discussion

In India lymphatic filariasis is caused by infection mostly with *W.bancrofti* parasite. Before starting our work we had to establish the nature of the parasite that was being transmitted in our study area. Many epidemiological studies had shown that *W.bancrofti* is the only parasite that is being transmitted in that area. (192).When we screened a large number of individuals from our study area in khurda district of Orissa state, we did not find a single individual infected with *B.malayi* parasite. Therefore we have taken our study area to be a bancroftian filariasis area.

16 villages, located within 10 Km. distance in which a large number of families were found to have filaria infection, were chosen as the study area. 200 families with similar socio-economic status were selected for initial screening. Out of 1100 individuals from all the families 258 individuals (163 males, 95 females) were found to be having chronic and acute symptoms of filariasis such as elephantiasis, hydrocele, lymphoedema, chyluria, periodic fever with attacks of adinolymphangitis and orchitis and were not included in the present study (Table-5.1). The rest of the individuals who had no clinical symptoms of filariasis were screened further for detection of microfilaria circulating in their blood by taking samples between 10.00 PM to 1.00 AM. When finger prick blood from each individual from this group was taken and stained for microfilariae to find out who had parasites, it was found that 720 (372 males, 348 females) individuals had no detectable microfilaria in their blood. These individuals were likely to be endemic normals (EN). The rest who had microfilaria but no apparent clinical symptoms (122 individuals) were categorised as asymptomatic microfilaremic (ASM) individuals. Recent studies using lymphoscintilography have clearly shown that ASM individuals are not really asymptomatic[193]. These individuals also have dilated lymphatics and compromised lymphatic function. They develop renal damage due to filarial infection. But they do not have usual filarial

pathology.Our study population, which was categorised as ASM, were observed for 3 months after detection of parasite in their blood to ensure that they are truly asymptomatic microfilaremic. For this we had to take informed consent of each individual because it is expected that after microfilaria is detected, these individuals should be treated. But to ensure that those who were included as ASM individuals in our study are not actually having transient infection which will clear or they are infected but have not started manifesting filarial symptoms, yet, we had to observe them for 3 months. Only those who continued to have parasite in their blood without appearance of any filarial symptoms were considered asymptomatic microfilaria carriers (ASM) and finally included in the study. The individuals who had no detectable microfilaria in their blood, collected between 10.00 P.M to 1.00 AM and had no filarial symptoms may not be true endemic normals. This is because detection of microfilaria by microscopic examination requires the presence of certain number of microfilariae per ml of blood. To make sure that an individual is really infection free more sensitive method of detection of parasite in the blood is required. Therefore, we have used two other criteria, which correlate with the presence of live parasites in the blood. One of them is detection of circulating filaria parasite antigens in the blood, and the other is detection of parasite antigen specific IgG4 antibodies. The individuals who do not have detectable circulating microfilariae but have adult worms in their body tissue would have parasite circulating in their blood. By using the commercially available antigen detection kits it has been possible to detect circulating antigens in the blood [194, 195]. Though the monoclonal antibodies used in these kits are directed against heterologous parasite antigens they cross-react with specific antigens of *W.bancrofti* parasite. We have used AD12 antigen assay system for detection of *W.bancrofti* parasite antigens in the blood. When parasite antigens circulate in the blood it indicates active infection. It is also known that the presence of live microfilaria induces high levels of

parasite antigen specific IgG4 antibodies. In a study conducted in Papua New Guinea by Kwan-Lim et al.,[196] when two groups of individuals were compared for their parasite antigen specific IgG4 status, it was observed that individuals positive for both phosphorylcholine (PC)-parasite antigen and microfilariae had higher antigen specific IgG4 response than that of individuals negative for both PC-antigen and microfilariae. Therefore we have also measured the levels of antigen specific IgG4 antibodies in the serum which gives an indication about whether there are live parasites in the body of individuals.

