CHAPTER 1

INTRODUCTION

Human lymphatic filariasis is a chronic parasitic disease prevalent in many tropical and sub-tropical countries. This disease causes significant morbidity, and male infertility resulting in social and economical hardships to some individuals. Affected persons suffer from general clinical symptoms such as irregular episodes of fever, chyluria, hydrocele and elephantiasis of extremities, with abnormalities of the lymphatic system.

Filariasis is caused by infection with nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* that are transmitted through blood feeding mosquitoes. The principal vector for *W. bancrofti* transmission is *Culex quinquefasciatus* while *B. malayi* and *B. timori* are transmitted by *Mansonella* species. It is estimated that around 130 million people worldwide are infected with filarial parasites (Hall et al., 1998). The distribution and transmission are closely associated with socio economic behaviour of the endemic population. The number of infected individuals globally is on the increase due to rapid urbanization and poor sanitation that serves as a good breeding ground for the growth of the vectors and disease. *W. bancrofti* alone accounts for 90% of the infection worldwide (Michael and Bundy, 1997) and the disease is endemic throughout the tropics, in Southeast Asia, Central and West Africa, South America, and South-pacific regions. *B. malayi* infection is prevalent in Southern China, India, Indonesia, Malaysia, Thailand and Vietnam, whereas *B. timori* occurs mainly in the Lesser Sunda Islands of eastern Indonesia. In India *W. bancrofti* and *B. malayi* infection are a major public health problem with 48 million infected cases (Michael and Bundy, 1997). *W. bancrofti* is the
main causative agent prevalent in Tamil Nadu, Pondicherry, Andhra Pradesh and Orissa whereas \textit{B. malayi} is mostly found in southern state of Kerala but scattered foci of low prevalence have been reported in Tamil Nadu, Andhra Pradesh, Orissa and Assam. The overlap of \textit{W. bancrofti} and \textit{B. malayi} infection has to be examined, so there is a need for development of immunodiagnostic methods capable of identifying mixed infection.

The other filariae that infect humans includes \textit{Onchocerca volvulus}, \textit{Loa loa}, \textit{Mansonella perstans}, \textit{Mansonella ozzardi} and \textit{Mansonella streptocerca}. Adult worms of \textit{O. volvulus} reside in the subcutaneous nodules and the presence of microfilaria (mf) in the skin and eye leads to severe lesions and visual impairment. \textit{Loa loa} provokes temporary inflammatory swellings (callabar swellings), hypereosinophilia and allergic manifestations.

\textbf{1.1 OVERVIEW OF THE THESIS}

The World Health Organization has recently re-emphasized an urgent need to develop diagnostic tests to detect prepatent and patent infection and also the drug targets against the parasite. This would facilitate the control program by reducing the morbidity and hence preventing further transmission of the disease. So it would be important to identify and study in detail the candidate genes to address these two areas.

The most common diagnostic method of lymphatic filariasis is microscopic examination of night blood smears for the presence of microfilariae. The major disadvantages of this method are:

1. The lack of sensitivity and labour intensive nature.
2. Blood has to be collected in the night due to nocturnal periodicity of mf, which is generally not acceptable to the endemic community.
Hence a simple, specific and sensitive serological antigen based test has to be developed that is affordable and acceptable at the community level. A number of diagnostic assays have been developed using recombinant filarial antigens to detect filarial specific antibodies (Dissanayake et al., 1992; Theodore et al., 1993; Chandrasekar et al., 1994 and Ramzy et al., 1995) or to detect Circulating Filarial Antigen (CFA) using monoclonal antibodies (Weil et al., 1987b; More and Copeman, 1990; Theodore and Kaliraj, 1996; Weil et al., 1997). The use of Polymerase Chain Reaction (PCR) to detect parasite DNA in the endemic population has been investigated (McCarthy et al., 1996; Zhong et al., 1996; Abbasi et al., 1996; Siridewa et al., 1996) and the use of PCR ELISA for diagnosis has been also reported (Nutman et al., 1994; Rahmah et al., 1998).

The treatment of lymphatic filariasis relies on the intake of Diethyl Carbamazine (DEC), an antifilarial drug (Hawking, 1979) discovered 50 years ago. Nausea and dizziness among the microfilarics often accompany its use. Another drug called Ivermectin, a semisynthetic product having antiparasitic activity, has been used as a chemotherapeutic agent. Both DEC and Ivermectin are largely microfilaricidal and emerging evidence suggest an acquired resistance to ivermectin by the parasites (Clark et al., 1995; Ming et al., 1998). Therefore there is an urgent need to develop new drugs with known mechanism of action that are safe and effective against adult parasite or larval stages.

The Og4C3 antigen detection system is commonly available for the identification of Circulating Filarial Antigen (CFA) in Bancroftian filariasis. However the Og4C3 assay system is specific only to Bancroftian filariasis and this assay can not be used to identify Brugian filariasis. The filarial specific recombinant antigen isolated from pRWbSXP-1 was sensitive and specific for identification of active filarial infection based on IgG4 assay. However, it has
been demonstrated that the use of the same homologous antigen enhances the diagnostic assay due to species specificity (Rao et al., 1999). Further the monospecific antibodies to pRBmSXP-1 recombinant protein was useful to demonstrate CFA from Brugian and Bancroftian filarial infection. The assay could be used to identify filarial infection in areas where there is an overlap of Brugian and Bancroftian filariasis (Lalitha, 1999).

The major areas of filarial research in our laboratory are to identify, clone and characterize the parasite specific antigen genes for the development of sensitive diagnostic methods, to study prophylactic efficiency of recombinant antigen in animal models and cellular immune response in various clinical groups of filarial patients. Further the identification of parasite specific biochemical pathways or enzymes, that are essential for growth and development of the parasite, will be important in developing chemotherapeutic agents against filarial parasites.

The specific objectives of the present study are:

1) Identification, cloning, expression and characterization of a SXP-1 cDNA homologue from *W. bancrofti* L3 cDNA library using the pRBmSXP-1 gene as a DNA probe, for the development of specific and sensitive diagnostic assays.

2) Isolation (by 3' RACE PCR reaction), cloning, expression and characterization by immunoreactivity and enzyme activity assay of recombinant transglutaminase from *Brugia malayi* adult female parasite, with an ultimate goal to develop effective and safe chemotherapeutic agents against filarial parasites.

One of the approaches adopted for the development of a specific and sensitive antigen based assay for diagnosis of filarial infection is using
recombinant clones isolated from *W. bancrofti* L3 cDNA library using *B. malayi* SXP-1 gene as a DNA probe. The SXP-1 gene was identified by differential immunoscreening from *B. malayi* adult cDNA library (Dissanayake *et al.*, 1992). The BmSXP-1 was recloned and expressed as a histidine fusion protein from pMAL vector for the diagnosis of *W. bancrofti* infection (Rao, 1998). In the present study, the pRBmSXP-1 gene was used as a probe to screen the *W. bancrofti* L3 cDNA library. Identification, cloning and expression of pBSWbSXP-1 cDNA was done in a T7 system of pRSET B as N-terminal histidine fusion protein and purification of recombinant antigen was done by Immobilized Metal Affinity Chromatography (IMAC). Antibody detection assay was developed using filarial specific recombinant antigen, isolated from pRWbSXP-1 for diagnosis of active filarial infection employing the serum and blood collected on filter strips. Circulating Filarial Antigen assay was done by sandwich ELISA using monospecific antibodies to pRWbSXP-1 recombinant protein. Further, this antigen detection assay was compared with commercially available Og4C3 kit (More and Copeman, 1990). The second part of the thesis deals with isolation, cloning and characterization of Transglutaminase (TGase) cDNA from *Brugia malayi* adult female parasite by 3'RACE PCR reaction. Subcloning and sequencing of the PCR product was done in pCR II TOPO TA cloning vector. Expression and characterization of recombinant Transglutaminase was done in pTrcHis B vector.

