CHAPTER 4

DISCUSSION

The major parasite causing lymphatic filariasis in the South Asian region is *Wuchereria bancrofti*. Affected persons frequently manifest acute symptoms of filarial fever, lymphadenitis, and lymphangitis. In some of these individuals the disease progression to chronic pathology is characterized by lymphedema and elephantiasis resulting from lymphatic blockage. A small percentage of individuals exhibit acute hypersensitivity reactions following infection, and present asthmatic symptoms, a syndrome designated as Tropical Pulmonary Eosinophilia (TPE). The majority of people living in endemic areas however, exhibit no symptoms of disease although an undefined fraction of these may harbor occult or subclinical infections (Partono, 1986). A number of researchers, most notably Hussain and Ottesen (1986), have conducted systematic analysis of the recognition of filarial antigens and have demonstrated qualitative and quantitative differences in the immune recognition. The pattern and the nature of the antigen recognized by these sera differed with each individual due to variation in immune response elicited by different helminthic and non-helminthic parasite antigens.

The non-availability of this human parasite in quantities required for antigen extraction has become an obstacle for progress in filarial diagnosis (Harinath, 1984). The use of heterologous antigens such as *Setaria digitata* (Dissanayake and Ismail, 1980), *Dipetalonema viteae* (Baschong et al., 1982), *Onchocerca gibsoni* (Forsyth et al., 1981), *Litmosoides carinii* (Dasgupta and Bala, 1978), *Dirofilaria immitis* (Sawada et al., 1969) etc. have been reported for filarial diagnosis. The *S. digitata* adult filarial parasites were easily obtainable from the peritoneal cavity of the cattle and were employed as the one of the sources for filarial antigens in this study. The filarial specific antigens of diagnostic
importance were more frequently reported from the surface component of the worms (Maizels et al., 1983; Kaushal et al., 1984; Philipp et al., 1984; Cabrera and Parkhouse, 1986).

Over the past few years, a number of filarial antigens have been identified from cuticular surface, somatic and secreted preparations (Maizels and Selkirk, 1988). However there was little information on the nature of such molecules. A combination of immunological, structural and functional data is clearly essential if appropriate parasite antigens are to be selected for diagnostic use. An understanding at this molecular level is also necessary to establish how filarial parasites are so successful at evading effective immune responses for long periods of time. With this context of prominent and potentially diagnostic values, the parasite surface have received particular attention in this study.

To begin with from the available source, viz the S. digitata adult cuticle, antigens were extracted with EDTA and the extracts showed the presence of proteins in the molecular weight range of 15 - 110 KDa in SDS-PAGE analysis on gel (Figure 3.6). The proteins of peptides with molecular weight range of 17-200 KDa range from the epicuticle of adult Brugia was reported by Maizels et al, (1988). An attempt was made to affinity purify the cuticular antigens using immobilized human filarial antibodies obtained from patients sera. The antibodies from the patients having chronic pathology of the disease was the choice for use as ligand for the purification. The reason being, in dot immunoanalysis of the S. digitata cuticular antigens, the sera from chronically infected subjects showed greater and intense reaction than the other two stages, of infections, such as microfilaremic and tropical pulmonary eosinophilia (Figure.3.4) Therefore the pooled sera of the chronically infected individuals were concentrated by ammonium sulphate fractionation and these filarial antibodies were coupled to CNBr activated Sepharose to affinity purify the specific cuticular antigen(s) of S. digitata. These affinity purified cuticular antigens on a SDS-PAGE showed 6 major peptides (Figure 3.6). (The bands were of the molecular weight 69, 49, 38, 35, 22 and 15 kDa).
The presence of antigens with different molecular sizes in filarial parasites have been already reported by several workers. In other studies a more systematic employment of a crude mixture of antigens from definite stages of the growth of lymphatic parasites such as Brugia and Onchocerca, gave a little clearer picture regarding cross reaction of the antigens as well as some information on the reasons behind immune unresponsiveness of the host system. This is reviewed here:

