CHAPTER 4

DISCUSSION

Effective control in transmission of filariasis by vector control and mass chemotherapy with DEC has been well documented (WHO, 1992). Logistical and financial issues have severely impeded the efforts of global elimination. Rapid re-emergence of infection has also been observed once the chemotherapy program has been relaxed (WHO, 1994). Vaccination has been used as an effective tool to control various viral, bacterial and parasitic infections. Immunological intervention by vaccination as a means to control filariasis is supported by various observations, the presence of a group of asymptomatic and amicrofilaremic individuals in an endemic area despite life long exposure to the parasite (Ottesen et al., 1977; Ottesen et al., 1982), induction of protective immunity in animal models using irradiated or chemically abbreviated worms, and whole or purified antigens.

Development of vaccines for parasitic diseases, mainly to lymphatic filariasis, is severely hampered due to the lack of suitable animal models and in vitro culture techniques, leading to the paucity of parasite material. An initiative to sequence the genome of the filarial parasites has been made by the Filarial Genome Project funded by TDR, WHO. This sequencing initiative has lead to the construction of cDNA libraries of the various stages of the parasite life cycle.
Differential immunoscreening of cDNA libraries have been exploited as a potential tool for identification of candidate antigens for immunoprophylaxis and diagnostic utility in various bacterial, viral and parasitic diseases. In the case of filariasis, differential immunoscreening of the adult cDNA library with sera from microfilaremic and amicrofilaremic donors led to the identification of SXP-1 gene (Dissanayake et al., 1995), a potential candidate with diagnostic utility. The *Wuchereria bancrofti* orthologue of *Brugia malayi* SXP-1 gene was identified and subcloned from the cDNA library of the adult stage of the parasite. This gene codes for an antigen of diagnostic importance in both brugian and bancroftian species (Rao et al., 2000). Immunoscreening of the cDNA libraries using sera from endemic normals (immune individuals) identified candidate antigens that can be protective in nature (Wang et al., 1997; Lizotte-Waniefski et al., 2000). McCarthy et al (1995) screened the *Brugia malayi* L3 cDNA library with putatively immune sera from Onchocerciasis endemic area and identified 22 recombinants of immunoprophylactic potential. A single pass sequence analysis revealed that 13 of them were distinct. One of the identified clones (TNPIBM3004) encoded a 20/22 kDa protein having significant similarity with developmentally regulated excretory/secretory protein of the larval stage of *Dirofilaria immitis* (Grieve et al., 1992). This protein showed prophylactic activity against dirofilarial infections in dogs. Hence an attempt was made to study the possible immunoprophylactic potential of TNPIBM3004 in experimental lymphatic filariasis. The results of the immunoprophylactic potential of the *Brugia malayi* ALT-2 gene was compared with the same of the multiple stage gene, WbSXP-1 as protein and DNA vaccines.
4.1 ANALYSIS OF BRUGIA MALAYI ALT-2 GENE

The nucleotide sequence of the clone TNPIBM3004, on analysis by BLAST family of programs revealed that the clone was identical to the one described earlier (Gregory et al., 1997). The sequence also showed significant homology to the *Dirofilaria immitis* 20/22 kDa, *A. vitae* (Av18) (Pogonka et al., 1999), and *Onchocerca volvulus* (Ov64) genes expected to be of prophylactic potential. Based on the FUNK system of classification proposed by the filarial genome project the recombinant clone TNPIBM3004 was renamed as ALT-2 gene.

The presence of a 21 amino acid long hydrophobic N-terminal and a signal peptidase cleavage site in the deduced amino acid sequence indicates that the protein might be excretory / secretory in nature. Immunofluorescence studies on the L3 stage of the parasite revealed that the ALT protein was not present on the surface of the larvae. The excretory/secretory proteins of the parasites have been observed to have enzymatic activity, which might be involved in the moulting of the parasite (Philipp et al., 1988). Interrupting the activity of the protein might lead to inhibition of larval moulting and transmission.

4.2 STAGE SPECIFIC EXPRESSION OF ALT GENE

Although several genes are conserved in the various life stages of the filarial parasite, the induction of different immune response by different stages of the parasite in humans might be possibly due to the stage specific gene expression. The blocks observed in the development of the parasite stages, namely L3 and mf before it switches hosts indicate that the parasite need specific stimuli in the respective host either man or mosquito for further
development. These observations clearly indicate that there is a stage-specific gene expression or at least an up-regulation of certain genes in the parasite for development or for evading the host immune response.