The outcome of the present study is summarized as follows:

a) A Homologue of *Brugia malayi* SXP-1 was identified from a *W. bancrofti* L3 cDNA library as pBSWbSXP-1. The cDNA of pBSWbSXP-1 was sub cloned and expressed in T7 expression system as an N-terminal histidine fusion protein. The recombinant antigen was purified under denaturing conditions by Immobilized Metal Affinity Chromatography. The recombinant antigen
preferentially reacted with MF sera infected with *W. bancrofti* at the IgG4 level. Monospecific antibodies to this recombinant protein were useful for the detection of Circulating Filarial Antigen in filarial patients. The antibody and antigen detection system could be used for the identification of individuals with either Bancroftian and Brugian filariasis.

b) The transglutaminase cDNA was isolated from *Brugia malayi* adult female parasite by 3’ RACE PCR reaction. The PCR product was subcloned in pCR II-TOPO TA cloning vector and the sequence was determined. The TOPO BmTGase cDNA was recloned and expressed in pTrcHis B vector as N-terminal histidine fusion protein. Recombinant transglutaminase was characterized by western blot probing with rabbit anti *Dirofilaria immitis* TGase antibody and by enzymatic assay using microtiter assay for transglutaminase.

1.2 REVIEW OF LITERATURE

1.2.1 Life cycle of filarial parasites

The life cycle of filarial parasite passes through a definite host, the human and an intermediate host, the mosquito (Fig. 1.1). The adult worms live in the human lymphatic system for 10-15 years where they produce millions of larvae known as microfilariae (mf) (Leeuwin, 1962). The mf circulates in the blood stream of the host and follow nocturnal periodicity. The microfilariae enter the secondary host mosquito, during the blood feeding. Inside the mosquito, the mf undergoes molting that leads to the development of larval stages, L2 and L3. The infective larvae, L3 move to thoracic region and proboscis of mosquito and the human host is infected when mosquito feeds. In humans the L3 develop into L4 and finally to sexually mature adults. The prepatent period (the time from infection to detection of microfilariae released
by the adult female worm) is approximately six to twelve months (Denham and McGreevy, 1977).

1.2.2 Morphology of filarial parasites

Adult *W. bancrofti* and *B. malayi* filarial worms are minute and threadlike with a smooth cuticle. Adult males measure 40mm in length and 0.1 mm in diameter, whereas females are 80-100 mm in length and 0.24-0.30 mm in diameter (Nanduri and Kazura, 1989). The mf of both *W. bancrofti* and *B. malayi* are sheathed. *W. bancrofti* mf range from 244-296 µm in length whereas *B. malayi* mf ranges from 177-230 µm. The mf of *B. malayi* have two terminal nuclei. The last terminal nucleus of *B. malayi* mf is small and is at the tip of the tail whereas the *W. bancrofti* mf has body nuclei (Fig 1.2). Localization and isolation of *W. bancrofti* adult worms by ultrasonography has facilitated ultra-structural studies (Smith et al., 1996; Faris et al., 1998) of the parasite surface by scanning electron microscopy (DeSouza et al., 1993; Araujo et al., 1995).

1.2.3 Periodicity

The microfilaria are absent in the peripheral blood during the daytime. Their appearance in the peripheral blood at night is known as nocturnal periodicity. The mf count in peripheral blood reaches the maximum between 10 p.m to 2 a.m (Gupta et al., 1990). The sub-periodic forms of *W. bancrofti* are seen in the pacific region and exhibit highest level of mf in the night, but 40 to 60% of mf persist during the day time also. When not in the circulation, mf generally reside in the capillaries and the blood vessels of the
Larvae migrate to lymphatics - develop to adult worms.

**Wuchereria bancrofti**

**Brugia malayi**

Female liberates microfilariae into blood.

Mosquito ingests microfilariae with blood meal.

Infective larvae develop in mosquito.

Infective larvae enter host when mosquito takes blood meal.

Adult worms in lymphatic channels.

**Humans**

**Fig.1.1 Life cycle of filarial parasites**

**Fig.1.2 Morphological features of W. bancrofti and B. malayi microfilaria**

Adopted from Eberhand and Lammie (1991)
lung, this may be due to differences in the oxygen tension between the arterial and venous blood in the lungs (Burren, 1972; Nanduri and Kazura, 1989). The reason for the parasite periodicity is not known. However, it has been observed that the periodicity can be altered by reversing the working and sleeping habits of the host (Mak, 1987).

1.2.4 Clinical manifestations
1.2.4.1 Asymptomatic amicrofilaremics (Endemic Normals)

Healthy individuals, those living in endemic area have no microfilariae in the blood circulation, with no symptoms of the disease. These individuals show no microfilariae or circulating filarial antigens in their blood stream (Ramzy et al., 1991). More importantly the immune response to parasite antigens is significantly greater in these individuals than that seen in individuals with microfilaria or lymphatic disease (Ottesen et al., 1977; Ottesen, 1984).

1.2.4.2 Asymptomatic microfilaremics (MF)

The asymptomatic form of microfilaremics is most often characterized by presence of thousands of larval parasites in blood and adult worms in the lymphatic nodules of the infected individuals. Studies using lymphoscintigraphy have shown diffuse structure of lymphatics with lymph vessel dilation in MF individuals (Freedman et al., 1994). Moreover studies demonstrated that asymptomatic microfilarial individuals have haematuria and proteinuria (Dreyer et al., 1992). In vitro filarial antigen specific lymphocyte hyporesponsiveness and decreased production of IL-2 and IFN-γ in response to parasite antigens are the major hallmarks of MF individuals (King and Nutman, 1991; Ravichandran et al., 1997; Mahanty et al., 1997).
1.2.4.3 Chronic pathology

In the endemic area, a small number of microfilaria positive people develop chronic lymphatic disease. This group suffers from either an acute attack of lymphatic inflammation such as lymphangitis, lymphadenitis or from a chronic obstructive form of the disease characterized by elephantiasis of the leg. This state is initially transient and reversible but later becomes permanent resulting in irreversible elephantiasis. Hydrocele is a common disease manifested in *W. bancrofti* infection. Microfilariae are often absent in majority of these patients and they are negative for circulating filarial antigen. However mf is observed in some individuals probably due to reinfection. Further these individuals have considerably more vigorous antigen specific T cell responses than do the MF (Ottesen *et al.*, 1977; Nutman *et al.*, 1987; Ravichandran *et al.*, 1997). There is also the nonfilarial elephantiasis. Non filarial elephantiasis may be produced by deposits of inorganic materials in the lymph node of the lower extremities. The prevalence rates of non-filarial elephantiasis is generally higher in males than in females, indicating sex differences in occupational linked trauma to the feet and cumulative effect of long term exposure to volcanic soils (Kloos *et al.*, 1992; Ruiz *et al.*, 1994). So due to prevalence of elephantiasis of filarial and non-filarial origin there is a need for diagnostic test to differentiate between the two.

1.2.4.4 Tropical pulmonary eosinophilia (TPE)

Tropical pulmonary eosinophilia is an interstitial lung disease characterized by wheezing that result from hyperimmune response to filarial antigen. These individuals have elevated levels of total serum IgE and filarial specific IgG (Paxton *et al.*, 1993). Most individuals with acute TPE have a rapid clinical response to DEC with reduced cough and dyspnea, in some individuals, the pulmonary form of the disease progresses to a chronic form.
that results in interstitial fibrosis and permanent loss of lung function (Neva and Ottesen, 1978; Ottesen and Nutman, 1992). Although the tropical pulmonary eosinophilia syndrome of filarial etiology has very distinctive clinical and immunological features, its clinical profile is not unique. Other helminths might even sometimes induce similar clinical presentations due to sharing of antigenicity (Rocha et al., 1995).

1.2.5 Diagnosis of Lymphatic filariasis

An early diagnosis facilitates reduction in transmission of infection and morbidity. Due to the broad spectrum of filarial disease no single diagnostic test can be expected to meet all the requirements. A variety of diagnostic tools available have been reviewed (Nanduri and Kazura, 1989; Wamae, 1994; Chandrasekhar, 1997). These include conventional parasitological diagnosis, lymphatic-imaging techniques, immunological methods and DNA based methods.