A 65-70 kDa peptide related to maturation from *B. malayi* microfilariae was reported by Fuhrman *et al.*, (1987), and a 55 kDa characteristic of tubulin was reported in all filaria by Maizels and Selkirk, (1988). A 36 kDa collagen probably type IV, was reported from adult Brugia by Selkirk *et al.*, (1988). A major surface glycoprotein of 22 kDa from adult Onchocerca was reported by Philipp *et al* (1984) and Taylor *et al.* (1986). A 20 kDa diagnostic somatic antigen from adult Onchocerca was reported by Cabrera and Parkhouse (1986) and Lucius *et al.*, (1986). A non-glycosylated cuticular antigen of 15 kDa from adult Brugia was characterized by Selkirk *et al.* (1986) and Maizels *et al* (1988). Thus there appears to be a great heterogenicity in the molecular weight and nature of the filarial specific antigens.

The most seroreactive bands of the *S. digitata* antigens which were specific for filarial sera (chronic and microfilaricmic) but not with endemic normal sera were the 89 and 15 kDa peptides (Figure 3.7). Of the two, on account of ease of isolation the 15 kDa peptide was further studied. This low molecular weight peptide was selectively extracted from the cuticle of *S. digitata* at higher EDTA concentrations (100 to 200 mM) (Figure 3.8). Its filarial specific nature was further confirmed in western blots, by its non reactive nature towards the non-filarial sera such as *Schistosoma mansoni*, *Trichinella spiralis* and *Plasmodium vivax*. (Figure 3.9).

In order to employ this low molecular weight antigens for immunodiagnosis, a continuous supply of this 15 kDa peptide becomes essential. Extraction from *S. digitata* can only be a temporary source due to reasons such as variation between extractions and the difficulty in the purification of co-extracted proteins.
Hence an attempt was made to isolate the filarial antibodies from patients serum that bound to the 15 kDa peptide specifically on preparative western blots. These monospecific human filarial antibodies were eluted from the preparative blots and employed to search for a similar antigen/epitope in a *W. bancrofti* genomic library constructed in λgt11 by Raghavan et al. (1991) in our laboratory. By differential screening at the IgG₄ subclass level, a recombinant clone λWbG7 was isolated and it reacted strongly and specifically with the filarial sera and not with non-filarial or non helminthic parasitic sera (Figure 3.12). Since the PC epitopes does not stimulate IgG₄ response, the screening by human IgG₄ antibodies had selectively identified clones having non-PC epitope.

Hybridization of the P³² labelled 2 kb *W. bancrofti* DNA of clone pGT7 with the non-filarial parasite DNA of *Ancylostoma duodenale*, *plasmodium vivax* and *Trichinella spiralis* showed no cross hybridization with all other parasitic DNA (Figure 3.17). Interestingly this 2 kb DNA showed no hybridization with *S. digitata* DNA also. It is likely that in our epitope search we had restricted ourselves to antigens that may resemble the 15 KDa at the immunological and not in the genomic level. This could be due to the codon usage and hence the non-recognition of the 2 Kb insert DNA with the DNA of *S. digitata*. It however hybridized with the *W. bancrofti* total DNA.

To over express this recombinant antigen and to have an access for purification, the DNA insert from the λgt11 vector (λWbG7) was recloned into the EcoRI site of the pMAL (pPR683) vector (Figure 3.15). It has been reported that pMAL vector expressed the cloned recombinant antigens to more than 5% of the total cell proteins of *E. coli* (Maina et al., 1988). The fusion could be generated with a protein (MBP) having high affinity for maltose (Maina et al., 1988). The expressed protein of the recombinant pGT7 was located in the cells in the form of an insoluble inclusion (Figure 3.18). The expressed proteins, were solubilized with Urea and refolded to obtain maximum reactivity with filarial sera. Western blot analysis of the expressed protein(s) with pooled bancroftian (microfilaremic) sera, showed a single strongly immunoreactive band at 105 KDa.
while with anti MBP sera the 105 KDa fusion product as well as the 43 KDa maltose binding protein were recognized.

When individual sera from filarial patients and endemic normals were screened in plate ELISA with the pGT7 recombinant antigen it was observed that at the total IgG level, there was seropositivity with all the filarial sera, and no cross reactivity was seen with the other parasite infection and non-endemic normal sera. However more than 50% of the endemic normal individual sera showed reactivity to this antigen. With the affinity column purified heterologous antigens from *S. digitata* the situation was similar except the non-helminthic parasite sera also showed cross reactivity. This indicated the danger of using a mixture of proteins for diagnostic assays.

