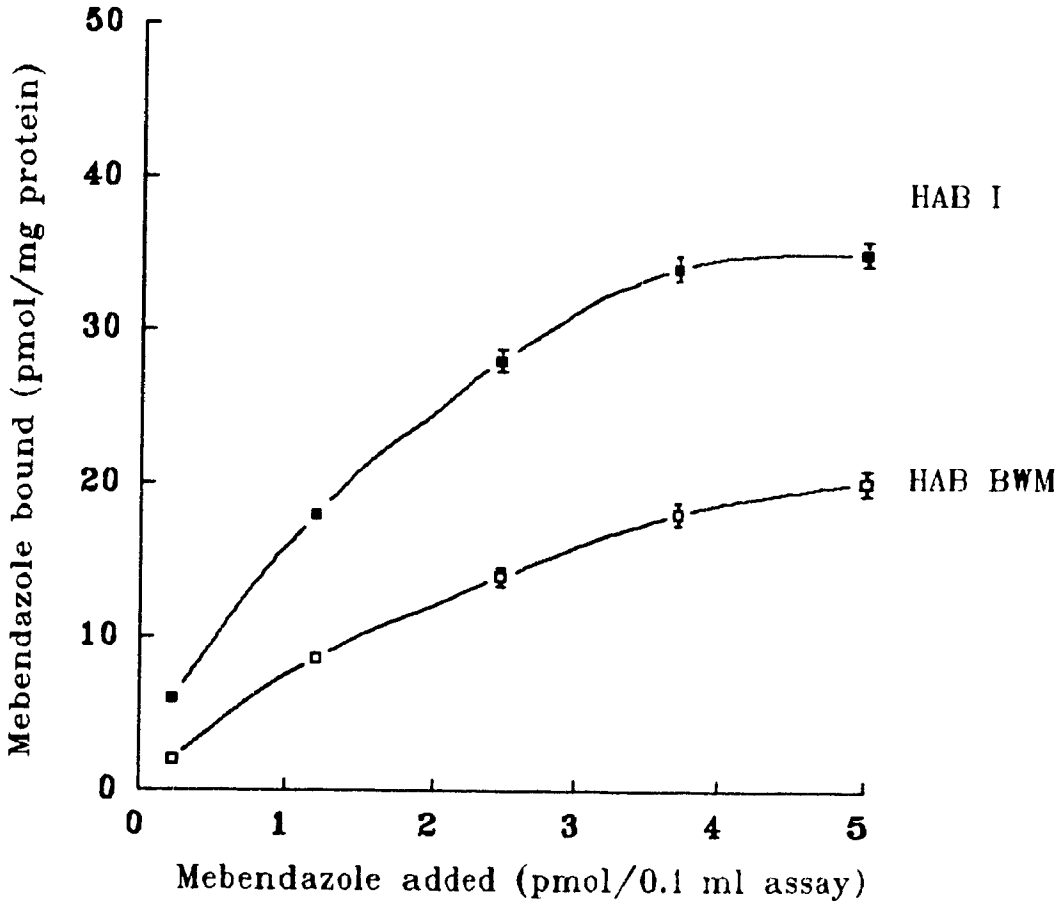
B


30
25
20
15
10
5
0
0
1
2
3
4
5
Mebendazole bound (pmol/mg protein)
Mebendazole added (pmol/0.1 ml assay)
TB BWM
LAB BWM

C


Mebendazole bound (pmol/mg protein)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Mebendazole added (pmol/0.1 ml assay)
LAB RT
TB RT

D


50
40
30
20
10
0
0
1
2
3
4
5
Mebendazole bound (pmol/mg protein)
Mebendazole added (pmol/0.1 ml assay)
HAB I
HAB BWM

those of the body wall muscle for MBZ (Table 4.1). The B_{max} was 24.3 ± 2.2 pmoles/mg of intestinal tubulin and 7.8 ± 1.2 pmoles/mg for body wall muscle tubulin.

The Scatchard plots for HAB of intestine and body wall muscle were linear and had similar slope but differed in their x-intercepts indicating that the K_a values of intestine and body wall muscle were similar but their B_{max} values were different. These data suggest the presence of only one type of high affinity receptor in these tissues.

Electron Microscopy

In this study, electron microscopy has been employed to examine the density of MTs in various tissues of *A. suum*. To preserve the ultrastructure of the various tissues of *A. suum*, the worms were fixed in gluteraldehyde immediately after removal from the host pig, processed and examined under an electron microscope. The ultrastructure of *A. suum* intestine, body wall muscle and reproductive tract has been described previously in detail (Chitwood & Chitwood, 1950; Sheffield, 1964; Bird, 1980).

A. suum has a straight intestine which is attached to the body wall at the mouth and rectum but it lies free throughout the rest of the body. The overall structure of the intestine is shown in Fig. 4.3A. The non-muscular walls of the intestine are composed of a single layer of columnar epithelial cells supported by a basement membrane. The nucleus is situated in the basal portion of the epithelial cell and large amount of glycogen and endoplasmic reticulum are also present in this area. Mitochondria are distributed throughout the cell. They are most abundant in the apical end of the cell just below the terminal web and are few in number in the areas basal to the nucleus. At high magnification, the MTs are observed throughout the cells, large amounts of MTs were found in the vicinity of the nucleus (Fig. 4.3B) and also near the basal region of the epithelial cell (Fig 4.3.C). The intestine of *A. suum* is a simple tissue in which the abundent MTs appear to be involved in the cytoskeletal processes of shape determination and secretion (Kohler & Bachman, 1981).

The body wall muscle consists of cuticle, hypodermal layer and somatic muscle

Table 4.1. K_a and B_{max} derived by saturation study from total binding for intestine and body wall muscle of *A. suum*. (180 µg protein per assay).

Drug	Tissue	K_a* ($\times 10^6 M^{-1}$)	B_{max}* (pmol/mg protein)
MBZ	Intestine	10.8 ± 2.3	24.3 ± 2.2[a]
	Body wall muscle	9.8 ± 3.0	7.8 ± 1.2

* = Mean of 3 experiments ± S.E.M.

a = B_{max} intestine greater than B_{max} body wall muscle, $P < 0.01$.

Fig. 4.3A. Electron micrograph of female *A. suum* intestinal epithelial cells stained with uranyl acetate.

mv = microvilli, bm = basement membrane, n = nucleus, v = vacuole, m = mitochondria.

Magnification: x 1323.

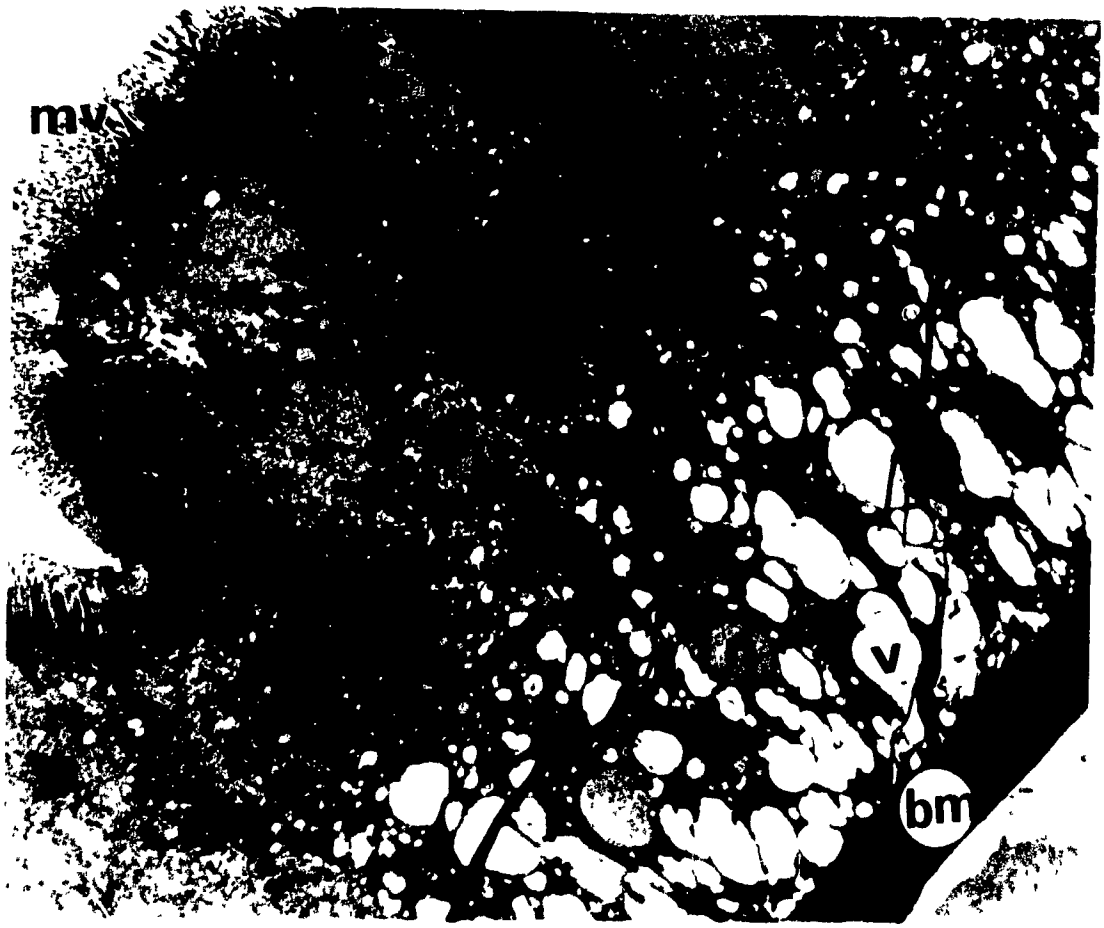

mv
v
bm

Fig. 4.3B. Electron micrograph of perinuclear region of female *A. suum* intestinal epithelial cell stained with uranyl acetate. Note the large amount of MTs in the vicinity of nucleus.

MTs are arrowed; m = mitochondria, v = vacuole,

Magnification: x 65 880.