Analysis of stage specific expression of the ALT-2 gene at the message level by PCR using ALT specific primers indicated that the transcripts of the ALT-2 gene were present only in the L3 stage of the parasite and not in other stages (Figure 3.4). This observation is in concurrence with that of Gregory et al (1997), where *Brugia malayi* ALT transcripts were confined to the L3 stage. However in the case of *Onchocerca volvulus* (Joseph et al., 1998) the ALT transcripts was not found to be stage specific. Further analysis of the protein extracts from the various stages of the life cycle of brugian filarial parasite by immunoblot using mouse polyclonal antiBmALT-2 antibodies revealed that the ALT protein is present only in the L3 stage of the parasite (Figure. 3.5). Similar observations have been made regarding stage specific up regulation of cuticular collagen gene expression in *Brugia malayi* (Scott et al., 1995) and *Brugia pahangi* (Hunter et al., 1999), cytidine deaminase in *Brugia pahangi* (Martin et al., 1996) and other genes in *Onchocerca volvulus* (Lizotte-Waniekewski et al., 2000). A gender specific gene expression of the eggshell protein has been reported in *Brugia malayi* (Michalski and Weil, 1999).

These observations indicate that the ALT-2 gene might exhibit a stage specific gene expression and is therefore a potential candidate for vaccine development for interrupting the transmission of filarial infection.

### 4.3 EXPRESSION OF *BRUGIA MALAYI* ALT-2 GENE IN PROKARYOTIC EXPRESSION SYSTEM

Identification of specific parasite antigens responsible for partial resistance and immunopathological consequences of the infection is necessary
for development of immunoprophylactic measures. The complexity of these multicellular organisms and the absence of any proper *in vitro* cultivation methods have hindered isolation and characterisation of such antigens. Advances in this area have become possible due to the cloning of several filarial proteins (Kazura *et al.*, 1992). The isolation of antigens by biochemical techniques is tedious and highly variable in the sense that the specific antigen present in the crude antigens are not defined; moreover the presence of innumerable antigenic determinants in the crude preparation might mask finer aspects of the immune response (Prahraj and Das, 1994). The nature of the stimulatory antigen is a critical factor in the induction of unresponsiveness (Unanue, 1989). The fractionated and unfractionated antigens of the *Brugia pahangi* have been reported to induce different proliferative response and cytokine profile (Dimock *et al.*, 1994). Hence it was decided to clone the BmALT-2 gene in a high expression system for production of the protein for further studies.

The BmALT-2 gene was subcloned in the T7 expression system, pRSET, transformed in *E.coli* host cells and expressed by induction with IPTG. The predicted molecular mass of the expressed BmALT-2 gene with N-terminal fusion tag was 17.2 kDa. Analysis of the expressed recombinant protein on a 12% SDS-PAGE in Tris-glycine buffer system indicated that the molecular mass of the recombinant protein was 24kDa. Similarly discrepancies in the molecular weight of the expressed recombinant protein when analysed on a Tris-glycine buffer system has been reported earlier. Expression of genes coding for Di20/22 kDa larval proteins an homologue of the ALT protein either in the prokaryotic expression system or in baculovirus expression indicated that the expressed recombinant proteins molecular mass on a Tris-glycine gel was 24 kDa whereas the predicted molecular mass is 17 kDa (Frank *et al.*, 1996). Tris-tricine buffer systems have been reported to be better systems for
determining the molecular weight of the protein. Analysis of the *Dirofilaria immitis* 20/22 kDa protein fraction in Tris-tricine buffer system revealed that the molecular mass was 17 kDa only (Frank *et al.*, 1996). Similarly in our study when the crude antigens of *Brugia malayi* were separated on a reducing Tris-tricine buffer system, transferred to a membrane and probed with mouse antibody against the purified recombinant protein, the molecular mass of the immunoreactive polypeptide was 14 kDa (Figure 3.5) which is in agreement with the estimated molecular mass of the deduced amino acid sequence.

Chelating imminodiacetic acid (IDA) has been exploited for purification of metal binding proteins as early as 1975 (Porath *et al.*, 1975). IDA charged with metals Ni, Cu and Zn has been used to purify a variety of proteins (Sulkowski, 1985). The expressed recombinant protein containing hexa-histidine fusion tag was purified by metal affinity chromatography using FPLC. The yield of the purified recombinant protein was found to be 2.5mg/l of induced culture.