However, when the screening was carried out with individual sera at the IgG4 level some clear cut observations had emerged. The response to pGT7 antigen by the TPE and Mf +ve sera were the highest followed by a low or non significant response by the sera from chronic, non-helminthic and other helminthic parasite sera. At the IgE level only the TPE patients sera showed high reactivity while the response of Mf +ve sera was lower and similar to that of the sera from chronic pathology or endemic normal individuals. Thus the recombinant antigen appears to be specifically recognized by the Mf +ve and TPE patients sera. However, as observed at total IgG4 level, more than 50% of the sera from endemic normal showed strong seropositivity to this antigens.

In order to understand the reactivity behind the response of the endemic normals, these individuals were followed up and it became clear that "normal" individuals did indeed harbored "Mf" making this test extremely valuable for the detection of the Mf in low number and also the occult status of the disease. More than 14% of the seroreactive endemic normals did indeed turn out to harbour Mf. It could further be argued that in other seropositive individuals the absence of Mf may be due to an occult status of the disease or the disappearance of
The strong recognition of IgG4 and IgE isotypes by the recombinant antigen by the TPE sera brings forth the earlier observations made by Hussain and Ottesen (1986), who reported a parallel antigen recognition by IgE and IgG4 isotypes. However Werner (1989) had observed that a \( \lambda g t11 \) fusion protein, containing part of the \( B. malayi \) myosin tail region, showed differences in the recognition profile with respect to antibody class. Also \( B. malayi \) myosin is weakly and infrequently recognized by IgE and IgG4 but is a major antigen with regard to recognition by all other IgG’s (except IgG4) (Werner, 1989). Isotype limited recognition by number of antigens were reported even in viral infections (Couteilier \textit{et al.}, 1987 and Kalife \textit{et al.}, 1988). Werner (1989) also reported that collagen and collagen like domain in a non-collagen proteins, present in the nematode cuticle was recognized primarily by antibodies of IgE class. This antigen may not posses any phosphorylcholine (PC) epitopes since these PC epitopes does not stimulate IgG4 antibody responses and it also fails to elicit IgE responses in humans (Lal and Ottesen, 1988). A recombinant clone isolated from \( W. bancrofti \) genomic DNA library by Raghavan \textit{et al.} (1991) showed restricted specificity to the major lymphatic filarial parasites \( W. bancrofti \) and \( B. pahangi \) and not to other filarial and non-filarial species tested. However it is reported that the cloned DNA was not a repetitive one and the protein expressed had homology to an intra-cellular molecule such as myosin, a highly immunogenic and prevalent molecule in many helminthic and non-helminthic parasitic organisms and hence its diagnostic validity is yet to be determined.

Diagnosis of the active filarial infection by detecting circulating filarial antigens is often a major step. Antigen detection assays have been shown to be accurate indication even of occult infections (Weil, 1990). As pGT7 antigen exhibited such specific immune response to the Mf +ve carriers, we decided to raise monoclonal and polyclonal antibodies against this recombinant antigen. Circulating parasite antigens which generally do not from circulating immune
complexes in human host will be the ideal antigen for detection. Such antigen may also have lesser chance for detection as they may be complexed and cleared by the host antibodies! In addition, the full antigenic identification of the circulating antigens cannot be generated on account of proteolysis.

A variety of techniques have been used to detect circulating antigens in humans infected with *B. malayi* (Au et al, 1981), *W. bancrofti* (Hamilton et al, 1984; Weil et al, 1986) and *Onchocerca volvulus* (Ouaissi et al, 1981; Das Moutis et al, 1983). Monoclonal (GlB13) antibodies developed to antigens such as egg antigen (PC) of *Onchocerca gibsoni* (Forsyth et al, 1985), 200 kDa *W. bancrofti* circulating antigen obtained from infected sera (Weil and Liftis, 1987), high molecular weight circulating glycoprotein isolated from bancroftian filarial patients (Paranjapee et al, 1986) and etc. have been shown to detect circulating antigen in *W. bancrofti* infections. Human sera and rabbit antisera to *L. carinii* adult worms have been used by others for the detection of antigens (Ouaissi et al, 1981; Hamilton et al, 1984). Circulating parasite antigens which generally do not form circulating immune complexes in human host will be the ideal antigen for detection. Such antigen may have lesser chance to get complexed and cleared by the host antibodies. However totally non-immunogenic circulating antigen molecule may not be available in nature due to distribution of the immunogenic epitopes on antigen moieties that may elicit a immune response.