1.2.5.1 Parasitological diagnosis

The most widely used method for diagnosis of filarial infection is the examination of blood smear for microfilariae. Counting chamber, membrane filtration, Knotts concentration techniques and DEC provocative day test have also become means of diagnosis (Denham, 1995). These methods of mf detection are less sensitive to identify low numbers of mf or mf sequestered in inaccessible sites (Wamae, 1994). The other disadvantage is the non-compliance of the population as the blood has to be collected during the night due to nocturnal periodicity of mf. Membrane filtration is a sensitive technique but it requires large volume of blood. Hence these techniques are difficult for large-scale field use in many parts of the world (Nanduri and Kazura, 1989). Even with these drawbacks, Geimsa staining of night blood smears continues
to be the most common method currently employed in many endemic areas for diagnosis of filarial infections.

1.2.5.2 Lymphatic Imaging

1.2.5.2.1 Lymphoscintigraphy

Lymphoscintigraphy is a simple, safe, noninvasive method to examine the peripheral lymphatic system, including truncal and nodal abnormalities, in endemic populations with occult and overt lymphatic filariasis (Freedman et al., 1994). In lymphoscintigraphy, radiolabeled albumin or dextran injected intradermally or subcutaneously is traced by gamma camera (Witte et al., 1993). Studies using conventional angiography or non invasive scintigraphy have revealed widespread abnormalities in the lymphatics of both legs of patients with bancroftian filariasis, regardless of whether clinical lymphoedema is present or not (Marchetti et al., 1998). Patients with elephantiasis show lymphstasis and significantly altered lymphatic vessel dilation dermal back flow and obstruction. Thus this technique provides clues about lymphatic system in-patients at risk. The advantages of this method are the ease of performance and non-invasive nature of the technique. However, the principal disadvantage of this method is its cost and unsuitability for field studies.

1.2.5.2.2 Lymphangiography

Alterations in the lymphatic anatomy of filarial patients were observed initially using lymphangiography technique (Sen and Ellappan, 1968). However the method is technically demanding, time consuming, invasive and uses oil based contrast material for imaging that can induce local morbidity aggravating the pathology. Thus, this technique is not useful for mass screening.
1.2.5.2.3 Ultrasonography

*W. bancrofti* adult worms were localized using a 50 MHz sectorial transducer by imaging the scrotal region of infected male patients. Using this technique live *W. bancrofti* adult worms have been isolated (Amaral *et al.*, 1994). These ultrasonographic findings revealed lymphatic dilation and tortuosity along with structures of peculiar aleatory movements known as “filarial dance” where the parasite were seemingly attached to the lymphatic endothelium (Suresh, 1996). However a main disadvantage of these methods is its cost and sophisticated instrumentation required for diagnosis and hence is not suitable for field studies.

1.2.5.3 DNA based diagnosis of filariasis

DNA of the parasite may be used in the identification of the filarial parasite, either adult or mf in host. Detection of parasite genetic material using nucleic acid probes, combined with PCR amplification techniques has been shown to be promising in the diagnosis of parasitic infections (Weiss, 1995). DNA probes are sensitive and specific but the sensitivity of DNA probes relies on the presence of repeat sequences in the parasite. The highly repeated DNA sequences are generally non-coding and evolve more rapidly than rest of the genome, thus making them potential targets for genus and species specific identification. Species specific DNA probes have been developed for *B. malayi*, *W. bancrofti*, *Onchocerca volvulus* and *Loa loa*. Such DNA based diagnostic methods have been developed for detecting parasite DNA in blood and vector population (Chandrasekhar, 1997).

From the *B. malayi* genome a 320 bp *Hha I* repeat sequence was identified and it was estimated that this repeat element is present in 30,000 copies and comprised 12% of the genome (McReynolds *et al.*, 1986).
Oligonucleotide probes derived from this repeat element was capable of specifically detecting 200 pg of *B. malayi* DNA (Williams *et al*., 1988). Another repeat element pBm15 was cloned from Sau3A digest of *B. malayi* (Sim *et al*., 1986a). When labeled with $^{32}$P the probe pBM15 was able to detect a single infective larva of *B. malayi* (Sim *et al*., 1986b). From the *W. bancrofti* genome, a repeat element of 969 bp generated by Sau3A digest was identified, and it was estimated that 450 copies per genome are present. The designated clone pWb12 could also detect DNA from single infective larva or one mf (Siridewa *et al*., 1994).

The conventional radiolabeled DNA probes are unsuitable for field studies because of the short half-life of radioactive materials and the problems associated with radioactive disposal and the need for stringent hybridization and washing conditions. Alternate methods of detection based on chemiluminescence combined with specific amplification of the target molecule by PCR have been studied (Nutman *et al*., 1994). A PCR assay has been developed using the *HhaI* repeat DNA sequence of *B. malayi* for detection of parasite in blood samples (Lizotte *et al*., 1994). A PCR assay based on the 195 bp genus specific repeat sequence from *W. bancrofti* was shown to detect one L3 in pools of mosquitoes (Nicolas *et al*., 1996). It has been reported that this assay could detect as few as one mf per ml from human blood samples (Zhong *et al*., 1996). This assay was further modified into a microtitre plate based method for rapid evaluation of field samples (McCarthy *et al*., 1996). Another PCR assay developed from a 969 bp repeat sequence pWb12 was also reported to detect *W. bancrofti* DNA in human blood samples, hydrocele fluid, and in mosquito vector (Siridewa *et al*., 1996). A PCR assay that amplifies a 2.3 kb repeat sequence designated as a Wb 19 from *W. bancrofti* patients using sputum samples (Abbasi *et al*., 1996). Recently PCR enzyme linked immunosorbent assay (PCR ELISA) has been developed to detect
Brugia malayi infected samples using finger prick samples collected on filter strips (Rahmah et al., 1998).

Thus several of these DNA based assays have been examined for the diagnosis of filarial infection. However, these assays are still in the process of development. Further, problems arising out of false positives, high cost, requirement of skilled personals, have to be studied and evaluated in endemic area before field applicability can be promoted.

1.2.5.4 Immunodiagnosis

Immunodiagnostic methods are generally based on the detection of antibody or circulating antigen in the serum. A variety of such diagnostic techniques based on heterologous or homologous antigens, crude, fractionated filarial worm antigens or recombinant filarial antigens and its monospecific or monoclonal antibodies have been developed and are discussed below. However their full diagnostic potential remains to be validated for various endemic population.

1.2.5.4.1 Antibody detection assay

The classical earlier methods were based on skin test or serological determination using complement fixation (CFT), diffusion in gel (GD), latex agglutination and indirect haemagglutination (IHAT), to assess cellular immune response or antibodies generated by the host against the parasite. Using W. bancrofti mf antigens IFAT, IHAT and ELISA (Dasgupta et al., 1984) have been developed for the diagnosis of filariasis and the efficiency of the tests was compared. They found that ELISA was simple and sensitive compared to other methods of detection (Kaliraj et al., 1981a). Using the B. malayi and B. timori parasite antigens the classical antibody assay was
replaced with labeled reagent assays such as immunofluorescent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA) and immunoradiometric assays (IRMA) and were highly sensitive in detecting very low levels of antibodies (Kaliraj et al., 1981b; Au et al., 1982). Most of these tests are based on the identification of filarial specific total Immunoglobulins.

1.2.5.4.2 IgG4 antibody based assays

The major immunological hallmark of the filarial parasites is the unusual high levels of filarial specific IgG4 antibodies (Ottesen et al., 1985b). Studies have shown in MF that levels of IgG4 are associated with levels of circulating filarial antigen (Dimock et al., 1996). The most significant differences were noticed in the levels of IgG4 in MF patients which were 17 times higher than those observed in CP (Hussain et al., 1987). Measurement of IgG4 against B. malayi antigens and Phosphorylcholine epitopes has been examined for the diagnosis of filariasis (Lal and Ottesen, 1989). It was possible to identify active filarial infection by demonstrating IgG4 antibodies.