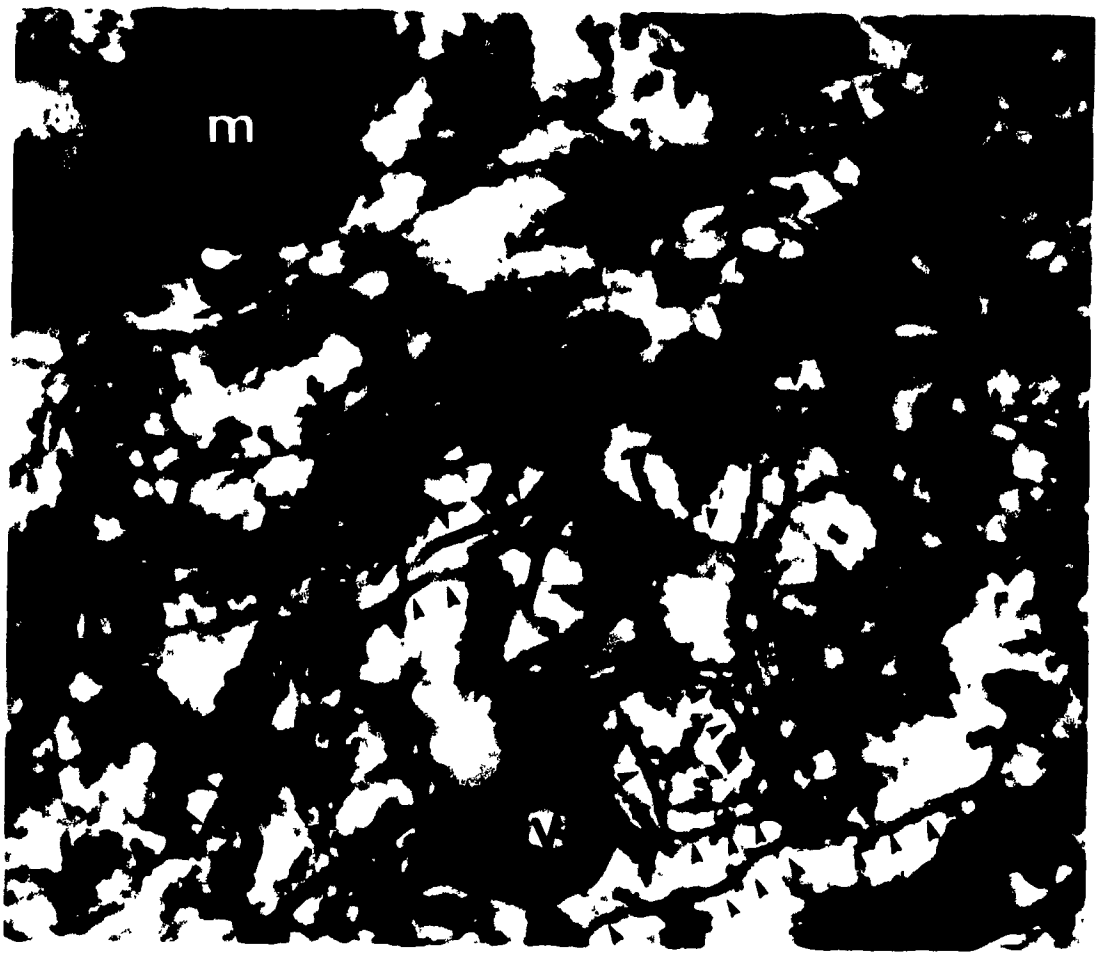
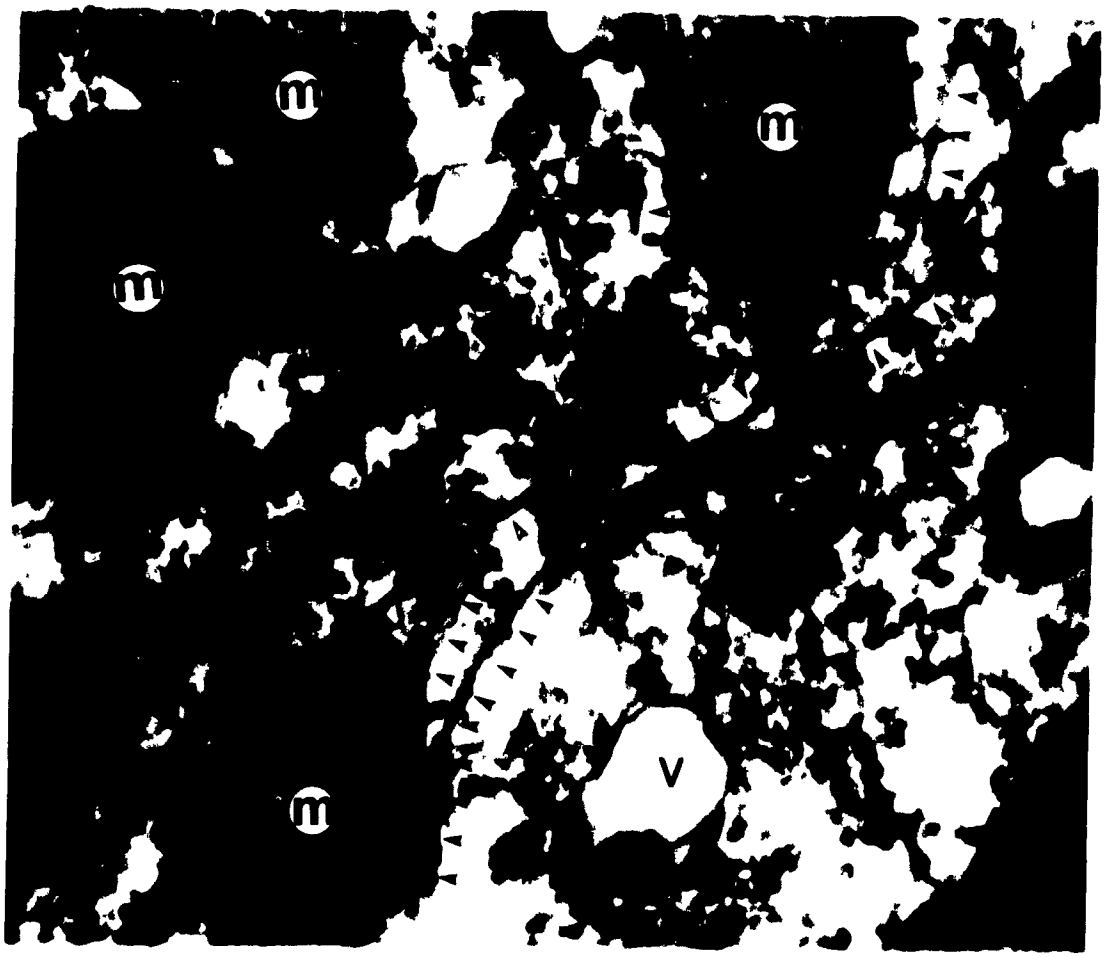

m

Fig. 4.3C. Electron micrograph of basal region of female *A. suum* intestinal epithelial cell stained with uranyl acetate. Note numerous MTs adjacent to mitochondria. MTs are arrowed; m = mitochondria, v = vacuole, g = glycogen.


m
m
m
m
V

cells (Fig. 4.4A). The cuticle of *A. suum* is a complex extracellular structure composed of proteins with trace amounts of lipid and carbohydrate (Bird, 1980). The cuticle is divisible into nine distinct layers (not shown). The hypodermis consists of a thin layer beneath the cuticle and has longitudnal thickenings protruding internally between the sectors of the longitudnal muscles in the form of bands These bands are known as the cords. Both cuticle and hypodermis are separated by cell membranes. The hypodermis is cellular in nature and contain a large diversity of protein species, glycogen, lipid and mitochondria (Fetterer & Wasiuta, 1987). It also contains large and small nuclei which are scattered throughout the intercordal hypodermis and the dorsal as well as the ventral cords. Underneath hypodermis lie the somatic muscle cells. *A. suum* has U-shaped type of muscle cells in which the fibers extend up the side of the cell and the sarcoplasm bulges into the pseudocoelom. Muscle cells are connected to each other by mean of cytoplasmic bridges which contains large amounts of glycogen, mitochondria and the nucleus (Fig. 4.4A). At high magnification, large numbers of MTs are clearly observed in the hypodermis, where they are closely associated with the mitochondria (Fig. 4.4B). Numerous MTs are also found in the sarcoplasmic core where they are in close contact with the fibrillar portion of the somatic muscle cells (Fig. 4.4C)

Female reproductive system mainly consists of ovary and uterus. For the electron microscopic study, sections of ovary were used. The ovary consists of a tubular sac in which germinal cells develop. This sac consists of an epithelial layer and a germinal cord. The epithelium consists of a single layer of greatly elongate, spindle-shaped cells (Fig. 4.5A). Each of these cells is multinucleate. The ovary is divisible into two regions: (1) The germinal region, an area of rapid division of relatively small cells, not showing cell boundries, rich in mitochondria, vacoules and glycogen and (2) the growth region, an area of gradual increase in size of the oogonia. Cell borders are often difficult to distinguish at the proximal or germinal end of the germinal cord, but sometimes distinct and this region is cellular rather than syncytial, since cell boundries gradually become more apparent as the cells move down the gonoduct. At higher magnification, many MTs were observed in the vicinity of the muscle cells (Fig. 4.5B), but the density of MTs was much less, in comparison to those

Fig. 4.4A. Electron micrograph of female *A. suum* body wall stained with uranyl acetate. At low magnification part of the cuticle, hypodermis, somatic muscle and sarcoplasmic core is shown.

n = nucleus, v = vacuole, m = mitochondria, s = sarcoplasmic core, smc = somatic muscle cells.

Magnification: x 1836


c
h
smc
m
n
v

Fig. 4.4B. Electron micrograph of female *A. suum* body wall muscle stained with uranyl acetate. Note the concentration of MTs in the hypodermis of the body wall muscle.

MTs are arrowed; m = mitochondria, v = vacuoles.

Magnification: x 56 160.

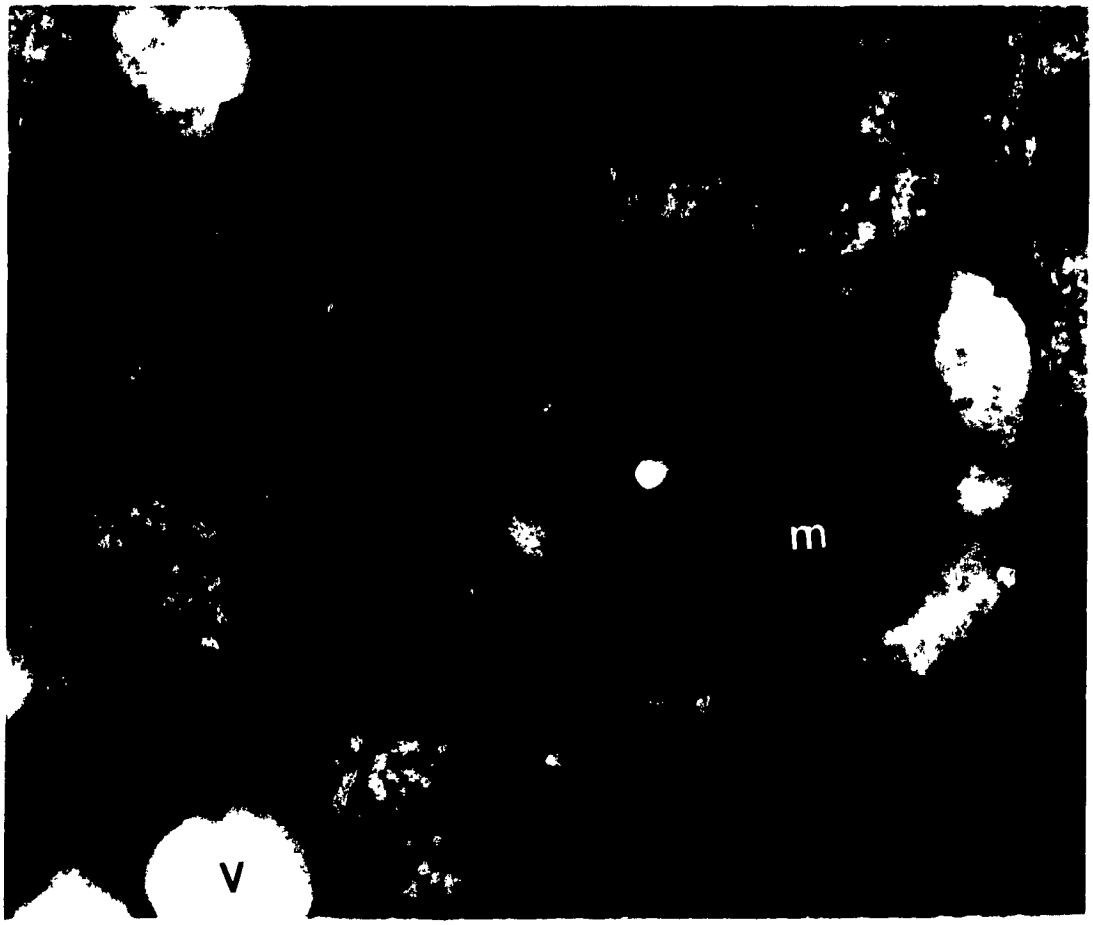

m
v

Fig. 4.4C. Electron micrograph of female *A. suum* body wall muscle stained with uranyl acetate. MTs are clearly observed in the sarcoplasmic core near the somatic muscle cells.

MTs are arrowed; smc = somatic muscle cells, m = mitochondria, v = vacuole.

Magnification: x 56 160.