By the use of the antibodies that we have raised, parasite antigen concentrations ranging from 30-3000 ng/ml was invariably detected in the sera of individuals from endemic regions of South India. However, the non-filarial parasitic sera from non endemic region did not show reactivity. This clear cut specificity may be attributed to the use of recombinant *W. bancrofti* antigen (pGT7) employed to raise the murine monoclonal and polyclonal antibodies. All microfilaraemic individuals either symptomatic, or asymptomatic showed positivity for circulating antigen. Similar results for microfilaraemic individuals were reported by Weil (1987), Zheng (1987), Lal (1987) and Forsyth (1985) as 98%, 95%, 93% and 91 respectively. Lal et al. (1967) reported a mean antigen
concentration of 308 ng/ml among microfilaremic subjects using a monoclonal antibody CA 101.

The monoclonal antibody, employed in the current study detected a mean antigen concentration of 440 ng/ml for the microfilaremic individuals whereas the polyclonal antibody showed higher levels of detection up to 700 ng/ml for the same set of sera (Figure 3.29). Hence the circulating filarial antigens were detected successfully and with greater sensitivity by the polyclonal antibodies, with no loss in specificity.

Asymptomatic, amicrofilaremic endemic normals showed antigenemia in 28% of the subjects while using polyclonal or monoclonal antibodies for detection. Whereas for the similar (endemic) normals, Weil (1987) reported 16% of the sera positive for antigen and Zheng (1987) reported it as 15-20% and Forsyth (1985) as 53%. The antibodies to recombinant antigen detected 28% of the endemic normals as positive (7/25) for circulating antigen in the assay as shown earlier. Of the 7 positive individuals 3 subjects (43%) showed Mf in peripheral blood after concentration of the night blood by membrane filtration. It should be recalled here that in the antibody detection assays (IgG4) 56% of the endemic normals were reactive, out of which 14% had Mf in the night blood, as confirmed by membrane concentration. This invariably outlines the efficiency and sensitivity of antigen detection. Though membrane filtration was employed in the definitive parasitological examination its wide use was restricted because of the aversion by the population for vein puncture and the cost of membrane filters when large epidemiological surveys have to be made. Therefore the detection of circulating filarial antigens or filarial subclass antibodies (IgG4) could replace the conventional direct, night blood observation.

The circulating antigen(s) present in TPE patients were detected efficiently by polyclonal antibodies (80% individuals antigen positive) than by monoclonal antibodies (40%). This discrepancy may be due to the specific epitope recognition by monoclonal antibody and the chance occurrence of such epitopes at this stage.
of infection. It was clear from the above data that the monoclonal antibodies though detected the circulating antigen at different stages of infection, its recognition of antigenemia over the TPE individuals was very low. However the polyclonal antibodies detected the maximum number of TPE individuals and it enlarges the sensitivity range (475 ng/ml) of the detection system enabling it to be manoeuvered comfortably as a tool in the field conditions. Moreover the different levels of antigenemia detected by the polyclonal antibody was in greater correlation with the peripheral blood mf counts (Figure 3.30). The mf -ve symptomatic (clinical filariasis) and asymptomatic (endemic normal) individuals showed antigenemia which were comparatively lower than the mf +ve individuals and one could substantiate the presence of antigen in chronic filariasis by resident adult worms and in endemic normals by abortive/unisexual infections, developing adults or undetectable microfilaria. Analysis of these normals from endemic regions for active infection using the monoclonal or polyclonal antibodies will lead to better monitoring and improved understanding of the epidemiology of the occult *W. bancrofti* infections.