1.2.5.4.3 Heterologous antigen based antibody assays

The non-availability of homologous antigens has led to the identification and purification of heterologous filarial antigens that show specificity to human filariasis. Parasite excretory-secretory products and Circulating Immune complex have shown to be potentially useful for the detection of antibodies because they appear more species specific than crude somatic extracts (Kaushal et al., 1984). Monoclonal antibodies K3AE7 and K3BDS raised against excretory-secretory (ES) antigens of S. digitata were shown to be promising in the diagnosis of W. bancrofti infection (Dhas and Raj, 1995). Filarial antigen detection system “Seva-Filachek”, developed based on B. malayi mf ES antigens has been shown to be useful in detecting occult filarial infections (Harinath et al., 1996). Secretory acetylcholinesterase
(75 kDa and 45 kDa) from *Setaria cervi* microfilariae, have been purified via affinity chromatography on edrophonium sepharose column and it was shown to be a potential antigen for diagnosis of human filariasis (Sharma and Rathur, 1999). The presence of Immune complex in filariasis containing a 200 kDa glycoprotein when probed with BmA antibody was observed (Lunde *et al*., 1988). Monoclonal antibody ELISA has been developed to detect Circulating Immune Complex (CIC) in filariasis patients. It has been found that this MAb reacts with three fractions of antigens of *W. bancrofti* mf sheath and it is stage specific and suggests that the mf play an important role in CIC formation in filariasis (Kobayashi *et al*., 1997).

### 1.2.5.4.4 Homologous antigen based antibody assays

The homologous antigens can be obtained either by fractionation of the crude worm extracts or by recombinant DNA technology. Several fractionated homologous antigens from *W. bancrofti* and *B. malayi* were studied for their potential diagnostic use in filariasis. These antigens are preferred over the heterogeneous antigens since they exhibit diminish cross reactivity among the different nematode species. Soluble antigens from *W. bancrofti* mf were fractionated by Sephadex G150 gel filtration and their utility in diagnosis was evaluated by indirect haemagglutination test (IHAT) and enzyme linked immunosorbent assay (ELISA) (Kaliraj and Harinath, 1982). The use of human filarial serum concentrated from clinical patients was evaluated for the detection of circulating antigen by counter immunoelectrophoresis (Kaliraj *et al*., 1981c). Fractionated and albumin adsorbed antigen of *W. bancrofti* from urine of filarial patients, UFA C2A was used in stick enzyme assay which indicated filarial antibody in 89% of MF, 84% of CP and 7% of EN (Ramprasad and Harinath, 1995). In inhibition ELISA using anti-*B. malayi* adult antibody and antigen fraction BmA-6, the presence of filarial antigen was demonstrated in 85% of MF, 35% of CP and
20% of EN individuals (Chenthamarakshan et al., 1995). It was observed that, the purified antigen showed sensitive and specific reactions in ELISA for the detection of antibodies in filarial sera and showed least cross reactivity with other parasitic infections compared with the crude antigens (Theodore and Kaliraj, 1990). Two antigens of molecular weights 17 kDa and 21 kDa were identified to be secreted or present as surface associated in *W. bancrofti* and *L3* by intrinsic and extrinsic radiolabeling (Maizels et al., 1986). A glycoprotein of 29 kDa and a ladder of proteins between 17-200 kDa have been identified on the surface of *B. malayi* by surface-labeling techniques (Maizels et al., 1989; Wamae, 1994).

**1.2.5.4.5 Recombinant antigen based assays**

Recombinant DNA technology provides an avenue for producing and purifying substantial quantities of specific parasite antigens. IgG4 based assays have been developed to identify individuals with active infection using the recombinant filarial antigens. Paramyosin cDNA has been isolated from *Brugia malayi* L3 stage and used for IgG4 assay and the reduction of anti IgG4 titer following combined chemotherapy with DEC and ivermectin was significantly correlated with a reduction in the adult worm burden (Langy et al., 1998). Differential immuno-screening of adult *B. malayi* cDNA library, a novel parasite antigen SXP-1 was identified (Dissanayake et al., 1992). It was observed that about 80% of the MF and 33% of CP contained IgG antibodies to recombinant SXP-1 (Dissanayake et al., 1994). In the SXP-1 antibody assay had 100% specificity and 100% sensitivity for the patients with bancroftian filariasis (Rao et al., 1999). A recombinant clone pGT7 selected from a genomic expression library of *W. bancrofti* mf in λgt11 (Raghavan et al., 1991) was shown to specifically recognize IgG4 antibodies in MF (Theodore et al., 1993). Similarly Chadrasekhar et al. (1994) identified and cloned an antigen BmM14 by screening a *B. malayi* cDNA library. The recombinant BmM
14 specifically recognized IgG4 antibodies in MF sera. Further the BmM14 antibody test was separately evaluated in Egypt and it was observed that >90% of sera from MF and 60% for CP were positive (Ramzy et al., 1995).

1.2.5.4.6 Antigen assays

Since most of the antibody detection systems could not differentiate between current and past infection, parasite antigen detection in blood and other body fluids has been the focus of research for the past 10 years (Dissanayake et al., 1982; Reddy et al., 1984; Hamilton et al., 1984). Several homologous and heterologous antigen preparations have been used and monoclonal antibodies have been produced which appear to be potentially useful for filarial antigen detection. Several investigators demonstrated circulating antigens in filariasis using monoclonal antibodies. Earlier workers have demonstrated the presence of circulating antigens in 93% of sera from humans with Bancroftian or Brugian filariasis by a double antibody sandwich enzyme linked immunosorbent assay, using rabbit antisera to *B. pahangi* adult worms (Au et al., 1981). The presence of circulating antigen in patients with bancroftian filariasis by immunoradiometric assay (IRMA) using rabbit polyclonal antisera labeled with $^{125}$I have been demonstrated (Paranjape et al., 1986). MAb Gib13 raised against *Onchocerca gibsoni* egg and mf antigens has been used in IRMA assay to detect circulating antigen in the sera of bancroftian filariasis (Forsyth et al., 1985). Another MAb,E34 rasied against *W. bancrofti* mf ES antigens was able to detect filarial antigen associated with active infection (Reddy et al., 1986). A MAb raised against a major 200 kDa circulating antigen was directed against phosphocholine (PC) epitopes. Though this PC determinant itself is not filarial specific its abundance in PC bearing filarial antigen in circulation makes it a potentially useful target for immunodiagnosis (Lai et al., 1987). A polyclonal rabbit anti filarial antiserum as capture antibody, and a monoclonal antibody as reacting antibody to identify,
circulating parasite specific antigen in bancroftian and brugian filariasis patients, it was observed that 95 % of the MF sera had circulating antigens whereas only 60 % of the CP patients with hydrocele or elephantiasis and 15 % of the EN were antigen positive (Zheng et al., 1987). Antibodies raised against *W. bancrofti* microfilarial SDS soluble antigen were used to develop filarial antigen dipstick ELISA and it was positive in MF patients (Cheirmaraj et al., 1992). Recently circulating filarial antigen (CFA) was detected by a MAb Og4C3 directed against antigen of *O. gibsoni* in a sandwich ELISA. This antigen was detected only in those patients infected with bancroftian filariasis and not *B. malayi, B. timori, O. volvulus or Loa loa* (More and Copeman, 1990; Lalitha et al., 1998). The poly and monoclonal antibodies derived from pGT7 were used in a sandwich ELISA for the detection of circulating parasite antigens (Theodore and Kaliraj, 1996). More recently a rapid form of filarial antigen card test was developed by ICT diagnostics, Australia, based on AD12.1 MAb and was found to be specific for bancroftian filariasis (Weil and Liftis, 1987a; Weil et al., 1997). Yet another MAb raised against *W. bancrofti* L3 larvae recognized 95 kDa antigen, this MAb did not react with nine other nematode species (*B. malayi, O. volvulus, B. pahangi, N. brasiliensis, Toxocara canis, O. gutturosa, Trichuris muris, A. lumbricoides, Trichurs triichiura*) and two vector species (*Aedes aegypti and Anopheles farauti*) and thus appears to be promising immunodiagnostic agent (Burkot et al., 1996). Several groups have used the circulating antigen levels as an index to study the efficacy of a drug or its combination with other drugs (Day et al., 1991; McCarthy et al., 1995) in various control programs.