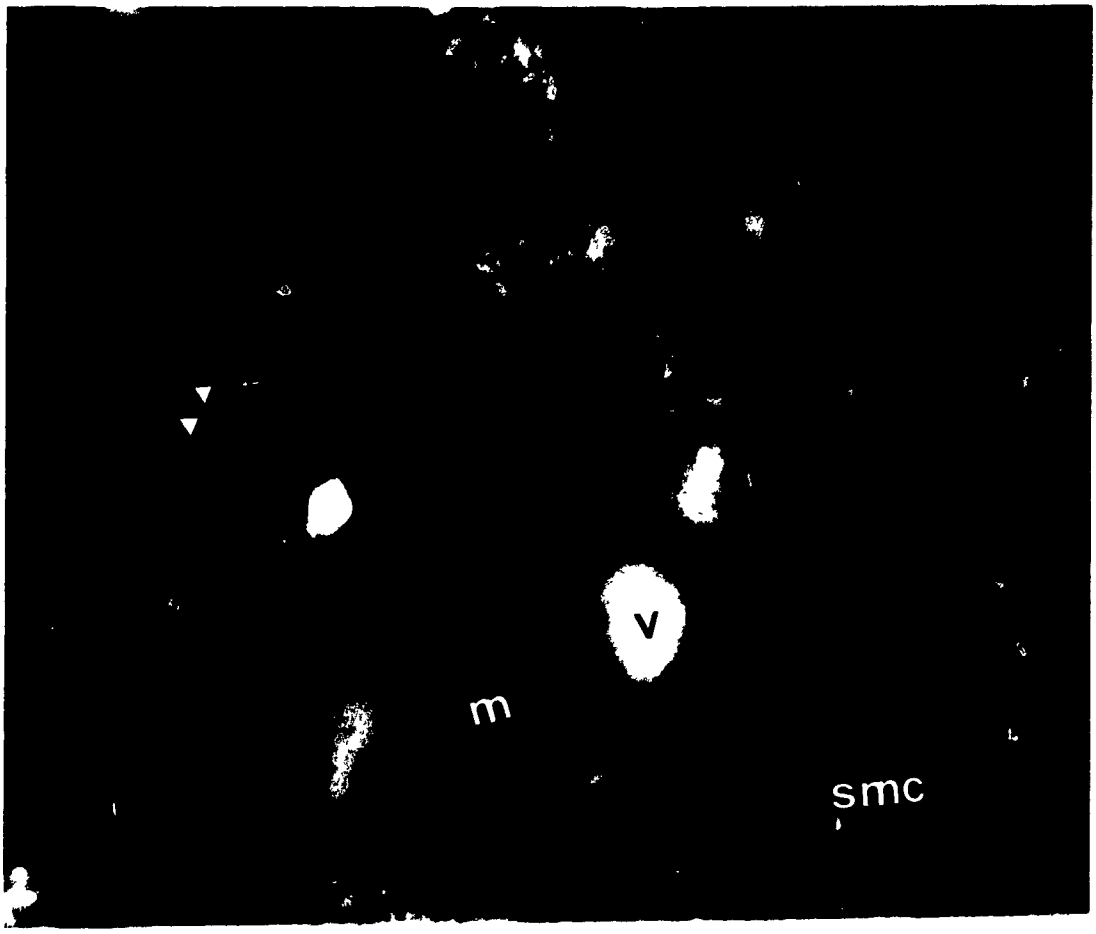

v
m
smc

Fig. 4.5A. Electron micrograph of a cross-section through the ovary of A. suum. This figure illustrates various degrees of constrictions of cytoplasmic bridges between oocytes and rachis.

mc = muscle cells, v = vacuole, o = oocytes, r = rachis or cytoplasmic network.

Magnification: x 1323.

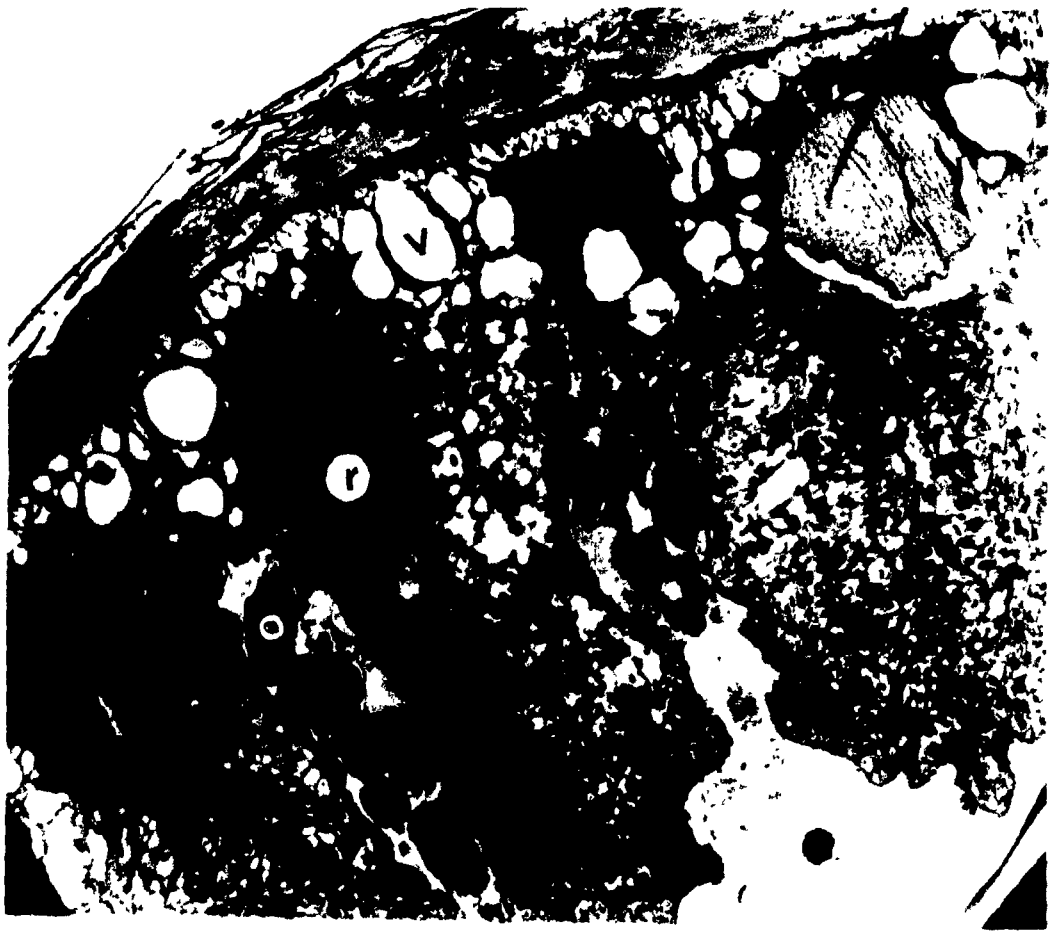
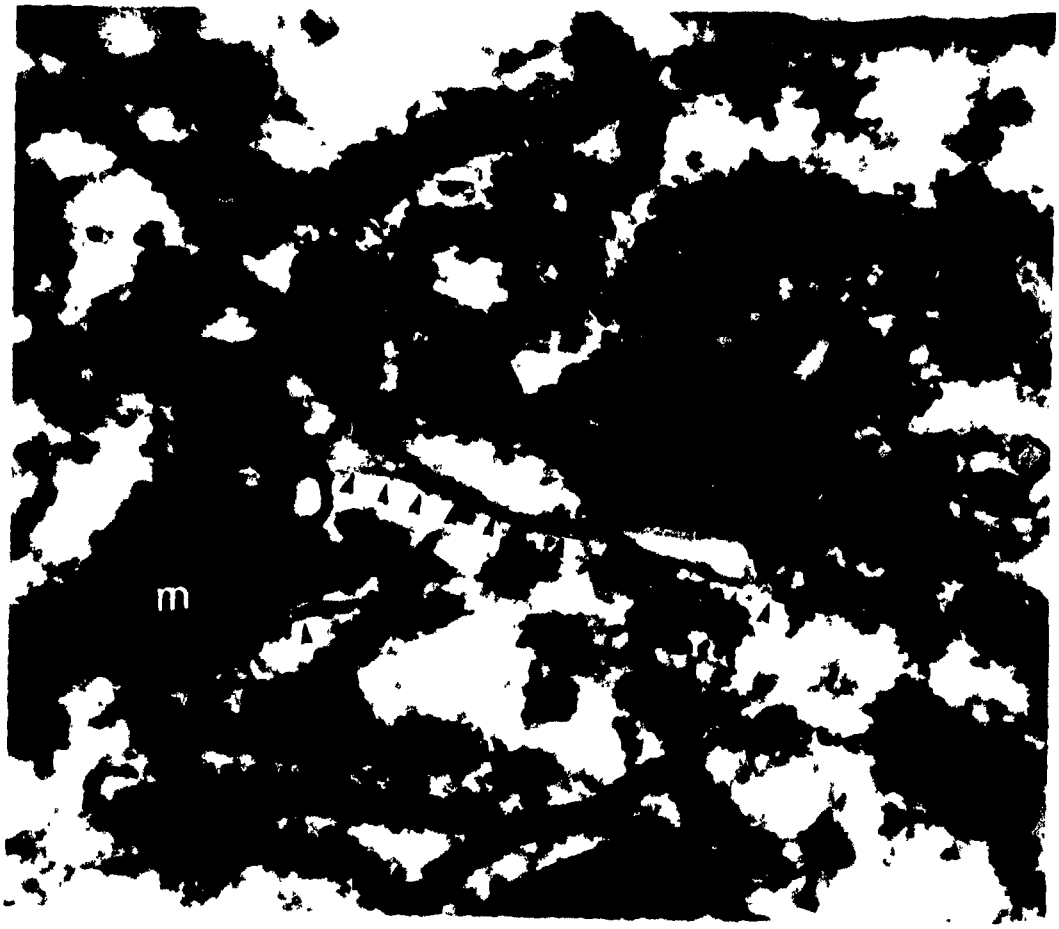

V
r
o

Fig. 4.5B. Electron micrograph of *A. suum* ovary. Note the numerous MTs in the afibrillar portion of the muscle cells.

MTs are arrowed; m = mitochondria, er = endoplasmic reticulum.

Magnification: x 56 160.


m

found in the intestine and body wall muscle. A small number of MTs were also found in the cytoplasmic bridges (Fig. 4.5C), where they provide physical support for the oocytes.

DISCUSSION

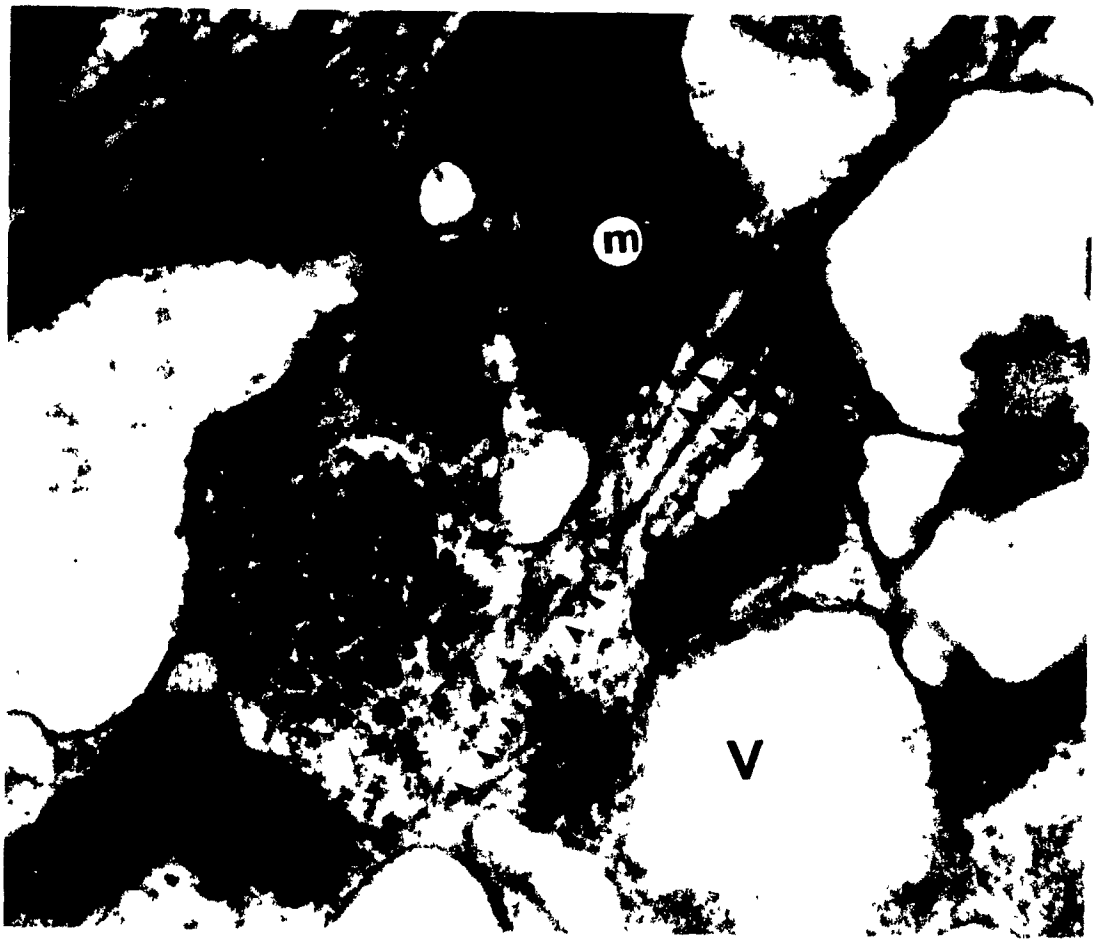
BZs are a broad spectrum anthelmintic, which are active against a wide variety of larval and adult helminths (Lacey, 1990). Several mechanisms have been proposed for the action of BZs in adult nematodes (1) interference with glucose uptake (Van Den Boosche & De Nollins, 1973); inhibition of fumarate reductase (Prichard, 1973) and disruption of cytoplasmic MTs in intestinal cells (Borgers et al, 1975) In nematodes intestinal tubulin/MT system appears to be of particular importance in this respect since one of the primary morphological changes observed, after MBZ treatment of host was the disappearance of the intestinal MTs of *A suum* (Borger et al, 1975) Several studies have demonstrated the direct interaction of MBZ with tubulin obtained from intestine of *A. suum in vitro* (Friedman & Platzer, 1980; Kohler & Bachmann, 1981; Barrowman *et al*, 1984) However, no data on the interaction of BZs to other tissues of *A. suum* were available Therefore, the aim of this study was to determine the interaction of MBZ with the tubulin from body wall muscle, reproductive tract and intestine, and determine if the other tissues of *A suum* were equally sensitive to the MBZ attack, and also to determine if the selective sensitivity of a particular tissue is associated with tubulin content in that tissue