### 4.4 ANTIBODY RESPONSES TO *BRUGIA MALAYI* ALT-2 IN AN ENDEMIC POPULATION

The fate of an infection in a host, either susceptibility or development of resistance to the infection is thought to be determined by the quality and the character of the immune response generated by the host. In lymphatic filariasis the development of immune response to the incoming infective L3 stage larvae determines the outcome of the challenge. Studies measuring the antibody response to parasite antigen in an endemic population have provided conflicting evidence regarding the role of humoral immune response in protective immunity. Parasite specific IgG and IgE levels were reported to play a critical role in the development of protective immunity (Ottesen *et al.*, 1982; Hussain
and Ottesen, 1986). an observation contradicted in other immune response studies in filariasis (Ward et al., 1988; Elson et al., 1995). Immunological recognition of the L3 stage of the parasite has been postulated to be critical in the development of protective immunity observed in an endemic area (Day et al., 1991a; Maizels and Lawrence, 1991). Significant differences in the humoral and cellular immune response to larval antigens have been reported in infected and immune subjects in an endemic area, indicating the presence of stage specific immunity (Day et al., 1991a; Nutman et al., 1991). Attempts to study the humoral immune response in an endemic area by Immunofluorescence using L3 larvae has revealed that a majority of individuals in an endemic area, irrespective of their infection status, recognise the surface antigens of the L3 larvae (Kurniawan-Atmadja et al., 1998).

Most of the studies on the humoral immune response against the L3 stage have been done with whole parasitic extracts and very little data exist using pure antigens. Analysis of antibody responses against a 43kDa L3 stage antigen of *Wuchereria bancrofti* by immunoblot led to the observation that the antigen was preferentially recognised by endemic normals and not by infected individuals (Freedman et al., 1989). In the present study, analysis of the antibody responses to rBmALT-2 by immunoblot analysis indicated that all the clinical groups irrespective of their infection status recognised rBmALT-2 (Figure 3.9). This observation is in concurrence with the earlier observations. Attempts to identify larval antigens recognised only by the endemic normals and not by other clinical groups has been unsuccessful (Day et al., 1991a; Zhang et al., 1999). Although all the clinical groups recognised rBmALT-2, a difference in intensity of reactivity was observed.

Individual sera from the various clinical groups were assessed by ELISA to determine the levels of reactivity to the rBmALT-2 protein. The level
of reactivity to the rBmALT-2 protein was observed to be significantly higher in the endemic normals tested when compared to the other groups. Though a subpopulation of the chronic patients and the microfilaremics showed reactivity to the rBmALT-2 protein, the levels of reactivity were low (Figure. 3. 10). The recognition of the rBmALT-2 by the microfilaremics and the chronic patients can be attributed to the acquisition of age specific or concomitant immunity. Concomitant immunity is a phenomenon broadly characteristic of helminthic infections, where the presence of an active infection induces an immune response against the incoming infective larvae leading to resistance (Steel et al., 1996), which has been corroborated by the epidemiological data (Day et al., 1991a; Day et al., 1991b). These observations clearly indicate that the BmALT-2 antigen can be a potential vaccine candidate.

Earlier reports indicated that treatment with DEC might partially reverse the immune hyporesponsiveness in the microfilaremics when the proliferative response and antibody titres to filarial antigens before and after DEC therapy (Piessens et al., 1981). Hence an attempt was made to study the antibody responses to rBmALT-2 after DEC treatment in the microfilaremic and chronic patients who did not show reactivity to rBmALT-2 before DEC therapy. It was interesting to observe that sera from 40% of the subjects recognised the rBmALT-2 antigen after DEC chemotherapy (Figure. 3.11). This observation gives an indication that the immune unresponsiveness in the microfilaremics can be partially reversed by chemotherapy leading to an increase in the larval specific immune response. Similar results regarding transient enhancement of antibody responses and recognition of new antigens have been observed in Onchocerciasis patients treated with DEC (Lee et al., 1990).
4.4.1 Isotype distribution

The degree and character of immune response elicited by the host may be the critical determinants of the result of infection leading to pathology or protection. Isotypes of IgG differ a great deal in their ability to interact with cell bound Fc receptors (van de Winkel and Capel, 1993). In humans IgG1 and IgG3 exhibit the highest affinity for the three classes of Fc receptor whereas IgG4 is ineffective in Fc mediated cell activation. This relationship is thought to be critical since the killing of filarial larvae has been observed to involve antibody-dependent cell-mediated cytotoxicity (Sim et al., 1983a; Sim et al., 1983b; Chandrashekar et al., 1985). Other than the IgG isotypes, IgM has been strongly implicated in the clearance of Brugia malayi microfilaria (Thompson et al., 1981). Involvement of IgE antibodies in filaricidal activity of adults (Baldwin et al., 1993) and microfilaria (Pancre et al., 1988) have also been reported.