**1.2.6 Treatment and disease control strategies**

**1.2.6.1 Chemotherapy**

Diethylcarbamazine (DEC), the chemical name being 1-diethylcarbamyl-4-methyl piperazine is one of the most important drugs for
the treatment of lymphatic filariasis (Duke, 1980). This compound is formulated and sold as a dihydrogen citrate under the names Hetrazan, Banocide and Notezine (Ottesen, 1985a). The standard dosage recommended by WHO for this drug divides 6 mg per kg of body weight in 3 equal doses for a period of 12 days. Mass treatment of filarial endemic areas using 0.2-0.4% (w/w) DEC fortified salt has been shown to be simple, cheap, and effective for reducing the incidence of disease (Gelband, 1994). DEC activates, cholinergic receptors in the muscle of the worm causing depolarization and muscle paralysis due to the hyperpolarizing effect of the piperazine moiety. This causes a dislocation of the parasite from the normal habitats in the host (Langham and Kramer, 1980). The advantages of DEC are that it is extremely stable, unaffected by cooking and can be used safely in pregnancy. A recent study has shown that annual single dose of 6 mg per kg of body weight is effective in reducing mf prevalence (Kimura and Mataika, 1996).

Another drug, ivermectin is a semi-synthetic product derived from a class of naturally occurring compounds with anti-parasitic activity. It binds to glutamate gated chloride channels in nematodes, interrupting neuromuscular activity. Extensive clinical trials have demonstrated its utility in treating onchocerciasis (Chodakewitz, 1995) and it has been shown to be effective at a single dose of 400 μg per kg of body weight (Moulia-pelat et al., 1995). Ivermectin and DEC combination therapy demonstrated additive effect on clearance of mf (Moulia-pelat et al., 1994). When ivermectin and diethylcarbamazine (DEC) are given simultaneously in a single dose to persons with W. bancrofti infection, the resulting suppression of microfilaraemia is more profound and sustained than when either drug is given alone (Dreyer et al., 1998). The spontaneous as well as filarial antigen stimulated expression of IL-2 and IL-10 cytokines in MF was found to increase significantly by day after treatment with DEC (Suba, 1997). Immunization with methyl piperazine carboxylic acid (MPCA), was found to potentiate
microfilaricidal activity in *S. digitata* infected *Mastomys coucha* (Mukhopadhyay and Ravindran, 1997).

**1.2.6.2 Disease Management**

Management of severe elephantiasis of male external genitalia by surgical excision of affected tissue and split skin graft was shown to be effective (Ollapallil and Walters, 1995). The understanding that local microbial super-infection results in exacerbating lymphatic pathology in CP has led to prescribing simple hygiene measures. Regular cleaning of the affected limb and topical application of antibiotics greatly reduced adeno-lymphangitis (Shenoy *et al.*, 1995). Use of pneumatic pumps with rhythmic cycles of pressure has been shown to be effective in the disease management of elephantiasis of the limbs and these devices are available from AMLA Mediequip, New Delhi, India. The other methods of management of lymphoedema include complex physical therapy, pneumatic pumps, compressive garment, heat treatment, nodo-venous anasthemosis and complex physical therapy (Manokaran and Jamal, 1996).

**1.2.6.3 Vector control**

Reducing the mosquito vector population is an effective complementary strategy for control of filariasis. The reduction in vector density has long-term effect in transmission interruption. As it is clear that mosquitoes have gained resistance to chemicals like DDT, the biological weapons like biocides from *Bacillus sphaericus* and *B. thuringiensis* play a major role in vector control (Porter *et al.*, 1993). Our Centre is actively involved in understanding gene regulation and bioprocess development for large-scale production and formulation of mosquito larvicides from *B. sphaericus* and *B. thuringiensis*. 
1.2.6.4 Other antifilarial drugs

Another drug, Coumarin (5,6 Benzo alpha pyrone) has been shown to reduce lymphoedema by stimulating macrophage activity. PHARM Products Pvt.Ltd, Thajavur, Tamilnadu are marketing this drug in India. The substituted compound of 9H-pyrido[3,4-b] indoles (beta-carbolines) was identified as a pharmacophores for designing macrofilaricidal agents, among the various compounds screened, methyl 1-(4-methylphenyl)-9H-pyrido[3,4-b] indole-3-carboxylatem has shown the highest adulticidal activity and methyl 1-(4-chlorophenyl)-1,2,3,4-tetrahydro-9H-pyrido[3,4-b]indole-3-carboxylate has shown the highest microfilaricidal effect at 50 mg/kg x 5 days (Srivastava et al., 1999). The other drug 2,3-Dimethoxy-5-methyl1,4-benzoquinone (Qo), an analogue of ubiquinone has been shown to produce irreversible paralysis in the adult parasites and microfilariae of the cattle filarial parasite Setaria digitata, and also paralyses the mf of the human filarial parasite W. bancrofti (Sivan and Kaleysa Raj, 1999).

The presence of intracellular bacteria in filarial nematodes B. malayi and O. volvulus and their elimination leading to growth retardation of the parasites has been reported. (Kozek and Figuerore, 1977; McLaren et al., 1975). Treatment with tetracycline for the elimination of these endobacteria limits growth of the filarial parasite (Hoerauf et al., 1999).

1.2.6.4.1 Benzimidazole (Albendazole)

Several benzimidazole compounds like mebendazole and flubendazole have been tested for antifilarial activity. Treatment of Onchocerca gibsoni infected cattle with mebendazole showed decrease in circulating mf numbers and embryogenesis was arrested at the larval stage. The embryonic effect of mebendazole has been confirmed in human
onchocerciasis by *in vitro* study (Kale, 1982). Flubendazole was shown to be more effective than mebendazole, however it exhibits teratogenic effects in mammals and causes a severe inflammatory reaction at the site of injection. Though the mode of action of benzimidazoles is not fully understood, they are known to bind avidly to parasite tubulin thus preventing their polymerization into microtubules (Lacey, 1990).

1.2.6.4.2 Isothiocyanates and derivatives

Isothiocyanates, developed by Ciba-Geigy Ltd., contain an isothiocyanic group as a characteristic structural element. Amocarzine, a synthetic analogue of anoscanate, was reported to have significant macrofilaricidal activity against experimental filarial infections at doses ranging from 50-100 mg/kg per day depending on the species. Treatment with amocarzine in patients infected with *O. volvulus*, showed a 75 % reduction in skin microfilariae in a single oral dose 20 mg/Kg (Subramanyam, 1987). Though Isothiocyanates seem to be the most promising new macrofilaricidal compounds available today, their success depends upon their yet unproven clinical efficacy.

Drugs that have proven beneficial against other diseases may subsequently be found to possess antifilarial activity. Chloroquinone, an antimalarial drug it has been tested with *O. volvulus* patients it has been shown that decrease of microfilaria counts in their blood circulation. Similarly patients infected with *W. bancrofti* demonstrated that Amodiaquine has adulticidal activity against filarial parasite (McMohon, 1979). *In vitro* studies show mefloquine having antifilarial activity against *Brugia pahangi, Brugia patei* and *O. volvulus* (Walter, *et al.*, 1987).
The possibility of investigating medicinal plants and their products to identify an effective macrofilaricidal drug has been studied (Comley, 1990). Extractions from several plants have been reported to possess antifilarial activity. 'Filacid', derived from the stem bark of *Strblus asper*, has been extensively studied and is highly effective in the treatment of filarial lymphedema, filarial chyluria and other filarial disorders (Singh & Singh, 1987).

However, the mode of action of these compounds is unclear and also their success depends upon their as yet unproven clinical efficacy. It will be interesting to watch the development and activity of this compound in secondary and tertiary screens.