The data from this study demonstrates that there are marked differences in the total binding of MBZ to the tubulin from intestine, body wall muscle and reproductive tract extracts of *A. suum*. Intestine, body wall muscle and reproductive tract extracts also differ in levels of HAB; more MBZ was bound by intestine than body wall muscle. However, in reproductive tract, HAB was not detectable After correction for non-specific binding of ligand in the total ligand binding, by the computer program LIGAND, the results indicated that the B_{max} of MBZ for the tubulin of *A. suum* intestine (24.3 ± 2.2 pmoles/mg protein) was about three fold higher than for that of body wall muscle (7.8 ± 1.2 pmoles/mg protein). The binding affinity of MBZ

Fig. 4.5C. Electron micrograph of *A. suum* ovary. Note that only few MTs are observed in the cytoplasmic bridge.

MTs are arrowed; m = mitochondria, v = vacuole.

Magnification: x 56 160.


m
V

to intestinal tubulin ($10.8 \pm 2.3 \times 10^6$) was similar that to body wall muscle ($9.8 \pm 3.0 \times 10^6$). These data suggest that tubulin from intestine binds more MBZ than does tubulin from body wall muscle, however, the MBZ binding occurs with the same affinity to the intestinal and body wall muscle tubulin. The greater extent of binding of MBZ to intestine compared with body wall muscle and reproductive tract may make intestine more susceptible to disruption by tubulin binding drugs than the other tissues. However, the pharmacokinetic differences may also play a role in the selective toxicity of MBZ to tubulin

LAB was similar in the intestine and body wall muscle. At the concentrations of MBZ used, all of the binding in the reproductive tract was LAB, and the binding contstants such as K_a and B_{max} were not calculated for reproductive tract. For intestinal tubulin, the data are consistent with several *in vitro* ultrastructural studies (Borgers *et al.*, 1975; Atkinson *et al.*, 1980) which suggest that intestine is more susceptible to MBZ than are the body wall muscle or reproductive tract.

Tubulin content have been shown to vary in different organisms and even within the different tissues of the same organism. Vertebrate brain is rich in tubulin which constitutes 42% of the soluble protein (Sullivan, 1988). However in nematodes, tubulin content is very low. Tubulin from *N brasiliensis* was estimated to be approximately 0 025% of the soluble protein (Tang & Prichard, 1988) and about 3% in *B. malayi* and *B. pahangi* (Tang & Prichard, 1989). There is no universal method for quantification of tubulin for all organisms and tissues. Depending on the organism, different investigators have used different methods to quantify tubulin, such as colchicine binding assays, SDS-PAGE, densitometry, radioimmunoassays or ELISA (Lacey 1988; Tang & Prichard, 1989). Lubega & Prichard (1991) using BZs binding and SDS-PAGE followed by Western blotting, demonstrated a reduction in high affinity binding per milligram protein from egg through larva to adult *Haemonchus contortus*. The authors suggested that this decrease may be due to a parallel reduction in tubulin content per milligram protein (Lubega & Prichard, 1991).

In this study, electron microscopy was used to quantitatively assess the tubulin in various tissues of *A. suum* by observing microtubial presence. At high

magnification, electron microscopy revealed the presence of MTs in the cross-sections of the intestine, body wall muscle and reproductive tract, tissues architecture did not appear to be disrupted. Interestingly, all these tissues were found to contain very different density of MTs. Intestine seemed to contain much more MT than body wall muscle which in turn contained more MTs than the reproductive tract. These findings are in agreement with the differences in the MBZ binding from highest in the intestine to less in body wall muscle to least in the reproductive tract. Therefore, it is suggested that the marked differences in the MBZ binding to the intestine, body wall muscle and reproductive tract could be due to the differences in the tubulin content of these tissues. However, MT content alone may not explain the differences in the sensitivity of these tissues to MBZ. The selective BZ binding may also depend on the type(s) of tubulin found in different tissues.

CONNECTING STATEMENT

In the following chapter 5, using one and two dimensional SDS-PAGE followed by Western blotting with anti-tubulin monoclonal antibodies, it is demonstrated that α and β tubulin from intestine, body wall muscle, and reproductive tract are different in the content, isoform numbers, electrophoretic mobilities, isoelectric points and peptide maps (Chapter 5). It is suggested that different types of tubulin exist in various tissues of *A. suum* and the selective toxicity of the MBZ to the various tissues could be due to either the existance of different tubulins or to different amounts of tubulin in these tissues.

CHAPTER 5

IDENTIFICATION OF TUBULIN ISOFORMS IN DIFFERENT TISSUES OF *Ascaris suum* USING ANTI-TUBULIN MONOCLONAL ANTIBODIES

INTRODUCTION

Microtubules (MTs) are proteinaceous organelles which perform a wide range of both essential and specialized functions in all eukaryotes. These functions include the segregation of chromosomes during mitosis and meiosis; the maintenance of cell shape; cell motility, as an integral part of cilia and flagella; and intracellular transport. The major structural component of MTs is the protein tubulin, a heterodimer composed of α- and β-subunits of molecular weight of 110 kDa (Ponstingyl *et al.*, 1981). Both α- and β-subunits are expressed as a heterogenous but closely related family of multiple isoforms in different organisms, tissues and even within single cells of the same organism (Fulton & Simpson, 1976; Gozes & Littauer, 1978; Sullivan, 1988; Birkett *et al.*, 1985; Tang & Prichard, 1988; 1989). The heterogeneous population of tubulin isoforms can result from both post-translational modifications and differential expression of often numerous tubulin genes (Cleveland *et al.*, 1980; Gard & Kirschner, 1985; Luduena *et al.*, 1988). Owing to the diversity of tubulin isoforms, it has been suggested that such differences may have implications for specific microtubule functions (Banerjee *et al.*, 1990). The precise nature or role of α- and β-tubulin isoforms have not yet been elucidated, although several groups have demonstrated that many in vivo functions of tubulin are to some extent, isoform specific (Gundersen *et al.*, 1984). Several chemicals such as colchicine, vinblastine,

podophylotoxin, benzimidazoles (BZs) have been shown to bind specifically to tubulin and to prevent its assembly into MTs. BZs, anti-mitotic or anti-fungal agents are widely used in the chemotherapy of parasitic diseases, particularly for nematodes.

Spence *et al.* (1982) demonstrated that mebendazole (MBZ), a BZ derivative, severely inhibited the growth and reproductive capability of *Caenorhabditis elegans* Sheir-Ness *et al.* (1978) and Burland *et al.*, (1984) reported that in both *Aspergillus nidulans* and *Physarum polycephalum*, BZ resistance resulted from alterations in the amino acid sequence of specific isoforms of β-tubulin which presumably reduce their affinity for BZs. In similar studies, May *et al* (1985), demonstrated that BZ resistance was due to the elimination of a specific β-tubulin isoform in *A. nidulans*, whereas, Foster *et al.* (1987) using *P. polycephalum* demonstrated that BZ resistance in mutant strain of myxamoebae was due to the presence of an extra β-tubulin isoform, compared to the wild type strain. Woods *et al.* (1989) reported that BZ resistance in *C. elegans* was due to the apparant loss or altered electrophoretic mobility of a single β-tubulin isoform. Borgers *et al.* (1975) reported that when MBZ was fed to pigs and turkeys infected with *Ascaris suum* and *Syngamus trachea*, respectively, MTs in intestinal cells in the nematodes were lost whilst the host cells remained unaffected. Intestinal tubulin from adult *A. suum* was reported to be the most sensitive target of BZ attack as lesions were observed in the intestine before any modifications occurred in other tissues (Borgers & De Nollin, 1975). Davis & Gull (1983) studied the protofilament number in *Ascaridia galli* and *T. colubriformis* and found that *A galli* contained 11 protofilament MTs and *T colubriformis* contained 11, 12, and 14, protofilament MTs. These nematodes lacked 13 protofilament MTs which are common among mammalian cells These authors postulated that the selective toxicity of the BZs for nematodes could be due to the differences in protofilament number found in mammals and nematodes. However, little is known of the structure, heterogeneity and distribution of tubulin in the different tissues of nematodes.

In this chapter, I report on the production of anti-*A suum* monoclonal antibodies. The aim of this study has been to investigate and compare the heterogeneity of α- and β-tubulin in the different tissues of *A. suum*, using tubulin

specific monoclonal antibodies

MATERIALS AND METHODS

Preparation of antigen

Adult *A. suum* were collected from the intestines of experimentally infected pigs into Minimum Essential Medium (MEM) salt solution and washed with 0.025 M MES buffer containing 1 mM EGTA, 0.5 mM $MgSO_4$ and 1 mM GTP. The worms were dissected and the intestine and reproductive tracts removed. Intestine, reproductive tract and body wall muscle were washed and homogenized (with a glass homogenizer with a Teflon pestle) separately in MES buffer. The extracts were centrifuged at 100 000 g for 1 h at 4°C. After centrifugation, the supernatant was retained and used for further purification Tubulin from pig brain was prepared by 2 cycles of polymerization-depolymerization, according to the method of Shelanski *et al.*, (1973).

A. suum tubulin from intestine, reproductive tract and body wall muscle was partially purified using polylysine affinity chromatography (Tang & Prichard, 1989). Five ml of supernatant was applied to the column and eluted sequentially with 0.025 M MES buffer, 1% $(NH_4)_2SO_4$, and 5% $(NH_4)_2SO_4$ in MES buffer. Fractions were collected for protein determination. The protein concentration was determined by the method of Bradford (1976), using BSA as the standard. The first protein peak was eluted with MES buffer, the second with 1% $(NH_4)_2SO_4$, and the third with 5% $(NH_4)_2SO_4$ in MES buffer. Fractions of each peak were pooled and concentrated in Centriflo (Amicon) at 400 g Polylysine-purified proteins were separated on SDS-PAGE, protein bands of the molecular weight corresponding to tubulin were excised, and the protein electro-eluted (Electro-eluter, Bio-Rad) (Blose, 1981). Eluted tubulin was precipitated three times with 80% acetone at -20°C. After 5 h, the tubulin was dissolved in 0.125 M Tris-HCl (pH 6.8), 0.1% SDS and 1 mM EDTA, dialysed overnight against this buffer at 4°C, and stored at -70°C until used.