Various stages of the parasite have been observed to exert different influence on the skewing of immune response in the host. In the case of Schistosomiasis the egg stage but not the adult worms has been implicated as a potent inducer of Th2 response characterised by high IgG4 levels (Pearce et al., 1991). In filariasis the isotype balance has been found to be greatly influenced by the category of disease (Hussain et al., 1987; Kurniawan et al., 1993) and tend to be dominated by IgG4 subclass against the adult antigens (Ottesen et al., 1985). The recognition of larval stage antigens is dominated by IgG1, followed by IgG2 and IgG3 but not by IgG4 (Kurniawan-Atmadja et al., 1998).

On analysis of isotype profile in individuals recognising rBmALT-2, it was observed that the antibody isotypes were predominantly IgG1, IgG2 and
IgG3 in all the clinical groups and IgG4 was undetectable (Figure. 3.12). Similar observation has been reported when rALT-1 specific isotype distribution was analysed in sera from subjects where brugian filariasis was endemic (Gregory et al., 2000). Although the humoral immune response in sera from filarial patients is dominated by IgG4 as assessed by ELISA using adult antigens, analysis using L3 larvae has indicated that the recognition is by IgG1, IgG2 and IgG3 and not by IgG4 even in elephantiasis or microfilaremic individuals indicating the existence of concomitant immunity (Kurniawan-Atmadja et al., 1998).

The pattern of recognition of filarial antigens has been observed to be strikingly different in the clinical groups studied. A high level of parasite specific IgG4 in microfilaremics has been postulated to be responsible for the immune hyporesponsiveness because of its functional monovalency and ability to decrease the complement fixing by IgG1 (Hussain and Ottesen, 1988). Increased levels of IgG3 observed in chronic patients have also been reported in various allergic reactions where the tissue damage is thought to be mediated by the IgG3 antibody (Stokes et al., 1981). Similar correlations have been established in Onchocerciasis where the levels of IgG3 were found to be enhanced in patients with severe localized dermal pathology (Parkhouse et al., 1987). IgG2 antibodies because of its weak complement fixing activity and low levels in various clinical groups have been speculated not to play a crucial role in pathology and immunity (Hussain et al., 1987). Analysis of the levels of IgG1 specific antibodies for L3 stage antigens in Loa loa infection indicates that the microfilaremics exhibited higher levels of IgG1 compared to the microfilaremics and a significant negative correlation between the IgG1 levels and microfilaremia was observed. These observations indicate that IgG1 may play a role in the effector mechanism(s) involved in resistance against L. loa and suggest that L3 antigens may be important in eliciting protective responses.
(Akue et al., 1997). Thus our study on isotype antibody assay indicate that the rBmALT-2 is recognised preferentially by the IgG1 isotype.

4.5 LARGE SCALE PRODUCTION OF DNA VACCINES

For large scale production of the BmALT-2 and WbSXP-1 DNA vaccines, the E.coli strain harbouring the recombinant plasmid (pVr1020, pVBmALT-2, pVWbSXP-1) were grown in a 2 litres bioreactor. In the preliminary experiments when LB media was used for cultivation the yield of the plasmids were 2.5mg/1 of culture. The use of a media rich in nutrients like TB has been reported to increase the cell density and plasmid yield (Sambrook et al., 1989). The plasmid yield (5.7mg/1 of culture) obtained on cultivation in TB, was lower than the expected plasmid yield, hence it was decided to check for plasmid stability (Yee and Blanch, 1993). Plating of the cells on media with and without kanamycin indicated at the end of the cultivation more than 70% of the culture was plasmid free cells, indicating plasmid instability. Pulsing of the media with antibiotics at the start of plasmid instability has been reported to increase the stability of the plasmid. The problem of instability was circumvented by pulsing the media with 50μg/ml of kanamycin every two hours resulting in a 5-fold increase of plasmid yield (12.5 mg/1 of culture).

4.6 IMMUNOPROPHYLACTIC STUDIES

The stage-specific nature of the ALT-2 gene and preferential recognition by the putatively immune individuals, predominantly at IgG1 level indicated that the gene might be a potential vaccine candidate. The prophylactic potential of the ALT-2 gene was studied in mouse model using micropore chamber method. The number of human trials with DNA vaccine for various bacterial, viral and parasitic diseases within a span of 6 years after the
conception of the idea is proof for the possible replacement of the conventional vaccines by DNA vaccine. Direct administration of plasmid DNA encoding an antigen has been observed to induce protective immunity in animal models in Schistosomiasis (Dupre et al., 1997), Leishmaniasis (Gurunathan et al., 1997), Trypanosomiasis (Costa et al., 1998) etc.