### 1.2.7 Biochemical pathways and drug design

A clear understanding of the mode of action of known antifilarial drugs and discovery of effective filaricides awaits greater knowledge of the biochemical pathways operating in filarial parasites. The main objective of biochemical studies on filarial parasites is to identify molecular targets (metabolic pathways or key regulatory enzymes that are essential to the parasite whose interruption or inhibition leads to instantaneous death) that may be exploited for developing a rational approach to antifilarial chemotherapy. The metabolic pathways in filarial parasites have been reviewed (Metha *et al.*, 1992) those which are used as putative target sites by antifilarial drugs are given in Table 1.1.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Target site in the parasite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levamisole</td>
<td>Glucose metabolism</td>
</tr>
<tr>
<td>Benzimidazoles</td>
<td>Folate metabolism</td>
</tr>
<tr>
<td>Suramin</td>
<td>Protein Kinase</td>
</tr>
<tr>
<td>Antimonial</td>
<td>Tubulin polymerization</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Neurotransmission and receptor functions</td>
</tr>
<tr>
<td>Menoctone</td>
<td></td>
</tr>
<tr>
<td>2,4-Diaminoquinazolines</td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td></td>
</tr>
<tr>
<td>Benzimidazoles</td>
<td></td>
</tr>
<tr>
<td>Pyrental and Morantel</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td></td>
</tr>
<tr>
<td>Ivermectin</td>
<td></td>
</tr>
<tr>
<td>Piperazine</td>
<td>Cholinergic activity</td>
</tr>
<tr>
<td>Diethylcarbamazine</td>
<td></td>
</tr>
<tr>
<td>Metrifonate</td>
<td></td>
</tr>
<tr>
<td>Mevinolin</td>
<td>Polyisopropenol synthesis</td>
</tr>
<tr>
<td>Synthetic retinoids</td>
<td>Retinoid binding proteins</td>
</tr>
<tr>
<td>Amoscanate analogues</td>
<td>Electron-transport pathway</td>
</tr>
<tr>
<td>Benzimidazoles</td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td></td>
</tr>
<tr>
<td>Trivalent organic antimonials</td>
<td>Energy-generating pathways</td>
</tr>
<tr>
<td>Glutamine antagonists</td>
<td></td>
</tr>
<tr>
<td>Antimycin analogues</td>
<td></td>
</tr>
</tbody>
</table>
The energy generating metabolism of filarial parasites will provide a good target, the number of energy metabolism pathways of filarial parasites that have been studied as potential targets for chemotherapy include glutamine dependent enzymatic reaction, mitochondrial respiration, Phosphoenolpyruvate carboxy kinase and glucose transport (Powell et al., 1989). Cell division is usually accompanied by a large increase in polyamine synthesis. Ornithine decoxybixlase (ODC) is the key enzyme involved in the polyamine synthesis. Since filarial parasites lack ODC, they depend on the mammalian hosts for supply of polyamines. So the uptake process and polyamine degradation can serve as good chemotherapeutic agents (Byers et al., 1990).

Proteases in filarial parasites could serve as good biochemical targets for potential drugs. For example cysteine protease has been identified and cloned from *B. malayi* and *Onchocerca volvulus*. These enzymes are structurally related to each other and are unlike mammalian counterparts. Similarly surface aminopeptidases have also been identified in *Brugia malayi* and this enzyme specific inhibitors, inhibits the molting of *Brugia malayi* L3 stage (Ginger, 1991).

Nucleoside diphosphate kinase (NDK) identified from *B. malayi* L3 stage (Ghosh et al., 1995), was found to be constitutively expressed during all stages of parasite development. NDK is a pivotal enzyme in the synthesis of Nucleotide triphosphates (NTP) other than ATP. They provide the dNTPs for nucleic acid synthesis, CTP for lipid synthesis, UTP for polysaccharide synthesis and GTP for protein elongation, signal transduction and microtuble polymerization (Parks and Agarwal, 1973). Molecular modeling of BmNDK showed several regions surrounding the conserved catalytic site that may be important in the design of drugs specific for the disruption of NTP (Nucleotide
triphosphate) synthesis in filarial parasites. Currently various pharmaceutical companies are actively involved in developing enzyme specific inhibitors.

1.2.8 Protective Immune responses in filariasis

Acquired immune responses can be divided into two broad functional categories humoral immune responses and cellular immune responses. Humoral immune responses are mediated by antibodies and are effective against extra cellular infectious agents. Cellular immunity is mediated by effector cells that destroy the infected host cells or release molecules that promote killing, thus effective against intracellular infections. Cellular immune responses play a significant role in modulating humoral responses through cytokines. Th1 cells produce Interleukin-2 (IL-2) tumour necrosis factor (TNF-α) and interferon gamma (IFN-γ) and Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Monocytes, macrophages and dendritic cells play a major role in the processing and presentation of antigen to B-cells and T-cells to mediate immune response. B-cells produce antibodies IgG1, IgG2, IgG3, IgG4, IgM and IgE. These antibodies play role either in protection or pathogenesis.

1.2.8.1 Humoral immune responses

The most striking feature of the humoral responses in filariasis is the elevation of total as well as filarial antigen specific IgG4 in the MF (Ottesen et al., 1985a). MF are characterized by high levels of antifilarial IgG4 (65% when compared with 4% in normals) subclass antibodies whereas CP generally have higher antifilarial IgG2, IgG3 and IgE levels (Maizels et al., 1991). Thus IgG1, IgG2 and IgG3 may be involved in the progression of pathology (Maizels et al., 1995) as seen in CP. A 62 kDa recombinant antigen from B. malayi with homology to aspartyl t-RNA synthetase has been shown to react at IgG3 level with sera from residents of areas endemic for W. bancrofti.
infection (Kazura et al., 1992). CP patients exhibit significantly higher levels of IgG3 antibodies to a recombinant antigen Bpa-26 that corresponds to C-terminal portion of the filarial heat shock protein 70 from B. malayi (Yazdanbakhsh et al., 1995). The pathological condition in Onchocerciasis is associated with parasite specific high IgG3 levels possibly implying a type III immune complex mediated hyper immune response (Cabera et al., 1988).

Another hallmark of infection with helminth parasites is the elevated levels of IgE and eosinophils. Individuals with TPE exhibit the highest levels of IgE (8630 ng/ml) whereas in other groups IgE levels ranged from 30-60 ng/ml (Hussain et al., 1981). Mast cells contain high affinity FceRI receptors for IgE and when they are bound by IgE cross-linked with antigen, they release vasoactive amines such as histamine, resulting in inflammatory reactions (Chen et al., 1995). Thus IgE may be involved in promoting inflammatory reactions. In schistosomiasis, a positive correlation between acquisition of resistance to re-infection and levels of IgE was observed (Capron and Dessaint, 1992).

IgG4 is a monovalent antibody incapable of complement fixation, but it is proposed that it acts as a blocking antibody for type III hypersensitivity reactions as well as IgE mediated hypersensitivity (Hussain and Ottesen, 1986). IgG4 and IgE recognize the same antigenic determinants and the relative amounts of each antibody may play a significant role in the IgE mediated hypersensitive reactions (Maizels et al., 1995). Higher parasite antigen specific IgG4:IgE ratio is characteristic of MF whereas clinical cases had reverse relationship (Kurniawan et al., 1993). The differential regulation of IgE and IgG4 has been puzzling. IL-4 is the key player in this system as it is a pre-requisite for both IgG4 and IgE class switching (Snapper et al., 1988). A recent observation has demonstrated that IL-12 inhibits IgE but augments IgG4 production (DeBoer et al., 1997). It is also possible that mf produce a
mediator to selectively inhibit IgE synthesis but not that of IgG4 (Kurniawan et al., 1993). Thus IgG4 reacts with the same filarial antigenic epitope as that of IgE and acts as a blocking antibody by competing with IgE for antibody binding site (Hussain et al., 1992).

1.2.8.2 Cellular immune responses
1.2.8.2.1 T helper cell dichotomy

Mosmann and Coffman (1989) identified two functionally distinct CD 4+ T-helper (Th) cell subsets namely, Th1 and Th2. Th1 cells produce Interleukin-2 (IL-2), tumour necrosis factor (TNF-α) and interferon gamma (IFN-γ) thereby activating macrophages and inducing delayed type hypersensitivity responses and cell mediated immunity. Th2 cells produce IL-4, IL-5, IL-10 and IL-13 that stimulate production of mast cells, eosinophils, and IgG1 and IgE antibodies and are involved in promoting humoral immunity, possibly suppressing the cellular immune response. Each sub-population regulates the other through their different cytokine profiles (Modlin and Nutman, 1993).

1.2.8.2.2 Parasite and Th 1/ Th 2- type cytokines

The two subsets of differentiated helper T cells, Th1 and Th2 are characterized by the contrasting profiles of cytokines they secrete. The Th1 produces cytokines (such as IFN-γ and TNF-β) that are inflammatory mediators which selectively activate macrophages, whereas the Th2 cytokines (such as a IL-4, IL-5 and IL-10) stimulate B-cell and eosinophil development and antibody production (Maizels et al., 1993; Modlin and Nutman, 1993).