By using criteria such as clinical symptoms, presence of microfilaria in the peripheral blood (detected by passing two ml of blood from each individual, collected between 10.00PM to 1.00AM through 3mm Nucleopore filter), and staining the filter for the parasite, measuring circulating parasite antigen and parasite antigen specific IgG4 titre in the serum we have tried to determine the true infection status of our study population. We have followed up the endemic normal individuals for 3 years before including them in the study because *W.bancrofti* parasite causes chronic infection and it takes several years for appearance of filarial symptoms. It is known that chronic filaria patients may not have circulating microfilaria in their blood and they may have low levels of antigen circulating in their blood. Therefore unless one observes an individual for a sufficiently long period of time it is difficult to say which individual is infection free. On the basis of these considerations we have selected 22 ASM individuals and 23 truly infection free endemic normals. We had to start with a study population of 1100 individuals to get adequate number of individuals in these two groups. This was mainly because, there were several drop outs, few cases got other chronic diseases during the study period and in some cases the endemic normals became infected or showed high antigen specific IgG4 titres. We have categorised the study population very carefully, as we wanted to study

the unique type of immune response, if any one associated with truly EN individuals and ASM individuals. Since a large number of people inspite of continued mosquito bite and exposure to infective larvae continue to be free of parasite and clinical symptoms it is likely that there may be protective immune response in human against filarial parasite.

In animal models protective immunity to parasite has been induced by injecting radiation attenuated infective larvae, which results in immunity to subsequent infection and mounting of immune response against the infective larvae of *Brugia pahangi* [197]. In jirds vaccination with recombinant paramyocin has been shown to induce immunity to *B.malayi* infection [198]. Though several studies have attempted to define immune response associated with immunity there is no direct evidence of such response in human filaria infection. The most convincing evidence of protective immunity in humans comes from age related epidemiological studies which demonstrate that in an endemic population younger people (16-20 year age group) get infection more readily than older people [199]. The reason may be the ineffectiveness of the immune system of the younger people to fight the parasite infection due to shorter duration of exposure to the parasite in comparison to what the older people get. It has also been observed that individuals living in non-endemic area get infection easily and develop pathology very quickly when they move to the endemic area for filariasis [200]. During our field study we have come across many such cases of transmigrants who developed filariasis very quickly after moving into filaria endemic area. The two groups in endemic area who are interesting in terms of immune response mounted against filaria parasite infection are endemic normals and asymptomatic microfilaria carriers. Those who remain truly infection free inspite of getting continuously exposed must be clearing infection by mounting immune response against the parasite. By contrasting them with ASM

individuals who have microfilaria circulating in their blood one would be able to identify what is the immune response that is responsible for eliminating microfilaria from circulation.

The nature and magnitude of the immune response an antigen elicits *in vivo* after its entry into a host is determined to a great extent by the cytokine milieu in which it encounters the antigen specific $CD4^+$ T cells and B cells [201, 202]. Therefore, the uniqueness of the immune response in truly EN or ASM individuals can be understood by looking at cytokine profiles induced in them by different parasite antigens. Barry Bloom in a study of leprosy patients has suggested that protective response can be analysed by measuring the cytokine profile in different clinical groups [203]. In filariasis eliminating the parasites would require activation of different cellular components of immune system. Allergic responses like high eosinophilia and IgE mediated immune response play major role in this infection which are under the control of Th2 cytokines. In other chronic infections like leprosy, leishmaniasis Th1 cytokines are known to be involved in eliminating infection by activating macrophages that harbour the parasites. Though filarial parasites are not intracellular organisms the Th1 cytokines may be activating some cellular components to fight the infection. But till now we do not have much idea about this.