The perfect animal model for any human infection would be one that mimics the infection’s parasitological, pathological and immunological manifestations. A surplus availability of parasite material and ability to study the immune system of animal in detail are crucial criteria in animal model studies. One of the best animal models for parasitic infections is *Schistosoma mansoni* in mice. where susceptibility, course of infection and pathology are similar to those in humans (Warren, 1982). The immune system of mice has been characterised in detail. This fortunate combination, i.e. the presence of animal model which resembles the natural infection and methods to study the details of the immune system in experimental animal model has undoubtedly facilitated the significant progress made toward the identification of antigenically defined vaccine against Schistosomiasis (James, 1987). However, the filarial parasites like *Wuchereria bancrofti* and *Onchocerca volvulus*, because of their strict host specificity reach reproductive maturity only in certain primates (Maizels et al., 1988). The lack of convenient animal model and paucity of the parasite material prohibits any extensive experimental immunity studies using human pathogens *Wuchereria bancrofti* and *Onchocerca volvulus*.

Though studies with animal filarial parasites, *Acanthocheilonema vitae*, *D.immitis* and *S.digitata* have been able to shed some light in the immune effector mechanisms involved, the zoophilic strain, *Brugia malayi*, a human parasite will be the parasite of choice for experimental prophylactic studies.
Though male jirds are fully susceptible to infection of *Brugia malayi* (Ash and Riley, 1970) the sequence of pathological and immune response are reverse of that postulated in humans (Maizels et al., 1991). The acute phase of infection in jirds exhibit lymphatic changes normally associated with the chronic state in humans exhibiting lymphadenopathy and amicrofilaremia (McVay et al., 1990). Features observed in the interim stage of asymptomatic, microfilaremia patients characterise the chronic infection in jirds: a stable microfilaremia and antigen specific hyporesponsiveness (Philipp et al., 1984).

Parasites of the genus *Brugia* rarely reach maturity in mice. The relative insusceptibility to infection is hypothesised to mimic the process by which endemic normals appear to resist infection, mainly by antilarval immune responses (Vickery and Vincent, 1984). The limited development of the early larval stages or the extended survival of mf in BALB/c mice have provided valuable insight into the mechanism of protective immunity as well as a model for studying the protective potential of vaccine candidates.

Several attempts have been made earlier to induce protective immunity in experimental animal models using radiation attenuated, chemically abbreviated infections and total parasite extracts from the various stages of the parasite. Protective immunity ranging from 25-90% has been observed. Very few studies have been carried out to study the immunoprophylactic potential of individual antigens. One of the first antigens to be studied was paramyosin; an antigen shared by the adult and mf stages of *W. bancrofti*. Mice immunised with paramyosin and challenged by intravenous injection of *Brugia malayi* microfilariae has been observed to enhance the clearance of blood-borne microfilarial stage of the parasite (Nanduri and Kazura, 1989b). The ability of paramyosin to induce protective immunity was further confirmed by parasite clearance studies in jird model, where a 43% reduction in adult worm
recoveries was observed (Li et al., 1993). Studies using the sera from mice protected by immunisation with radiation-attenuated larvae identified a 62kDa antigen from Brugia malayi (Nilsen et al., 1988). Immunisation with the 62kDa antigen from Brugia malayi has been observed to reduce 40-60% of microfilaremia in a mouse model (Kazura et al., 1990).

Immunity against the infective or early developing larval stages has been predicted to be potential targets for immunological intervention (Nutman, 1989). Immunoscreening of the larval stage cDNA libraries using sera from animals protected by immunisation with radiation attenuated larval stage has lead to the identification of OvB20 (Abdel-Wahab et al., 1996) and chitinase (Adam et al., 1996) as potential vaccine candidates. Studies in experimental animal model revealed that immunisation with OvB20 resulted in 49-60% reduction in adult worm recoveries (Taylor et al., 1995) whereas immunisation with chitinase reduced the microfilarial density but not the adult worm burden (Wang et al., 1997).

Though the subcutaneous route of infection resembles the natural course of infection, intraperitoneal inoculations (Carlow et al., 1987a) or implantations in micropore chamber (Bianco et al., 1989) has resulted in the easier and accurate estimation of the parasite burdens. In the present study, the prophylactic potential of the recombinant protein and DNA vaccine of a L3 stage specific gene and a multiple stage gene was assessed in mouse model using micropore chamber method.