Human T lymphocyte clones with a characteristic Th2 cytokines production profile can be derived from parasitized hosts by stimulation with
worm antigen or mitogen. This evidence increasingly supports a protective function for Th2 response in certain helminth infections. Parasite specific hyporesponsiveness is seen in MF patients; it is also observed that these patients have greater quantities of IL-10 secretion spontaneously and in response to parasite antigens (Mahanty and Nutman, 1995). Further parasitic stage specific T cell response was demonstrated in patients with lymphatic filariasis using antigens derived from the microfilarial, adult male alone, mixed adult male and female worms (Mahanty et al., 1996). They have reported that the MF individuals had impaired proliferative response compared to individuals with CP to microfilarial, mixed male and female adult worms antigens whereas the proliferative response was same with the adult male derived antigens in both groups. Moreover, the antigen driven cytokine secretion by peripheral blood mononuclear cells revealed significantly lower IL-2 and IFN-γ production by MF individuals in response to microfilarial and mixed antigens. This emphasizes that the MF individual exhibit preferentially impaired Th1 type response to microfilarial antigens and that microfilarial induced IL-10 may be critical in the down regulation of specific Th1 response.

This is supported by the studies done by various groups (Urban et al., 1991; Urban et al., 1992) that IL-10 is involved in the down regulation of antigen responsiveness, in parasitic infection. It has been demonstrated in human lymphatic filariasis that Th2 cytokines and relatively low lymphocyte proliferative response to filarial antigens are found in MF individuals (Ravichandran et al., 1997). The mechanism of IL-10 suppression of lymphocyte proliferation may occur either through the ability of IL-10 to inhibit expression of MHC class II molecules on antigen presenting cells or by inhibiting the expression of certain costimulatory molecules (De Waal Malefyt et al., 1991). Pearlman et al. (1993) have studied the change in the cytokine levels following addition of neutralizing anti-10 antibodies to antigen driven lymphocyte. They have observed an enhanced T cell proliferation and
subsequent Th1 cytokine production. Recent studies shows that CD45RA+CD4+ cells from normal individuals were stimulated with soluble microfilarial antigens (mf Ag) in vitro in the presence of APC and it was observed that the (mf Ag) by itself induced proliferation, IFN-γ and IL-5 production, suggesting that the filarial antigen by themselves can prime the CD45RA+ CD4+ cells in vitro and thus deviate the immune responses either to the type I or type II cytokines production (Steel and Nutman, 1998).

It has been well documented that the filarial antigen driven IgE production is upregulated by IL-4 and downregulated by IFN-γ, suggesting that amount of IgE production depends on the relative quantity of IL-4 and IFN-γ generated by filarial antigen specific T cells (King et al., 1990). The effect of IL-12 on IgG4 and IgE production was examined with cells derived from filarial patients. Studies were done to determine the role of IL-12 in Ag-driven polyclonal IgE production using rIL-12, anti IL-12 and endogenous IL-12. Recombinant IL-12 inhibits IgE synthesis by IL-4 stimulated lymphocytes from healthy persons and influenced the development of Th subset selection involved in IgG isotype selection. It was demonstrated that IL-12 modulates helminth Ag driven IgE production, by regulating the relative quantities of IFN-γ and IL-4 generated by Ag-specific lymphocytes (King et al., 1995). The presence of antigenemia, which is an indicator of current active infection, is closely associated with the frequency of IFN-γ and IL-4 producing cells in lymphatic filariasis (DeAlmeida et al., 1998). Th2 type response is a necessary prerequisite for the induction of suppression in filarial infection. Host IL-4 production is indeed essential for the induction of nonspecific suppression of cell population (MacDonald et al., 1998). Monocytes and macrophages play a major role in the processing and presentation of antigen and in the release of cytokines like IL-1 and TNFα, which act on T-cells to mediate immune response. MF patients exhibit low number of parasite antigen responsive T cells (King et al., 1992; Nutman et al., 1987). The increased levels of TNFα
may be responsible for the lymphatic damage and the subsequent maintenance of the inflammatory responses seen in CP patients (Raman et al., 1999).

Infection with the third-stage larvae (L3) of the filarial nematode *Brugia malayi* results in a Th 2 immune response in mice and humans. The production of interleukin - 4 (IL-4) is critical for down regulating polyclonal Th1 responses in L3 infected mice. IL-10 and APCs also contribute to the suppression of mitogen driven Th1 responses of spleen cells from infected mice (Osborne and Devaney, 1999). Brugia reactive Th1 cells are primed following infection with L3, but are actively suppressed in vivo by a mechanism that involves IL-10 and resident APC population, but not IL-4. In Jird model, down regulation of immune responses to *B. pahangi* was shown to be associated with deficient IL-2 production (Leiva and Lammie, 1989). In rat model of *B. pahangi* infection, it was shown that factors present in the normal serum could reverse the anergy of IL2Rα expression on PBMC from microfilaraemic rat (Satoh et al., 1997). Mice infected with *B. pahangi* L3 exhibited dramatic reduction in Con-A driven IL-2, IFN-γ levels. However, anti-IL-4 antibody or rIL-2 restored the Con-A driven proliferation, IL-2 and IFN-γ production (Osborne et al., 1996).

### 1.2.8.2.3 Proliferative responses

It is well established that MF are hyporesponsive to parasite antigens in vitro in terms of peripheral blood mononuclear cell (PBMC) proliferation whereas proliferative responses are strong in CP (Nutman et al., 1987). However proliferative responses were found to be higher in those CP that are free of mf whereas those with circulating mf were hyporesponsive (Lammie et al., 1993). This differential proliferative response correlated with the levels of circulating parasite antigen (Dimock et al., 1996), In *B. pahangi* infected
Meriones unguiculatus (Gerbil) model. It has been demonstrated that mf alone is not responsible for hyporesponsiveness but the degree of down-regulation is related to total parasite burden (Bosshardt et al., 1995).

It is not clear how Th1 and Th2 balance is involved in immunity to various life cycle stages of the parasite or pathogenesis seen in CP. The preferential expansion of Th2 dependent antibody isotypes may be due to the dynamics of antigen release in persistent infections and also may be due to the nature of extra-cellular parasitism and antigen presentation (Yazdanbakhsh et al., 1995). In filariasis, Th1 response may be a double edged immunological weapon that promotes protection if the Th1 response is against L3 as in the case of EN but causes pathology if the response is against adult or mf (Wilson, 1993). Using animal models it has been demonstrated that Th2 responses associated with elevated IgE and eosinophilia and are involved in the establishment of chronic infection whereas Th1 responses confer resistance (Pearce et al., 1991). On the other hand, in Trichuris muris infection, Th1 like response mediates chronic infection while IL-4 seems to promote worm expulsion (Else et al., 1994). Thus it would be appropriate to define the immune responses in terms of combination of cytokines and effector cells instead of Th1 type or Th2 type (Allen and Maizels, 1997).

1.2.9 Parasite defense strategies

Adult forms of W. bancrofti and B. malayi reside unaffected for years in the lymphatics, an environment that is especially enriched with immune cells and effector molecules. The other important feature of parasite persistence is its ability to enhance down-regulatory mechanisms of host immune system to induce tolerance or anergy (Maizels et al., 1995). Patients with clinical symptoms are without mf whereas individuals with large number of circulating
mf are asymptomatic. Thus the mechanism of immune evasion and resulting host-parasite interactions are the central questions to be addressed (Maizels et al., 1993). The main task would be to identify the immune responses that are protective and those that tolerate high parasitic load without any clinical symptoms as seen in MF.

Helminths block the Immuneresponse induction by molecular mimicry and uptake of host antigens. An other tactic employed by the parasites is the secretion of the hapten phosphorylcholine. For example Schistosomes absorb host MHC, as defence against NK cells, Contrapsin (anti thrombotic serum serine protease inhibitor) (Modha et al., 1988) and LDL proteins possibly for causing the progressive loss of antibody binding sites with maturation (Chiang and Caulfield, 1989).