When one uses a mixture of antigen to stimulate lymphocytes to produce cytokines one may not get the right picture as different antigens in the complex mixture would induce different cytokine profile at the same time [204]. Some of the antigens may even suppress immune response. Therefore, one has to use defined parasite antigens. To understand this, 24 recombinant filaria antigens from *Onchocerca volvus, Loa loa, Brugia malayi*, etc., were screened [204]. Using lymphocytes from infected individuals it was observed that two antigens,

namely, Ov27 and OvD5B induced IgE and IgG4 responses in the lymphocytes of infected individuals *in vitro* which could be abrogated by the addition of anti-IL-4 neutralising antibodies to the culture. These two antigens were also able to induce secretion of IL-4, IL-5, and IL-10 by the lymphocytes from the same persons. Thus it became apparent that certain epitopes present on filaria parasite antigens can preferentially elicit a Th2 type of immune response in human lymphocytes. In another study, using two recombinant antigens of *Brugia* spp., Bpl-4 and Bpa-26, it was found that only those antigens which are chronically exposed to the immune system may cause a Th2 cytokine-dependent isotype switch to produce IgE and IgG4 antibodies. It was felt that such an immunoglobulin isotype imbalance, is responsible for the pathogenesis of filaria disease [205]. When SDS-PAGE-fractionated antigens of *Brugia pahangi* adults were used, it was observed that differential proliferation and secretion of IL-10 could be induced in the lymphocytes of individuals with chronic filariasis [206] In our study we have focused on antigens from two parasites namely *W.bancrofti* microfilaria and *Setaria digitata* adults. Out of the three different stages of *W.bancrofti* parasite it is easier to get antigens from microfilaria. Since the excretory secretory antigens of *W.bancrofti* microfilaria has been shown to be immunogenic [207] we have used excretory secretory antigens of *W.bancrofti* microfilaria in the present study. The hallmark of helminth infection of humans is the induction of differential IgG isotype response against parasite antigens. In order to identify the antigens reacting with a particular isotype antibody one has to analyse the reaction pattern of antigens by immunoblot. Since immunoblot requires large amount of antigens and *W.bancrofti* microfilaria antigen is scarce we have selected *S.digitata* adult antigen for analysis.

*S.digitata* adult antigen is known to cross react with filaria sera [208]. This antigen has also been shown to be strongly cross reactive with all types of filaria

sera in our study [209]. By competitive ELISA we found that *S.digitata* adult soluble antigens at 100 mg/ml inhibits the reactivity of *W.bancrofti* filaria sera at 1/5000 dilution with *W.bancrofti* mf antigen by 85% .

Using *W.bancrofti* microfilaria ES antigen as well as *S.digitata* adult soluble antigens we have analysed the various IgG isotype antibodies circulating in the blood of EN, and ASM individuals included in the present study. This is mainly because the nature of IgG isotype produced against any parasite antigen reflects the cytokine profiles that parasite may be inducing in an individual [210, 211]. By measuring the titre of different IgG isotype antibodies against filaria parasite antigens we would have an idea about the cytokine profiles in different individuals with lymphatic filariasis. We find that in the two groups of individuals the IgG1 titres are not very different from each other against both the antigens tested (Figs.-5.2 &5.6). IgG2 titres were lower in microfilaria carriers, than the antigen negative endemic normals (Fig.-5.3&5.7). This indicates that the absence of microfilariae is correlated with the elevation of IgG2 isotype antibodies in humans which is under the control of Th1 cytokines. In the case of EN and ASM individuals there is not much difference in the levels of IgG3 antibodies. (Figs. 5.4 & 5.8). The titres of antigen specific IgG4 responses in EN and ASM individuals are significantly higher than the other three IgG isotypes. The ASM individuals produced the highest titre of IgG4 antibodies than that of EN individuals (Figs. 5.5 &5.9). We do not understand why the EN individuals should produce so much parasite specific IgG4 antibodies. The high titre of IgG4 antibodies in truly EN individuals needs to be explained. We think it may be arising due to constant exposure of EN individuals to live parasites through repeated mosquito bite. It will be interesting to follow the IgG4 level of EN individuals when they move out of endemic area. We have not been able to do this. To support this hypothesis, besides the Papua New Guinea study [212], it