Immunization and preparation of MAb P3D6

Six week old female BALB/c mice (Charles River Canada Inc., St. Constant,

Québec) were injected subcutaneously at three week intervals with purified eluted *A suum* intestinal tubulin (100 µg/injection) using equal volumes of complete Freund's adjuvant for the first injection and incomplete adjuvant for the second injection. The third immunization with 100 µg tubulin in PBS was administered intraperitoneally (i.p.). At this stage, mice were bled and serum was tested for anti-tubulin antibodies by ELISA. The spleen cells from the mouse giving the highest titre were fused with the non-secreting myeloma cell line P3-x63-Ag8.653 (ATCC, Rockville, MD), as described by Hurrell (1982). Positive cultures as determined by ELISA were cloned twice by limiting dilution.

In addition to MAb P3D6, anti-chick brain MAb 356, which cross-reacts with α-tubulins, and anti-chick brain MAb 357, which cross-reacts with β-tubulins from a spectrum of eukaryotic cell types, were purchased from Radiochemical Centres (Amersham, England) and used to confirm the identity of tubulins.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as described by Voller *et al* (1976). Microtiter plates (96 well, Falcon) were coated with crude or polylysine- purified tubulin at a concentration of 10 µg/ml in PBS. Horseradish peroxidase-labelled anti-mouse IgG or IgM (Bio-Can, Mississauga, Ontario) at dilutions of 1:5 000 and 1:20 000, respectively, were used. The substrate was (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). The plates were read on a Titertek Multiskan plate (Flow Lab, Irvine, Ayrshire, U K.) at 414 nm. Hydridoma supernatants with absorbances of above 0.5 were considered positive. Normal mouse serum or culture medium used to grow hybridoma cells (Iscoves modified Dulbecco's medium (IMDM) with 20% fetal calf serum (FCS)(Gibco), 10% NCTC 135 (Gibco) and hypoxanthine thymidine (HT) (Gibco)) was used as a negative control.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were run in a Mini Protean II dual slab cell (Bio-Rad, Richmond CA) using the discontinuous system of Laemmli (1970) consisting of 4% polyacrylamide as

stacking and 12% polyacrylamide as separating gel.

Isoelectric focusing and two-dimensional electrophoresis (IEF-2-D SDS-PAGE)

IEF gels were prepared and run according to the method of O'Farrell (1975) as modified by Chung (1987). IEF was performed in tube gels (1.5 x 8 cms) containing 9.5 M urea (LKB) and 2% (w/v) ampholines (LKB) (1.6% pH 4-6 and 0.4% pH 3.5-10). IEF was conducted at 400 V for a period of 16 h and then at 800 V for 3 h. Two-dimensional electrophoresis was performed in 4% polyacrylamide stacking and 12% polyacrylamide separating gels, running at 50 V for 30 min and at 150 V for 60 min, in the Mini Protean II slab cell. After 2D electrophoresis, gels were either stained with silver stain (Bio-Rad), or the proteins electrophoretically transferred onto nitrocellulose (NC) sheets (Towbin *et al.*, 1979) for Western blot analysis.

Western blotting

The NC sheets were cut into several strips containing an identical pattern of separated proteins. To visualize protein bands, one NC strip was stained with amido black while other strips were washed in PBS and incubated in 10% newborn calf serum (NBCS)(Gibco) in Tris-buffer saline: 140 mM $NaCl_2$, 50 mM Tris-HCl, pH 7.4, with 0.1% (v/v) Tween 20 (TBS-T) for 2 h at room temperature, to saturate the unoccupied protein binding sites. After washing, the strips were incubated overnight at 4°C with anti-tubulin MAbs or IMDM (negative control). The NC strips were then washed 6 x 5 min with TBS-T, immersed in peroxidase-conjugated goat anti-mouse IgM or IgG (Bio-Can) diluted 1:500 with high salt buffer (1 M $NaCl_2$, 10 mM Tris-HCl pH 7.4, 0.5% (v/v) Tween 20 (HSB-T) with 10% NBCS), and incubated for 2 h at room temperature. After washing the NC strips with TBS-T for 30 min, the bound peroxidase was detected by substrate 4-chloro-1-naphthol (Sigma), at 3 mg/ml of methanol/PBS, 1:5 (vol/vol), containing 0.075% of hydrogen peroxide (Birkett *et al.*, 1985).

Limited proteolysis of tubulin

Limited proteolysis of tubulin in gel slices was performed as described by Cleveland *et al.* (1977) and modified by Chung (1987). Gel pieces corresponding to the tubulin were cut from the polyacrylamide gels and placed directly into the sample well of a second 15% SDS-polyacrylamide gel Gel pieces were overlayed with α-chymotrypsin from bovine pancreas (Boehringer, Mannheim). Limited proteolysis of tubulin proceeded directly in the stacking gel during the subsequent electrophoresis SDS-PAGE was performed at 50 V until bromophenol blue dye reached the bottom of the stacking gel and then increased to 150 V for the remainder of the electrophoresis After SDS-PAGE, the digested peptides were either stained with silver stain or transferred onto NC sheets in the same way as described for the Western blot analysis and immunostained either with anti-*A. suum* tubulin MAbs or anti-chick tubulin MAb 356 and 357.

RESULTS

Preparation of antigen

After centrifugation, the supernatant of adult *A. suum* intestine was fractionated on a polylysine agarose column. Several fractions were obtained from the polylysine chromatography The protein content of each fraction was determined and it was observed that the elution profile consisted of three distinct peaks as reported in chapter 2.

The protein samples of pooled fractions from polylysine column were subjected to SDS-PAGE. The results showed that the first and second peaks contained little if, any tubulin. However, the third peak was enriched with tubulin, as detected by Western blotting with commercial anti-tubulin MAbs (tubulin could not be detected in the first and second protein peaks)(see Fig. 2.2) Third peak proteins were concentrated and then subjected to SDS-PAGE, respectively. The tubulin band was cut out of the SDS-gel and subjected to electro-elution for further purification which resulted in a single tubulin band.

Monoclonal antibodies

Forty MAbs were obtained against *Ascaris* tubulin, all of them were of IgG isotype. Twenty five MAbs recognized tubulin as well as other high and low molecular weight proteins in Western blots, whereas the remaining fifteen were strictly specific for tubulin. In this chapter the characterization of one tubulin specific MAb P3D6 is described, as all other tubulin specific MAbs gave very similar results. MAb P3D6 cross-reacted with the intestinal, body wall muscle and reproductive tract tubulins, but it did not cross-react with mammalian tubulin

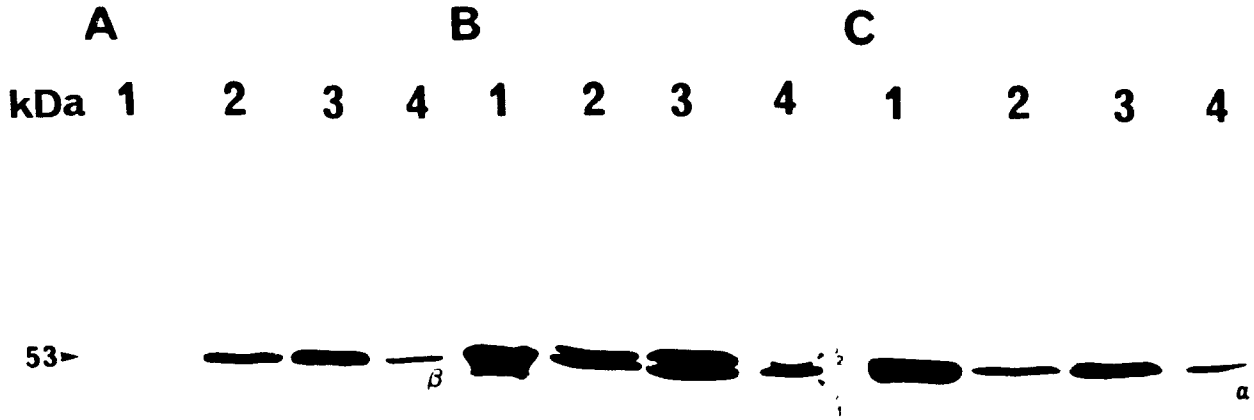
Specificity of MAbs 356, 357 and P3D6

Analysis of amido black stained blots revealed that crude extract of various tissues of *A. suum* contained many bands in the tubulin region (not shown) Tubulin from *A. suum* intestine, reproductive tract and body wall muscle separated into three bands, designated α, β_1, and β_2. Anti-*A. suum* tubulin MAb P3D6 recognized specifically β_1-tubulin band from the three tissues of *A. suum*. No cross-reactivity was detected with pig brain tubulin (Fig. 5.1A). The anti-chick brain β-tubulin MAb 357 reacted strongly with the major β_1- as well as the minor β_2-tubulin in all tissues of *A. suum* (Fig. 5.1B). The width and intensity of β_1- and β_2- were visually similar in all tissues From the width and intensity of the SDS-PAGE bands it would appear that β-tubulin is twice as abundant as α-tubulin in *A. suum*. The intestine seems to have the highest amount of tubulin and reproductive tract the least. Moreover, the anti-chick brain α-tubulin MAb 356 recognized α-tubulin from the three tissues of *A. suum* and it also cross-reacted with mammalian tubulin (Fig. 5.1C).

Identification of tubulin isoforms

Anti-*A. suum* tubulin MAb P3D6 and anti-chick brain tubulin MAbs 356 and 357 were used to characterize the α- and β-tubulin isoforms in the three tissues of adult *A. suum*. Cross-reactive anti-chick β-tubulin MAb 357 recognized an isoform pattern which differed with tissue type and from that recognized by anti-*A. suum* β-tubulin MAbs. Anti-*A. suum* β-tubulin MAb P3D6 and commercial anti-chick β-tubulin MAb

Fig. 5.1. Tubulin bands, visualised from total protein extracts of, pig brain (Lane 1), *A. suum* body wall muscle (Lane 2), Intestine (Lane 3) and reproductive tract (Lane 4), by immunostaining with anti-*A suum* β-tubulin MAb P3D6 (panel A), anti-chick brain β-tubulin MAb 357 (panel B) and with anti-chick α-tubulin MAb 356 (panel C). Prior to staining, extracts were electrophoresed on 12% SDS-polyacrylamide gels followed by transblotting onto NC. Twenty five µg of each protein were loaded per lane.


A
B
C
kDa
1
2
3
4
1
2
3
4
1
2
3
4
53
β
α

357 recognized different isoform patterns from *A. suum* reproductive tract and body wall muscle. MAb P3D6 recognized two β-tubulin isoforms in the intestine (Fig 5.2), one in the reproductive tract, and one in the body wall muscle extracts of *A. suum*. MAb 357 recognized two β-tubulin isoforms in the intestine, two in the reproductive tract, and two to three in the body wall muscle. Anti-chick brain MAb 356, recognized one α-tubulin isoform in the intestine, two in reproductive tract, and four to five isoforms in the body wall muscle of *A. suum*. To determine whether the β-tubulin isoforms observed with MAb 357 correspond to one of the β-tubulin spots observed with the MAb P3D6, SDS-polyacrylamide gels containing intestinal, reproductive tract or body wall muscle extract were probed with a mixture of MAb 357 and MAb P3D6. It was observed that the isoforms recognized by MAb 357 did not correspond to the isoforms observed with MAb P3D6 (not shown). β-tubulin isoforms were in the pH range of 5.0–5.4 whereas α-tubulin isoforms were in the pH range of 4.9–5.6. The same isoform pattern was found in samples prepared in the presence or absence of protease inhibitor phenyl methyl sulfonyl fluoride (PMSF).