Antigen processing generally requires cysteinyl and aspartyl proteases such as cathepsins B and D (Diment, 1990). Filarial worms release a cystatin like molecule, which may block cathepsin B. Parasites can directly or indirectly block the effects of antibody with surface or secreted proteases capable of degrading host Immunoglobulin molecules. Physical effects include thickening of the tegument during maturation. The increasing invulnerability of maturing helminths may contribute towards the operation of concomitant immunity. Many nematodes have a loose coat, which can be readily sloughed off under immune attack. A number of parasite genes, encode enzymes whose primary function, is to detoxify the intermediate products resulting from reduction of molecular oxygen. Adult B. malayi, D. immitis and O. volvulus secrete CuZn superoxide dismutases (Tang et al., 1994). Parasites do not rely solely on enzymatic mechanisms of defense. Cuticular lipids are relatively resistant to, lipid peroxidation due to the low unsaturation indices of the constituent fatty acyl residues, but complete protection is afforded by the
presence of a tocopherol, presumably assimilated from host extracellular fluid (Selkirk et al., 1998).

It has been suggested that a subset of developmentally regulated components in filarial parasites may be important in protective immune response (Bianco et al., 1990). The synthesis of Bm serpin at elevated levels just prior to and during the early stages of parasite development in the vertebrate host and its release as part of the ES products suggest that it may have an important function in parasite survival (Yenbutr and Scott, 1995). In a number of systems, serpins have been identified as factors that modulate or inhibit host immune responses (Ray et al., 1992; Macen et al., 1993). Filarial parasites release number of ES derived immune modulations, such as proteases and protease inhibitors that have the potential role in immune evasion (Tang et al., 1994; Lu et al., 1998). Parasites survive immune attack by adopting a variety of strategies to evade or modify immune responses. One of the approaches adopted by parasites is to produce homologues of host molecules that are important in immune signaling to blunt or divert inflammation. *B. malayi* secrete Macrophage migration inhibition factor (MIF) like protein that has the potential to modify host immune responses to promote parasite survival (Pastrana et al., 1998). Filarial infection influences NK cells to down regulate some component of innate immunity responsible for host defense. NK cells produce some factors that is nutritional or is trophic for *B. malayi*. The growth of *B. malayi* is dependent on host NK cell function (Babu et al., 1998).

1.2.10 Parasite transglutaminase

Transglutaminases (TGase) (EC 2.3.2.13) are a family of enzymes that catalyze the post translational modifications of proteins through the exchange of primary amines for ammonia at the γ-carboxy amide group of
glutamine residue. Peptide bound lysine residues or polyamine serve as the primary amines to form either ε-(γ-glutamyl) lysine or (γ-glutamyl) polyamine between or within the proteins. The resulting bonds are covalent, stable and resistant to chemical, enzymatic and histological degradation (Folk, 1980). A number of TGase from different species that have been described under various names are listed in Table 1.2.

1.2.10.1 Significance of TGase catalyzed reactions in filarial parasites

Micromolar concentration of monodansylcadaverine (MDC), a TGase pseudo substrate affected the production and release of microfilariae by female adult worm of the human filarial parasite *B. malayi*. Significant inhibition (84%) was observed with 50 μM MDC and complete inhibition was seen in 200 μM MDC. The phase contrast microscopic examination of uterine contents from MDC treated female worms revealed that in *utero* development of MF in these worms was severely impaired. The *utero* from untreated control worms contained numerous embryos at various developmental stages with a well-defined sheath surrounding microfilariae (Metha *et al.*, 1996). TGase catalyzed reactions also play an important role during molting of infective stage larvae (*Lustigman*, 1993). This was studied using specific inhibitors on *in vitro* molting of *O. volvulus* third stage larvae L3 into fourth stage larvae (L4). The synthetic TGase inhibitor N-benzyloxycarbonyl-D,L-β -(3-bromo-4,5- dihydroisoxazol-5-yl)-alanine benzylamide reduced the *in vitro* molting of *Nippostrongylus brasiliensis* L4 to adult stage and affected the viability and molting of the adult parasite. Taken together these results suggest that TGase catalyzed cross-linking reactions are critical for the successful molting and development of the parasite nematodes.
1.2.10.2 Evidence for TGase enzyme and TGase catalyzed products in nematodes

Evidence for the presence of TGase activity and TGase catalyzed products in nematodes has been accumulating. Adult worm of *B. malayi*, *B. pahangi* and *O. volvulus* contain high level of TGase activity (Mehta *et al.*, 1990; 1992b; Lustigman, 1993). The enzyme activity could be detected in both male and female worms of *B. malayi*, though enzyme levels in female worms were generally much higher than male worms (Mehta *et al.*, 1992b). The distribution of TGase expression in *B. malayi* female worms was analyzed by *in situ* incorporation of MDC a high affinity fluorescent pseudo substrate of TGase (Mehta *et al.*, 1992b). The results revealed incorporation of large amounts of MDC in the early embryonic stages in the uteri of *Brugia malayi* female worms. TGase activity in parasite extracts was Ca\(^{2+}\) dependent and was inhibited by EDTA or EGTA (Mehta *et al.*, 1990; 1992b). More recently TGase activity was detected in various developmental stages of the free-living nematode *Caenorhabditis elegans* (Madi *et al.*, 1998). As in filarial nematodes, the TGase activity was highest in adults, but also substantially present in L1 stage. Using an anti-ε(γ-glutamyl) lysine isopeptide selective antibody, the product of TGase catalyzed reaction was localized in *O. volvulus* L3 at regions where the separation between L3 and L4 cuticles occur. Similarly TGase catalyzed ε(γ-glutamyl) lysine crosslinks have been detected in wild type and cell death mutants of *C. elegans* (Madi *et al*, 1998).

1.2.10.3 Transglutaminase as a target for therapy

A clearer understanding of the mode of action of known antifilarial drugs and the discovery of effective filariacides require greater knowledge of the biochemical pathways operating in parasite. Such targets may be exploited for developing a rational approach to anti nematode chemotherapy. Several
studies have focussed on enzymes involved in the energy generating metabolism and antioxidant defense in nematodes as a target for chemotherapy (Kohler, 1991; Mehta et al., 1992a; Devaney et al., 1996).

**Table 1.2 Transglutaminases from different sources**

<table>
<thead>
<tr>
<th>Type</th>
<th>Other names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Factor XIIa, fibrin-stabilizing factor</td>
</tr>
<tr>
<td>Epidermal</td>
<td>TGase$_e$, callus TGase, hair follicle Tgase</td>
</tr>
<tr>
<td>Prostate</td>
<td>TGase$_p$, Vesiculase, dorsal prostate protein 1</td>
</tr>
<tr>
<td>Tissue</td>
<td>TGase$_c$, TtG, liver TGase, Type II TGase, cytoplasmic Tgase</td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>TGase$_k$, particulate Tgase, TGase type I</td>
</tr>
<tr>
<td>Erythrocyte band 4.2</td>
<td>B4.2, erythrocyte membrane protein band 4.2</td>
</tr>
<tr>
<td>Haematocyte</td>
<td>TGase$_h$, <em>Limulus</em> Tgase</td>
</tr>
<tr>
<td>Annulin</td>
<td>Grasshopper Tgase, limb protein, TGase$_k$ invertebrate homologue</td>
</tr>
<tr>
<td>Ascidian</td>
<td><em>Ciona intestinalis</em> TGase, CiTGase</td>
</tr>
<tr>
<td>Microbial Tgase</td>
<td><em>Streptoverticillium</em> TGase.</td>
</tr>
<tr>
<td>Parasite Tgase</td>
<td>pTGase, <em>Dirofilaria immitis</em> TGase, DiTGase</td>
</tr>
</tbody>
</table>

pTGase catalyzed reaction may play a role in the biosynthesis of new cuticle during the growth and development of filariae L3, L4 and adult stages. TGase catalyzed reaction appear to be critical for the growth, development and maturation of nematode because of its role in cuticle biosynthesis. Its lack of homology to mammalian TGase makes it target for developing an effective chemotherapeutic drug that is not toxic to the host.
(Chandrasekhar et al., 1998). Such a drug would specifically inhibit the parasite growth and development. One strategy for developing a novel inhibitor of pTGase enzyme is to determine the three dimensional structure of the enzyme by X-ray crystallography method using large amounts of pure enzyme. In this context the present study was designed to clone and express the transglutaminase from adult female *B. malayi* parasite.

Hence there is a need to evaluate the possible application of filarial proteins to develop simple and more specific diagnostic agents such as the pRWbSXP-1 antigen, identification and characterization of parasite specific enzyme such as transglutaminase for the prophylactic studies using the recombinant DNA technology.