In our study, mice were immunised with 5µg of recombinant protein emulsified in Freund's adjuvant intraperitoneally or 100µg of DNA vaccine intramuscularly. Five doses with a week interval were administered. Immunisation of mice with recombinant larval stage antigen or multiple stage
antigens resulted in induction of partial protection. The level of reduction in the recovery of viable larvae was observed to be higher with the larval stage recombinant antigen, rBmALT-2 protein (75%) when compared to the multiple stage antigen, rWbSXP-1 protein (67%) (Table 3.2). Induction of partial protection with SXP-1 of *Brugia malayi* has been reported in jird model (Wang *et al*., 1997). A similar reduction (76%) in parasite survival on immunisation with the recombinant *Brugia malayi* ALT-1 protein has been reported (Gregory *et al*., 2000). The level of reduction in parasite survival on immunisation with the larval stage antigen of the ALT gene family is the highest level of protection reported so far for an individual antigen (Gregory *et al*., 2000). The protective potential of the ALT genes in lymphatic filariasis is also supported by the protection conferred by an orthologue of OvB20 in *A.vitae*, which induces a partial protection (49-60%) (Taylor *et al*., 1995).

Higher levels of protection have been observed with the radiation attenuated or chemically abbreviated infections when compared to individual antigens in filariasis. The need for cold chain, high cost of production and purification have severely limited the use of recombinant proteins and live attenuated vaccines for control of diseases particularly in developing countries. DNA vaccines have been easier to produce compared to recombinant proteins. The stability of the DNA vaccines do not necessitate a cold chain during transportation and have been observed to mimic the effects of the live attenuated vaccines. These observations lead to determine the prophylactic potential of the DNA vaccines of ALT-2 and SXP-1 genes.

Nucleic acid vaccination has been used an effective method to induce protective immunity in animal models for various bacterial (Alarcon *et al*., 1999), viral (Gebhard *et al*., 2000) and parasitic diseases (Kalinna, 1997). In filariasis, DNA vaccines of chitinase (Harrison *et al*., 1999), paramyosin (Li *et
glutathione S-transferase (Catmull et al., 1999), tropomyosin and OvB20 (Harrison and Bianco, 2000) have been observed to induce partial protection in animal models by induction of both humoral and cellular immune response. An attempt was made to generate DNA vaccines of the ALT-2 and SXP-1 genes and analyse their protective potential by micropore chamber technique in mouse model. Analysis revealed that the DNA vaccine of the larval stage gene, pVBmALT-2 (46%) conferred better protection than the multiple stage gene, pVWbSXP-1 (35%) as observed in the case of immunisation with respective recombinant proteins.

Analysis of humoral immune response induced by vaccination with recombinant protein and DNA vaccine indicated that immunisation with protein induced stronger antibody responses than DNA vaccine with both the larval stage as well as multiple stage antigens. The level of antibody titer was ~40 folds lower in pVBmALT-2 (1:220) immunised mice when compared to that immunised with recombinant BmALT-2 protein (1:8000) (Figure 3.15A). Whereas in the case of SXP-1 a ~50 fold lower level of titer was observed in pVWbSXP-1 immunised mice when compared to the recombinant WbSXP-1 protein immunised mice (Figure. 3.15B). Detectable levels of antibodies were observed in mice immunised with DNA vaccine only after third dose of immunisation whereas detectable antibody levels was observed in mice immunised with recombinant protein even after the first dose.

The results observed in our study are consistent with those observed in the case of merozoite surface protein-1 from Plasmodium yoelii, where the antibody responses were found to be lower in mice immunised with DNA vaccine than that of the mice immunised with protein (Kang et al., 1998). Detectable levels of antibody were observed only after third immunisation with DNA vaccine whereas antibodies were detected after the first immunisation in
protein vaccinated mice (Kang et al., 1998). A similar observation has been reported when the C-terminus of PyMSP-1 encoding DNA vaccine (PyC2) was used to immunise mice (Gardner et al., 1996).

The humoral arm of the immune response has been observed to play a crucial role in the development of protective immunity in lymphatic filariasis by antibody-dependent cell-mediated adhesion and cytotoxicity (Mehta et al., 1981). L3 larvae when incubated with sera from vaccinated jirds, the larvae were found to be completely and uniformly covered by specific antibodies (Yates and Higashi, 1985). The rejection of L3 larvae by jirds immunised with radiation-attenuated larvae has been attributed to antibodies against the surface antigens of L3 larvae (Lucius et al., 1986). A positive correlation was also observed between the degree of immune protection seen in mice and antibody levels to soluble larval antigen (Abraham et al., 1988). Passive transfer of sera from immune dogs to BALB/c mice has been observed to confer partial protection (Abraham et al., 1988).

The possible reason for the higher level of protection conferred in mice immunised with recombinant proteins when compared to that of DNA vaccines could be attributed to the difference in levels of antibody titres induced (Figure 3.16). The use of Freund's adjuvant in the recombinant protein formulations used for immunisation might possibly be the reason for higher level of antibody titres observed.