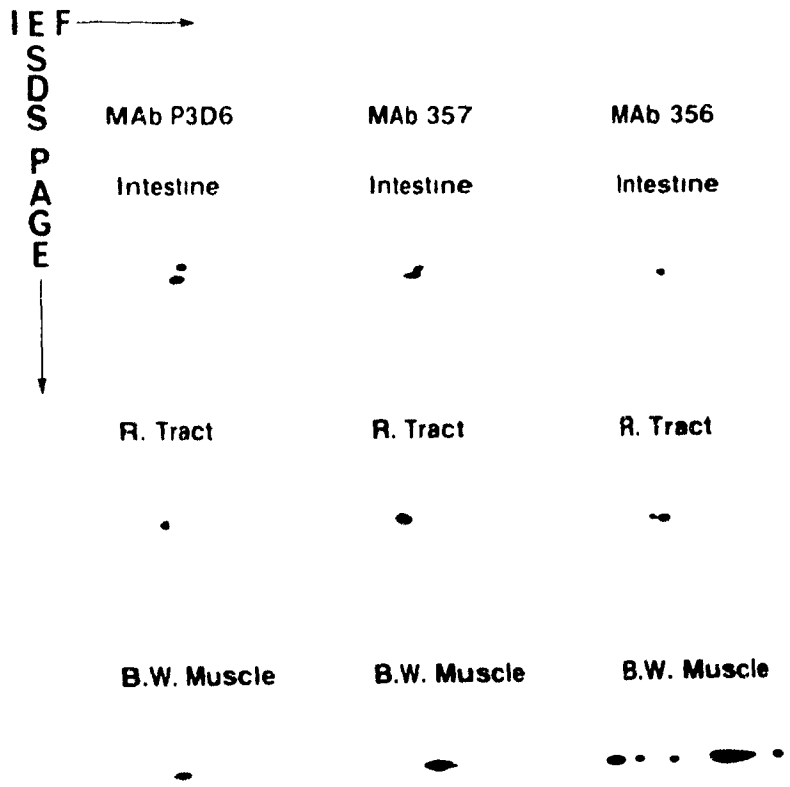
Interaction of MAb P3D6 with tubulin proteolytic fragments

In order to localize the antibody epitopes on tubulin fragments, Western blotting experiments using anti-α- and β-tubulin specific MAbs, following limited proteolysis of tubulin with chymotrypsin, were performed. Western blot analysis of tubulin peptides showed that after chymotrypsin digestion of intestinal tubulin, anti-*Ascaris* MAb P3D6 reacted with large β-tubulin peptide fragments of 35, 30 and 24 kDa (Fig 5.3, panel A, lane 1). In the reproductive tract this MAb recognized a β-tubulin fragment of 35 kDa (lane 2). However, this MAb did not react with body wall muscle chymotryptic peptides, and reacted only with undigested tubulin (lane 3-4).

With the intestinal tubulin, MAb 357 reacted with the large β-tubulin fragments of 35 and 24 kDa (Fig. 5.3, panel B, lane 1). However, in the reproductive tract and body wall muscle peptides, the same MAb reacted only with undigested tubulin. It did not recognize any chymotrypsin-digested peptides (lane 2-5). Anti-chick α-tubulin MAb 356 reacted with the large α-tubulin fragments of 30 and 24 kDa from intestinal

Fig. 5.2. Western blot analysis of *A suum* intestinal, reproductive tract, and body wall muscle homogenate, after 2-dimensional SDS-PAGE. The gels were transblotted onto nitrocellulose sheets and immunostained with anti-*Ascaris* β tubulin MAb P3D6, anti-chick β tubulin MAb 357 and anti-chick α tubulin MAb 356. Thirty five µg of each protein was loaded per tube gel

R. Tract = Reproductory Tract, B W. Muscle = Body wall muscle.


IEF
SDS PAGE
MAb P3D6
MAb 357
MAb 356
Intestine
Intestine
Intestine
R. Tract
R. Tract
R. Tract
B.W. Muscle
B.W. Muscle
B.W. Muscle

tubulin (Fig. 5.3, panel C, lane 1) but only the 30 kDa fragment of reproductive tract and body wall muscle tubulins (lane 2-3).

DISCUSSION

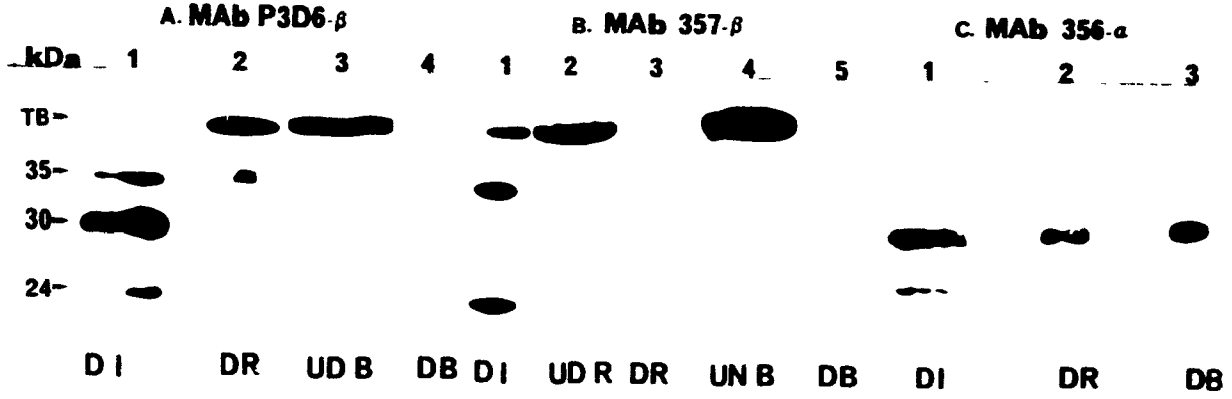
In this chapter, anti-*A. suum* β-tubulin MAb and commercial cross-reactive anti-chick brain α- and β-tubulin MAbs were used to characterize the α- and β-tubulin recovered from intestine, reproductive tract and body wall muscle of adult *A. suum*.

One dimensional gel analysis of patterns of *A. suum* intestine, reproductive tract and body wall tissues revealed that the relative mobilities of the tubulin subunits were consistent with the relative molecular size with α-tubulin the largest and β- tubulin the smallest (Cleveland *et al.*, 1980; Tang & Prichard, 1988). Anti-chick β- tubulin MAb 357 recognized both β_1- and β_2- tubulin bands in all tissues of *A. suum*. However, the anti-*A. suum* tubulin MAb P3D6 bound specifically to β_1- but not β_2- tubulin in the three tissues of *A. suum* and did not react with pig brain tubulin.

Intestinal tubulin of adult *A. suum* has been reported to be the most sensitive target for BZs (Borgers *et al.*, 1975; Borgers & De Nollin, 1975). However, it is still not clear if the sensitivity of intestinal tubulin to BZ is due to the increased drug availability in the intestine or due to the differences in the amino acid sequences which would result in different isoform patterns. Some authors have postulated that the selective toxicity of the BZs for nematodes is due to the differences in the structure of nematode tubulins compared with mammalian tubulins (Davis & Gull, 1983). This study has demonstrated that the number of tubulin isoforms, electrophoretic mobilities and patterns of expression were different among the three tissues of *A. suum*. β-tubulin spots observed by MAb 357 in all tissues, did not correspond to the spots observed with MAb P3D6 in the three tissues, however, these isoforms were within the same pH range. It should be noted that detection of tubulin isoforms within similar pH range and migration pattern in all tissues does not necessarily mean that the α- and β-tubulin isoforms in one tissue are the same as those found in other tissues. The number and electrophoretic mobilities of β-tubulin isoforms found in the intestine were distinctly different from body wall muscle or

Fig. 5.3. Limited proteolysis and Western blots of *A. suum* tubulin. The electrophoretically separated peptides were immunostained with anti-*A. suum* β tubulin MAb P3D6 (Panel A). Lane 1: digested intestinal tubulin; lane 2: digested reproductive tract tubulin; lane 3: undigested body wall muscle tubulin; and lane 4: digested body wall muscle tubulin; (Panel B) immunostained with anti-chick β tubulin MAb 357. Lane 1: digested intestinal tubulin; lane 2: undigested reproductive tract tubulin; lane 3: digested reproductive tract tubulin; lane 4: undigested body wall muscle tubulin; and lane 5: digested body wall muscle; (Panel C) immunostained with anti-chick brain α tubulin MAb 356. Lane 1: intestinal tubulin; lane 2: reproductive tract tubulin; and lane 3: body wall muscle tubulin. Twenty to thirty μg protein was loaded onto each lane.

DI = digested intestine, DR = digested reproductive tract, UD R = undigested reproductive tract, DB = digested body wall muscle, UD B = undigested body wall muscle.


A. MAb P3D6-β
B. MAb 357-β
C. MAb 356-α
kDa
1
2
3
4
1
2
3
4
5
1
2
3
TB
35
30
24
D I
DR
UD B
DB
D I
UD R
DR
UN B
DB
DI
DR
DB

reproductive tract. It is of particular importance since a number of studies (Borgers *et al.*, 1975; Borgers & De Nollin, 1975) have suggested that intestinal tubulin is the primary target of BZs attack. The differences in the isoform patterns observed in the various tissues of *A. suum* might contribute to the understanding of the selective toxicity of BZ anthelmintic drugs to the different tissues of *A suum*. Anti-*A suum* MAb demonstrated a remarkably high degree of species and isoform specificity.

Limited proteolysis of various tissues of *A. suum* with chymotrypsin showed that anti-tubulin specific MAbs bind in distinct patterns to tubulin. These data confirm the two- dimensional observations that different tubulins are found in different tissues of *A. suum*. Proteins of the size of tubulin are generally built of several structural domains that have distinct functions. In the case of tubulin, such functions include binding of GTP and of microtubule-associated proteins, the association between monomers, dimers or protofilaments and the binding of anti-microtubule drugs. Nematode and isoform specific anti-tubulin MAbs can serve to characterize the structure and distribution of the tubulin molecule in the various tissues of *A. suum*

In this chapter, using *A. suum* β-tubulin specific MAbs, is reported that *A. suum* contains multiple tubulin isoforms, and that these isoforms are differentially expressed within different tissues of the parasite. Furthermore, the knowledge of differences in tubulin isoforms from different tissues may be important in determining their selective sensitivity to BZ attack and in relating isoform expression to functions. The specificity of the different β tubulin isoforms in *A. suum* tissue may relate to different biological functions for the tubulins, and microtubules formed from these tubulins, and may be related to the differential tissue sensitivity to benzimidazole (BZ) drugs.

CHAPTER 6

GENERAL DISCUSSION

The studies described in this thesis were undertaken to examine: (1) the immunological and drug binding differences in the tubulins from the intestine, body wall muscle and reproductive tract of *A. suum*, and (2) the immunological differences between the tubulins from filarial nematodes and gastrointestinal nematodes. In order to address these questions, the properties of tubulins from a filarial nematode *B. pahangi* and a gastrointestinal nematode *A. suum* were investigated and compared.

Tubulin, the main structural component of MTs, has particular importance in nematodes as MTs are targets for anthelmintic chemotherapy and also because nematodes present many unusual structural and biochemical features. It is important to determine the pattern of expression of the tubulin family members because different tissues have various functions and behaviours. If a protein is expressed differentially one might gain insight into its functions and importance in the various tissues in which it is found. It is therefore necessary to localize the protein within the cell, as sorting of different isoforms of a single protein type within the cell implies that the different isoforms have different functions. In previous studies, antibodies specific for tubulin have been employed as specific probes for the study of tubulin isoforms, post-translational modifications of tubulin, molecular mapping of its functional domains and for the study of the orientation of α- and β-subunits of MTs in a number of species. In order to understand better the heterogeneity and sensitivity of tubulin in this nematode, my study focused on the production of MAbs to nematode tubulin and their use in the characterization of tubulin from the intestinal and tissue dwelling nematodes *A. suum* and *B. pahangi*.

B. pahangi

The first set of specific objectives of this study were to produce monoclonal antibodies against tubulin from the tissue nematode *B pahangi* and to use them to (1) investigate the heterogeneity of tubulins from nematodes and mammals; (2) to determine their effects on the viability of adult *B. pahangi*; (3) to localize β-tubulin in adult *B. pahangi*; and (4) to detect β-tubulin in the sera and peritoneal fluid from infected gerbils and sera from infected humans, dogs, and cats

The data from my study on the filarial nematode *B pahangi* demonstrate that tubulin was successfully purified by polylysine chromatography, SDS-PAGE and electroelution. Five IgM and forty nine IgG isotype MAbs were obtained after immunization of mice with purified *B. pahangi* tubulin Although mice were immunized with both α- and β-tubulins, MAbs were produced against β-tubulin only One dimensional SDS-PAGE followed by Western blotting demonstrated that all anti-*B. pahangi* tubulin MAbs recognized β_1 tubulin, with the tubulin subunits being differentiated by molecular weight (Chapter 2)

Anti-*B. pahangi* MAbs P3D and 1B6, both IgG isotype were selected for more extensive characterizatio Anti-chick brain tubulin MAbs 357 was used as a positive control in most of the experiments Anti-*B pahangi* MAbs recognized β-tubulin from a number of filarial nematodes (*B. pahangi, B. malayi* and *D immitis*) and an intestinal nematode (*H contortus*). However, these MAbs did not cross-react with tubulin from pig brain, 3T3 mouse fibroblast cells or the parasitic protozoan *G. muris*. On the other hand, anti-chick β-tubulin MAb 357 reacted with pig brain, 3T3 mouse fibroblast and *G. muris* tubulins as strongly as it did with filarial and other nematode β-tubulins. These results indicated that the anti-*B pahangi* tubulin MAbs recognized epitopes that are conserved between filarial and intestinal nematode β-tubulins, but are not present in protozoan and mammalian β-tubulins However, the cross-reactive anti-chick MAb 357 recognizes an epitope that is conserved among filarial and intestinal nematodes, as well as protozoan and mammalian β-tubulin. The epitope of MAb 357 has been localized to a region of β-tubulin between amino acids 339-417 in the proteolytic fragments of pig brain tubulin (Serrano *et al.*, 1986) (Chapter 2).

Several other anti-tubulin MAbs raised against parasitic protozoa and nematodes have been isolated but these have been found to cross-react with tubulin from diverse species (yeast to human). Helm *et al.* (1989) have raised MAbs against microfilariae of *Brugia* species. Contrary to my anti-*B. pahangi* β-tubulin MAbs, their MAbs cross-reacted with mammalian tubulin.

Different isoforms were recognized by different anti-*B. pahangi* MAbs. MAb 1B6 appears to be specific to one β-tubulin isoform, whereas MAb P3D shows an isoform pattern similar to the one observed with the anti-chick β-tubulin MAb 357. In addition, MAb P3D recognized an isoform pattern which is similar to that recognized by the anti-peptide polyclonal antibody raised against the 18 amino acid sequence of the extreme C-terminal region of *B. pahangi* β-tubulin (Guénette *et al.*, 1991). MAb 357 and the anti-peptide antibody recognize two β-tubulin isoforms in *B. pahangi* extract (Chapter 2) Whether these isoforms are the product of different tubulin genes or the result of post-translational modifications, is not known.

Limited proteolysis of *B. pahangi* tubulin followed by Western blot analysis, demonstrated that anti-*B. pahangi* MAb P3D reacted with the C-terminal fragment of the chymotryptic and V8 protease digests. It has been suggested by Mandelkow *et al.* (1985) that the strong reactivity with the C-terminal fragments could be due to the fact that this region is exposed in native tubulin. MAb 1B6 reacted with the N-terminal fragments of the chymotrypsin-digested β-tubulin. This MAb reacted only with the intact tubulin, no reaction was observed with V8 protease proteolytic fragments. Using MAb 357, chymotrypsin or V8 protease digested proteolytic fragments did not show any reaction. Yet MAb 357 recognized undigested β-tubulin, suggesting that the epitope is destroyed by proteolytic cleavage (Chapter 2). The results observed with MAb 357 are in agreement with those obtained by Mizuno *et al.* (1985), using V8 protease-digested fragments of sheep brain and mung bean β-tubulin.

Measurement of the *in vitro* activity of anti-*B. pahangi* tubulin MAbs P3D and 1B6 and anti-chick brain tubulin MAb 357 against female *B. pahangi* was carried out to determine whether anti-*B. pahangi* or anti-chick tubulin MAbs could independently affect the live adult worms. MBZ was included in the study to determine whether the

presence of MBZ drug alone or together with MAbs has a differential effect The results in this study demonstrated an apparent decline in motility when the worms were cultured with anti-*B. pahangi* tubulin MAbs P3D and 1B6 However, no noticeable reduction in the motility was observed when the worms were treated with anti-*B. pahangi* MAb 1A5, anti-chick MAb 357, MBZ or IMDM/FCS medium without MAbs. The viability of the worms was assessed by MTT assay Anti-*B pahangi* MAbs P3D and 1B6 significantly reduced the viability of adult *B. pahangi in vitro* However, no significant reduction in the viability was observed when adult *B. pahangi* were exposed to anti-*B. pahangi* MAb, anti-chick MAb 357 and/or MBZ (Chapter 2)

Filarial species are unique amongst nematodes in the different morphology (Franz, 1988) and increased permeability of the cuticle Howells & Chen (1981) demonstrated that atypical methods of nutrient uptake occur in filarial nematodes, where the nutrients are taken up transcuticularly rather than via the mouth and intestine. It was postulated that the gut of the filarial nematodes is non-functional and that the cuticle serves as an absorptive surface. Adult *B. pahangi* has been shown to be able to take up glucose, amino acids and certain filaricidal compounds through the cuticle (Howells & Chen 1981). The easy accessibility of the cuticle to chemical agents makes it an attractive target for chemotherapy. Damage to the cuticle could render the worm more permeable, but this might upset the ionic balance of the tissues, render the parasite more vulnerable to the host's immune response and increase the penetration of certain anthelmintic drugs and chemicals (Ottesen, 1987) Dick & Wright (1973) demonstrated microtubule-like structures in the cuticle of a non-filarial pin worm, *Syphacia obvelata*. Subsequently, Rogers *et al.* (1974) reported the presence of tubulin between the hypodermis and the basal stratum of the cuticle, where it may serve a morphological function or may function in intracellular transport or nutrition.

It is suggested that the mechanisms by which the anti-*B pahangi* β-tubulin MAbs reduce the viability of adult worms may be due to their interference with microtubules in the body wall muscle of *B. pahangi*. The antibody may interfere with certain microtubule functions, but not with cellular distribution For example,

antibodies may block several MT-dependent motility processes, by aggregating MTs. The antibody may interfere with cellular architecture by disrupting links between MTs and other cytoplasmic structures. The antibody may interfere with microtubular integrity by binding to epitopes on the tubulin molecule which are essential for microtubule polymerisation or maintenance of structure. In a recent study, Draber *et al.* (1990) reported that five MAbs against N-terminal domains of tubulin inhibited the assembly of tubulin into microtubules *in vitro* from porcine brain. Similarly, Mandelkow *et al.* (1985) reported that some polyclonal anti-tubulin antibodies were able to inhibit tubulin assembly *in vitro*.

Reduction in the viability of adult *B. pahangi* worms, by antibodies raised against an internal or non-surface protein is an interesting finding, but the mechanism by which these MAbs reach their target, is not known. The data showing that an internal protein can have a significant inhibitory effect on the parasite are in agreement with the study of Pearce *et al.* (1988). The authors have demonstrated that the non-surface schistosome antigen paramyosin, when administered intra-dermally, conferred significant resistance against *Schistosoma mansoni* challenge infection in mice. Their study suggests that non-surface antigens can be protective.

Several investigators have raised MAbs against different filarial nematodes and used them as probes for the detection of target antigens. In this study, anti-*B. pahangi* and chick brain β-tubulin MAbs were used to detect β-tubulin in the sera and peritoneal fluid of *B. pahangi* infected gerbils and in the sera of *B. pahangi* infected dogs, *B. malayi* infected cats by Dot-ELISA. All gerbil sera, peritoneal fluids and dog sera from *B. pahangi* infected animals reacted strongly with anti-tubulin MAbs. The strong reactivity of the gerbil sera, peritoneal fluid and dog sera may be due to the continuous release of tubulin from the damaged or dead adults or circulating Mf.
To localize the distribution of β-tubulin in the various tissues of *B. pahangi*, immunogold electron microscopy was performed. Immunogold labelling of female *B. pahangi* revealed the presence of β-tubulin in the median and basal layers of cuticle, hypodermal layer, somatic muscle cells and developing microfilariae (Chapter 3). Whether the MTs serve a morphological function or whether they function in

intracellular transport or nutrition, as observed in the intestinal nematodes (Atkinson *et al.*, 1980), is not known. On the basis of these data it could be suggested that the MTs found in the cuticle and body wall of *B. pahangi* may play a morphological function or may function in intracellular transport or nutrition, which are routinely performed by MTs in the functional intestines of *A. suum*, *A galli* etc. The presence of β-tubulin in the cuticle and hypodermal layer of *B. pahangi* could serve as a source of circulating filarial β-tubulin.

A. suum

BZs are broad spectrum anthelmintic, which are active against a wide variety of larval and adult helminths. Several mechanism have been proposed for the action of BZs in adult nematodes: (1) interference with glucose uptake (Van Den Boosche & De Nollins, 1973); inhibition of fumarate reductase (Prichard, 1973) and disruption of cytoplasmic MTs in intestinal cells (Borgers *et al.*, 1975) In nematodes, the intestinal tubulin/MT system appears to be of particular importance in this respect since one of the primary morphological changes observed, after MBZ treatment of the host was the disappearance of the intestinal MTs of *A suum* (Borgers *et al* , 1975) Several studies have demonstrated the direct interaction of MBZ with tubulin obtained from intestine of *A. suum in vitro* (Friedman & Platzer, 1978; Kohler & Bachmann, 1981; Barrowman et al., 1984). However, no data on the interaction of BZs to other tissues of *A. suum* are available. Therefore, the second set of objectives of this study were to explore: (1) if the tubulins in the intestine, body wall muscle and reproductive tract of *A. suum* were equally sensitive to MBZ, and to determine the binding constants K_a and B_{max}; (2) to compare the tubulin content in the intestine, body wall muscle and reproductive tract to MBZ binding; and (3) to produce monoclonal antibodies against the intestinal tubulin of *A. suum* and use them to investigate the heterogeneity of α- and β-tubulin contents, isoforms, and proteolytic maps in the different tissues of *A. suum*.

The data from my MBZ binding studies demonstrate that there are marked differences in the total binding of MBZ to the tubulin from intestine, body wall muscle

and reproductive tract extracts of *A. suum*. Intestine, body wall muscle and reproductive tract extracts also differ in levels of HAB; more MBZ is bound by intestine rather than body wall muscle, however, in reproductive tract HAB was not detectable. After correction for non-specific binding of ligand in the total binding, by the computer program LIGAND, the results indicated that the binding affinity of MBZ to intestinal tubulin (10.8×10^6) was similar to that of body wall muscle (9.8×10^6). The B_{max} of MBZ for tubulin of intestine (24.3 pmoles/mg tubulin) of *A suum* was about 3 fold higher than that of body wall muscle (7.8 pmoles/mg tubulin) (Chapter 4). These data suggest that the receptor(s) for the MBZ may be similar in the intestine and body wall muscle (K_a). However, the concentration of the receptor(s) per milligram of protein (B_{max}) was greater in the intestine than in the body wall muscle. In other words, the tubulin from the intestine binds more MBZ than does the tubulin from body wall muscle However, MBZ binding occurs with the same affinity to the intestine and body wall muscle tubulin. This higher binding ratio of MBZ to tubulin dimer from the intestine compared with body wall muscle and reproductive tract may contribute to the higher sensitivity of the MTs of this tissue to the MBZ attack. However, the pharmacokinetic behaviour of the drug in the intestine may also play a role in this selective toxicity of MBZ to this tissue. LAB was similar in the intestine and body wall muscle. However LAB binding in the reproductive tract was similar to that of the total binding Because MBZ binding was not saturable in the reproductive tract, parameters such as K_a and B_{max} were not calculated for this tissue. For intestinal tubulin, the data are consistent with several *in vitro* ultrastructural studies (Borgers *et al* , 1975; Atkinson *et al* , 1980; Franz *et al* , 1990a,b) which suggest that the intestine is more susceptible to MBZ than are the body wall muscle or reproductive tract.

Electron microscopy demonstrated that intestinal cells contain more MTs than body wall muscle which in turn contain higher number of MTs than the reproductive tract. The marked differences in the effects of MBZ on the various tissues could be due to differences in the tubulin content (Chapter 4). However, tubulin content alone may not explain the differences in the sensitivity of these tissues to MBZ. The selective BZ

binding may also depend on the type(s) of tubulin found in different tissues

Tubulin, which is the major structural component of MTs, is a heterodimer composed of two distinct polypeptide chains, α and β Both α and β subunits are expressed as heterogenous but closely related families of multiple isoforms in different organisms, tissues and even within single cells of the same organism (Fulton & Simpson, 1976; Gozes & Littauer, 1978; Sullivan, 1988, Birkett *et al*, 1985) The precise nature or role of α- and β-tubulin isoforms has not yet been elucidated, although several investigators have demonstrated that many *in vivo* functions of tubulin are to some extent isoform specific (Gundersen *et al.*, 1984; Banerjee & Luduena, 1987).