However differential immune responses were reported regarding the use of adjuvants for enhancement of protective immunity. The use of adjuvants in the formulation, especially Freund's adjuvant, for immunisation has been reported to enhance the level of protective immunity observed on immunisation with different extracts of *Onchocerca spp* in mice (Carlow and Bianco, 1987).
An enhancement of protective immunity was observed with the use of Freund’s adjuvant even in case of immunisation with radiation attenuated larvae or chemically abbreviated infections (Yoshida et al., 1997) whereas in contradiction to the above observation enhancement was not observed in any of the adjuvant formulations tested with microfilarial stage of *Onchocerca linealis* (Townson et al., 1984).

In the present study DNA vaccines of the larval stage and multiple stage antigens were observed to induce significantly lower levels of specific antibodies in comparison to the recombinant protein. Adjuvants play an important role in augmenting the immune response generated by acting as a depot and slowly releasing the antigen molecules, preventing it from degradation. The heat killed Mycobacterium in Freund’s complete adjuvant also acts as a nonspecific stimulant of immune responses. This lower level of immune response generated by the DNA vaccine could be attributed to the absence of adjuvants in the formulation, the dose, and route of delivery and codon bias.

The general belief is that the level of gene expression *in vivo* after DNA vaccination correlates with the immune response elicited. Several attempts have been made to increase the level of expression by modifications to the construct. Codon bias has been observed in several species, and the use of selective codons in a particular gene correlates with the efficiency of gene expression. The substitution of native codons in listeriolsin (Uchijima et al., 1998), HIV (gp120) (Andre et al., 1998), gp160 (Vinner et al., 1999) with codons frequently used in murine species was observed to enhance CTL and protective immunity.
Administration of adjuvants like alum (Ulmer et al., 1999), monophosphoryl lipid A (Sasaki et al., 1997), QS-21 (Sasaki et al., 1998), have been observed to augment the immune response induced by DNA vaccines. The nature of the antigen encoded by the gene has been observed to have an impact on the type of immune response, secreted antigens have been found to induce higher levels of IgG1 when compared to membrane bound antigen (Boyle et al., 1997a).

Optimisation of codon usage, dose, delivery or co administration of immunostimulatory molecules might aid in the enhancement of immune response elicited by DNA vaccine, thereby increasing the level of protective immunity conferred.

Evidence suggests that the site and mode of DNA delivery may affect the nature of immune responses induced against the antigens encoded by plasmid DNA (Feltquate et al., 1997; Torres et al., 1997). The route of immunisation has been observed to influence the magnitude of immune response in that intradermal delivery elicited higher antibody levels compared to intramuscular injection (Boyle et al., 1997b). The N-terminal domain of stage-expressed serine repeat antigen (SERA) of Plasmodium falciparum induced higher level of antibodies when administered intradermally by gene gun method compared to the same construct administered intramuscularly (Boyle et al., 1997b). Use of biojector for intramuscular immunisation in monkeys was found to induce a better immune response when compared to intramuscular immunisation of Hbs antigen (Gramzinski et al., 1998).
4.6.1 **Isotype distribution in immunised mice**

The protective value of the immune response is highly dependent on the types of cytokines produced by CD4+ Th cells. In mice, the predominant cytokines produced by Th1 cells, \textit{(i.e.)} IL-2 and IFN-\(\gamma\) induce activation of macrophages, delayed-type hypersensitivity and production of IgG2a antibodies. In contrast, Th2 cells produce IL-4, IL-5, IL-10 and IL-13 and promote development of eosinophilia, generation of IgG1 and IgE antibody responses (Mosmann and Coffman, 1989; Abbas \textit{et al.}, 1996;).

The development of respective Th1 or Th2 effector cell as a critical determinant of protective immunity was first depicted in murine model of Leishmania (Sher and Coffman, 1992). The distribution of antibody isotypes post immunisation is considered to be reliable indicators of the cytokine types produced \textit{in vivo}. IgG2a is thought to be produced as a consequence of Th1 cell stimulation and IFN-\(\gamma\) secretion; in contrast IL-4 suppresses IgG2a production and enhances IgG1 production (Mosmann and Coffman, 1989). The antibody subtypes produced by DNA vaccination include IgG, IgM and IgA. The presence of CpG motifs in the bacterial DNA have been reported to bias the immune response towards Th1, even if not complete skewing (Raz \textit{et al.}, 1996; Shiver \textit{et al.}, 1996). Observation of IgG2a as the predominant isotype after DNA vaccination provides evidence that DNA vaccination favours Th1 response after intramuscular or intradermal immunisations (Roman \textit{et al.}, 1997). The route of administration has been found to have a significant role, where the immunisation by gene gun has been observed to induce Th2 response (Feltquate \textit{et al.}, 1997).