Using *A. suum* β-tubulin specific MAbs, it is reported that *A. suum* contains multiple tubulin isoforms and that these isoforms are differentially expressed within different tissues of the parasite The knowledge of differences in tubulin isoforms from different tissues may be important in determining their selective sensitivity to BZ attack and in relating isoform expression to function

Forty IgG isotype MAbs were obtained against intestinal β-tubulin of *A suum* One of these, anti-*A suum* MAb P3D6, and two commercial cross-reactive anti-chick brain α-356 and β-357 tubulin MAbs, were used to characterize the isoforms of α and β tubulin recovered from the intestine, reproductive tract and body wall muscle of adult *A. suum*. Analysis of one dimensional gels of *A suum* intestine, reproductive tract and body wall tissues revealed that the relative mobilities of the tubulin subunits were consistent with relative molecular size, α-tubulin being the largest and β-tubulin the smallest (Cleveland *et al*, 1980, Tang & Prichard, 1988) Anti-chick β-tubulin MAb 357 recognized both β_1- and β_2- tubulin bands in all tissues of *A suum* However, the anti-*A. suum* tubulin MAb P3D6 recognized specifically β_1- but not α or β_2-tubulin in the three tissues of *A suum* MAb P3D6 is highly species-specific. It cross-reacts with tubulin from different tissues of *A. suum* but does not recognize mammalian tubulin (Chapter 5) From the width and intensity of α- and both β-tubulin subunits, using SDS-PAGE followed by Western blotting, it was observed that intestine has the highest amount of tubulin and reproductive tract the least This

finding confirms and is in agreement with the results obtained from electron microscopic studies on the three tissues (Chapter 4).

Intestinal tubulin of adult *A suum* has been reported to be the most sensitive target for BZs (Borgers et al , 1975; Borgers & De Nollin, 1975). However, it is still not clear if the sensitivity of intestinal tubulin to BZ is due to the increased drug availability in the intestine or due to the differences in the amino acid sequences which would result in different isoform patterns. Some authors have postulated that the selective toxicity of BZs for nematodes is due to the differences in the structure of nematode tubulins compared with mammalian tubulins (Davis & Gull, 1983). This study has shown that the number of tubulin isoforms, electrophoretic mobilities and patterns of expression were different among the three tissues of *A. suum*. β-tubulin isoforms detected by MAb 357 in all tissues, did not correspond to the isoforms detected with MAb P3D6 in the tissues, however, these isoforms were within the same pH range It should be noted that detection of tubulin isoforms within similar pH range and migration patterns in all tissues does not necessarily mean that the α- and β-tubulin isoforms in one tissue are the same as those found in other tissues. The number and electrophoretic mobilities of β-tubulin isoforms found in the intestine was distinctly different from body wall muscle and reproductive tract. The differences in the isoform patterns observed in the various tissues of *A. suum* might contribute to the understanding of the selective toxicity of BZ anthelmintic drugs to the different tissues of *A. suum* This is of particular importance since a number of studies (Borgers et al , 1975; Borgers & De Nollin, 1975) have suggested that intestinal tubulin is the primary target of BZs attack. Anti-*A suum* MAb demonstrated a remarkably high degree of species and isoform specificity The specificity of the different β-tubulin isoforms in *A. suum* tissue may relate to the different biological functions of the tubulins and microtubules formed from these tubulins, and may be related to the differential tissue sensitivity to benzimidazole (BZ) drugs. Limited proteolysis of various tissues of *A. suum* with chymotrypsin showed that anti-tubulin specific MAbs bind in distinct patterns to tubulin. These data confirm the 2-dimensional observations that different tubulins are found in different tissues of *A. suum* (Chapter

5).

The following may be summarized or concluded from this study.

1. Species-specific MAbs were raised against β-tubulin of adult *A. suum* and *B. pahangi*. These MAbs recognize tubulin from a number of filarial and intestinal nematodes but do not recognize tubulin from mammalian cells

2. β_1- and β_2-tubulin peptides were recognized in body wall muscle, intestine and reproductive tract of *A. suum*, for the first time, in the nematode using anti-chick brain MAb In *B. pahangi* only β_1-tubulin is recognized by anti-*B. pahangi* tubulin MAbs. Anti-*A. suum* tubulin MAb is also specific to β_1 tubulin but not to α- or β_2 in all tissues of *A suum* investigated

3. α and β-tubulin from body wall muscle, intestine and reproductive tract of *A. suum* are different in their electrophoretic mobilities, isoform numbers, isoelectric points and drug binding abilities. Differential expression of α- and β-tubulin isoforms, may lead to changes in tubulin binding of various ligands β-tubulin isoforms in the intestinal extract of *A suum* were different from those of body wall muscle, and reproductive tract, in number and electrophoretic mobilities. Anti-α MAb 356 recognized an isoform pattern which is different in various tissues and different from that recognized by anti-β MAb 357 and P3D6, in the number of isoforms, electrophoretic mobility of various isoforms and pH range; MAb P3D6 recognized an isoform pattern which is different in various tissues and from that recognized by MAb 357. Tubulin isoforms recognized by anti-*B. pahangi* MAbs differ from those found in *A suum*, in their electrophoretic mobility, isoform number and pH range

4. Differences in the electrophoretic mobility and number of isoforms in the intestine may account for the sensitivity of this tissue to BZ attack, compared

to body wall muscle, and reproductive tract.

5. Limited proteolysis followed by Western blotting of tubulin from various tissues of *A. suum* showed that anti-tubulin MAbs bind in distinct patterns to tubulin. Limited proteolysis analysis also indicate that the antigenic sites recognized by anti-*A. suum* MAb differ from those recognized by anti-*B.pahangi* MAbs. These data are consistant with the two dimensional SDS-PAGE observations that different tubulins are found in various tissues of *A. suum*. It is possible that differences in the α- and β-tubulin peptide maps are due to extensive post-translational modifications or they may be the products of different genes.

6. Different levels of specific MBZ binding were observed for intestine, body wall muscle and reproductive tract tubulins. These differences could be due to the differences in the quantities of tubulin or the types of tubulins found in these tissues

7. Anti-β-tubulin specific MAbs P3D and 1B6, significantly reduced the viability of adult *B. pahangi, in vitro*. No significant reduction was observed when the *B. pahangi* were exposed to anti-chick β-tubulin MAb 357 and/or MBZ

8. Anti-B. pahangi tubulin MAbs were able to detect β-tubulin in the sera and peritoneal fluid from infected gerbils, sera from cats, dogs and humans

9. Immunogold labelling of *B. pahangi* female with anti-*B. pahangi* MAbs revealed that β-tubulin is present in the median and basal layers of cuticle, hypodermis and in the somatic muscles. β-tubulin was also found in the uterine wall and the developing embryos; however, tubulin was not seen in the intestine of the worm.

In conclusion, it is believed that MAbs specific for the β-subunit of tubulin would allow the subcellular localization and the function of each subunit to be studied. Proteins of the size of tubulin are generally built of several structural domains that have distinct functions. In the case of tubulin, such functions include binding of anti-microtubule drugs, GTP or microtubule-associated proteins and the association between monomers, dimers or protofilaments. Nematode-specific anti-tubulin MAbs may serve to characterize the structure and distribution of the nematode tubulin molecule, and to define microtubule stability and functional domains.

The use of nematode specific MAbs in antigen detection assays have demonstrated that they can identify individuals with pre-patent or occult infections which are undetected by classic parasitologic tests; they give a more accurate indication of active infection than traditional serodiagnostic tests; they also circumvent the problem of specificity. MAb P3D-based Dot-ELISA could be useful as a specific and potentially practical test for detecting active infection of *B. malayi* and *O. volvulus*. Detection of *B. malayi* tubulin with Dot-ELISA in conjuction with anti-*Brugia* β-tubulin MAb P3D, could be considered as a useful addition to the diagnosis of lymphatic filariasis, leading to improved understanding of the epidemiology of filariasis.

FUTURE DIRECTIONS

* Anti-nematode tubulin specific MAbs could be used as a ligand to purify β-tubulin subunit or β-tubulin isoforms;

* These specific MAbs offer a tool to study the mechanisms of microtubule assembly and disassembly and the interactions between microtubules and other proteins or assembly modifying ligands;

* These specific MAbs could be used to identify the products of cDNA expression libraries and at this level it would be possible to match tubulin isoforms with their individual genes;

* These MAbs can be used to investigate changes in the numbers as well as the changes in the distribution of tubulin isoforms in different developmental stages of various nematodes;

* These MAbs can be used as immuno-diagnostic tools, for the detection of circulating antigen, which would be particularly useful in epidemiological surveys of lymphatic filariasis;

* These MAbs could be used to localize the binding site of different proteins and assembly-modifying ligands.

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APPENDIX I

Index Descriptors and Abbreviations

ABTS	2,2'-AZINO-bis(3-ETHYLBENZTHIAZOLINE-6-SULFONIC ACID)
MAbs	Monoclonal antibodies
ELISA	Enzyme-linked immunosorbent assay
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MES	2[N-morpholino]-ethanesulfonic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
GTP	Guanosine-5'-triphosphate
BSA	Bovine serum albumin
$MgSO_2$	Magnesium sulfate
$(NH_4)_2SO_4$	Ammonium sulfate
EDTA	Ethylenediamine tetraacetic acid
PBS	Phosphate buffered saline
IEF	Isoelectric focusing
2D	Two-dimensional
MTs	Microtubule(s)
BZs	Benzimidazole
MTT	[3-(4,5 dimethy(thiazol-2-y1)-2,5-diphenyl tetrazolium bromide]
DMSO	Dimethylsufoxide
MBZ	Mebendazole
kDa	Kilodalton
PMSF	Phenylmethyl sulfonyl fluoride
Pen/Strep	Penicillin/streptomysin
IMDM	Iscove's modified Dulbecco's mediun
HT	Hypoxanthine thymidine
HAT	Hypoxanthine aminopterin thymidine
FCS	Fetal calf serum
Ig	Immunoglobulin
1D	One-dimensional
B_{max}	Maximum BZ binding at infinite ligand concentration
K_a	Apparent association constant at equilibrium
IN	Intestine
BWM	Body wall muscle
RT	Reproductive tract
I	Intestine
LAB	Low affinity binding

HAB	High affinity binding
TB	Total binding
dpm	Disintegration per minute
EIA	Enzyme immunoassay
pmol	Picomole(s)

APPENDIX II

PUBLICATIONS

PUBLISHED

* Identification of tubulin isoforms in different tissues of *Ascaris suum* ***International Journal for Parasitology, 21/8 (Dec. 1991) p. 913-918.***

SUBMITTED

* *Brugia pahangi*: characterization and biological activities of monoclonal antibodies specific to nematode tubulin. ***Immunology, 1991.***

* Detection of β-tubulin in animal and human Filariasis: Use of anti-β-tubulin specific monoclonal antibodies for immunodiagnosis. ***Parasitology Research, 1991.***

IN PREPARATION

* Biochemical characteristics of the interaction of the mebendazole anthelmintic drug binding to body wall muscle, intestine and reproductive tract of *A. suum*.

ABSTRACTS SUBMITTED TO SCIENTIFIC MEETINGS

1991. *In vitro* effects of anti-tubulin Monoclocal antibodies on the viability of *Brugia pahangi. The 40th Annual Meeting of the American Society of Tropical Medicine and Hygeine. Dec. 4-8, 1991, Boston, U.S.A.*

1990. Tissue and ultrastructural localization of tubulin in the various tissues of *Ascaris suum*, using an anti-*A. suum* tubulin specific monoclonal antibodies. *The 65th Annual Meeting of The American Society of Parasitologists, June 26-30, 1990, Michigan State University, East Lansing, Michigan, U.S.A.*

1990. Localization of tubulin isoforms in various tissues of *Ascaris suum*, using anti-tubulin monoclonal antibodies. *The Fourth Spring Meeting of the Canadian Society for Immunology, March 9-12, 1990, Mont Roland, Quebec, Canada.*

1988. Monoclonal antibodies for *Brugia pahangi* tubulin *The 37th Annual Meeting of the American Society of Tropical Medicine and Hygeine. Dec 4-8 1988, Washington D.C., U.S.A.*