Analysis of isotype distribution in the recombinant ALT-2 protein immunised mice indicated that the levels of IgG1 were higher than IgG2a. The
difference in the levels of IgG1 and IgG2a were not of statistical significance (p>0.05). In contrast, mice immunised with pVBmALT-2 had higher levels of IgG2a than IgG1. No significant increase in levels of IgM and IgA were observed in mice immunised with recombinant protein whereas significant increase in the levels of both IgM and IgA was observed in mice immunised with DNA vaccine pVBmALT-2 (Figure 3.17). High levels of parasite specific IgM and IgG subclass (IgG1, IgG2a and IgG3) was implicated in the protective immune responses generated by vaccination with irradiated larvae of *Litomosoides sigmodontis* in mice (Le Goff *et al.*, 2000).

The isotype distribution of mice immunised with recombinant WBSXP-1 and pVWbSXP-1 also did not show any bias towards IgG2a or IgG1. Though higher levels of ALT specific IgM and IgA antibodies could be observed in mice immunised with pVBmALT-2 (p<0.05) no such difference could be observed in mice immunised with pVWbSXP-1(p>0.05).

Although a degree of bias was observed towards Th1 type of response in pVBmALT-2 immunised mice and Th2 type of response in rBmALT-2 immunised mice, no complete skewing was seen. However it has been reported that administration of DNA vaccine along with various co-stimulatory molecules like IL-1 (Boraschi and Tagliabue, 1999), IL-2 (Barouch *et al.*, 1998), IL-12 (Sin *et al.*, 1999), IFN-γ (Kim *et al.*, 1999), GM-CSF (Kim *et al.*, 2000), B7-1 and B7-2 (Tsuij *et al.*, 1997) could bias the immune response towards either Th1 or Th2 type of immune response. Co-administering cytokines and biasing towards a specific T helper immune response could aid in gaining valuable insight in determining the type of immune response required for protective immunity.
The results of the present study clearly demonstrate the immunogenicity and the prophylactic potential of the DNA vaccines and recombinant proteins of the *Brugia malayi* ALT-2 gene and *Wuchereria bancrofti* SXP-1 gene. Further studies optimising the codon usage, dose, route of delivery, adjuvants and co stimulatory molecules might aid in improving the protective efficacy of these candidate antigens.

4.7 CONCLUSIONS AND FUTURE DIRECTIONS OF THE WORK

Sequence analysis of the clone TNPBM3004 showed a significant homology at the nucleotide and amino acid level to the Abundant Larval Transcripts (ALT) from various filarial parasites. The gene was identical to the ALT-2 gene of *Brugia malayi*, hence renamed as BmALT-2. Analysis of the transcripts in parasite cDNA libraries from various stages of the parasite by PCR using gene specific primers indicated that the *Brugia malayi* ALT-2 gene was transcribed only in the L3 stage of the parasite. Analysis of the crude extracts of the parasite using BmALT-2 specific antibodies indicated that translation of the ALT-2 gene occurs only during the L3 stage of the parasite.

The *Brugia malayi* ALT-2 gene was cloned in a prokaryotic T7 expression system (pRSETB), expressed by induction with IPTG and purified by metal affinity chromatography using FPLC. The purified recombinant protein, rBmALT-2, was used to study the immunogenicity of the protein in human subjects with various clinical presentations.

Analysis of the recognition of the rBmALT-2 by various clinical groups of filarial patients indicated that the endemic normals or the putatively immune individuals showed a higher antibody reactivity compared to the microfilaremics or chronic patients. The predominant isotype showing
reactivity to the rBmALT-2 was observed to be in the order of IgG1, IgG2 and IgG3.

The L3 stage specific gene, BmALT-2 and a multiple stage gene WbSXP-1 were cloned in the DNA vaccine vector, Vr1020, to study their immunoprophylactic potential. Immunisation of mice with respective recombinant proteins or DNA vaccines of BmALT-2 and WBSXP-1 indicated that both were immunogenic in animal models.

The prophylactic potential of the BmALT-2 and WbSXP-1 recombinant proteins and DNA vaccine was studied in mouse model using micropore chamber method. ALT-2, the L3 stage specific gene of Brugia malayi conferred a statistically significant level of protection both as a recombinant protein (75%, p =0.02) or DNA vaccine (46%, p=0.01) compared to the multiple stage gene, WbSXP-1 (rWbSXP-1 67%, p=0.08; pVWbSXP-1 35%, p=0.04).

Further studies to optimize the dose and route of delivery are in progress. Studies to augment the immune responses by co-administering cytokines will aid in increasing the protective efficacy of the vaccine candidate as well as in understanding the mechanism of protective immunity.