may be reported here that when mf level goes down due to DEC treatment, IgG4 level also goes down. Our own study reported in this thesis, supports this hypothesis. The IgG1 and IgG2 antibodies present in the sera of EN and ASM individuals reacted with *S.digitata* adult antigens of approximate mol. wt. 25 kDa and above. The IgG3 antibodies present in the sera of EN and ASM individuals reacted with antigens of approximate mol. wt. 30 kDa and above. The IgG4 antibodies present in the sera of EN, and ASM individuals reacted with a wide range of antigens (25- to 210-kDa) with exception of ASM cases who also recognised additional 20 kDa and 14 kDa antigens of *S.digitata* adult. The fact that low mol. wt. antigens are recognised by IgG4 antibodies has been reported earlier[213]. It has also been reported that the IgG isotype antibodies present in the sera of filaria parasite infected individuals recognised different antigens of *B.malayi* adult parasite [214]. It appears from this study that the different antigens recognised by different isotype antibodies, which are under the control of different cytokines, may be playing an important role in modulating the immune response of different individuals. These observations prompted us to use different fractions of antigens and particularly the 14-20 kDa *S.digitata* adult antigen (fraction 6) to stimulate the lymphocytes of EN and ASM individuals.

The endemic normals do not have detectable microfilariae parasite or parasite antigen circulating in their blood. The asymptomatic microfilariae carriers do not have any apparent symptoms of filariasis but have circulating microfilariae. We have used the lymphocytes from these two categories of individuals for stimulation with antigens. We have tested different mol. wt. fractions of *S.digitata* adult soluble antigens as well as PPD, *W.bancrofti* mf-ES antigen and *S.digitata* adult total soluble antigen for their ability to induce proliferation and secretion of IL-2, IL-4 and IFN-g by the PBMC of EN (negative for *W.bancrofti* parasite antigens) and ASM (positive for *W.bancrofti* parasite antigens) individuals(Tables-5.8 and 5.9).

PPD induced maximum proliferation and to the same extent in the lymphocytes of EN and ASM individuals (Tables-5.8). Thus both EN as well as ASM individuals had comparative number of immunocompetent cells to respond to PPD. But, the response to *W.bancrofti* mf-ES antigen and soluble total antigen of *S.digitata* adults were poor. The lymphocyte from EN individuals responded to these antigen better than the lymphocytes from ASM individuals. Out of all the fractions of *S.digitata* antigens tested the fraction-3 (66-45 kDa) and fraction-4 (45-36 kDa) induced best proliferation of lymphocytes but to different extent in EN and ASM /individuals. On the whole the lymphocytes of endemic normals responded better to all the antigens tested when compared with lymphocytes from ASM individuals(Table 5.8). When secreted IFN-γ induced by different antigens was quantitated (Table 5.9), it was observed that PPD caused maximum secretion of IFN-γ, but of similar quantity in the lymphocytes of both EN and ASM individuals. The fraction 3 and 4 of *S.digitata* adult soluble antigens induced better secretion of IFN-γ than the *W.bancrofti* mf-ES antigens, total soluble antigens of *S.digitata* adults and other molecular wt. fractions (Table-5.9).

When the ability of different antigens to induce IL-4 secretion was tested under similar condition PPD was found to induce very low levels of IL-4 secretion (28.0 pg/ml). The filaria parasite antigens induced slightly higher levels of IL-4 secretion (37.0 pg/ml). Interestingly fraction-6 (20-14 kDa) of *S.digitata* adult soluble antigen induced differential IL-4 secretion in the lymphocytes of EN (23.0 pg/ml) and ASM (50.0 pg/ml) individuals. When we calculated the ratio of IFN-γ to IL-4 secreted by lymphocytes from EN and ASM individuals, induced by different antigens (Table 5.9) it was observed that the endemic normals had greater scatter and higher IFN-γ/IL-4 mean values for all the

antigens tested than the asymptomatic microfilariae carriers who had much lower mean values for all the antigens except PPD. The similarity of IFN-γ /IL-4 values induced by PPD in the lymphocytes of both EN and ASM individuals showed that only filaria antigens are perceived differently by them. The fractions-3,-4 and -6 showed significant differences in their mean IFN-γ/IL-4 ratio values when compared between EN and ASM individuals (Fr-3, Fr-4 and -6, $P<0.001$). *W.bancrofti* mf-ES antigens also behaved like fraction-6 of *S. digitata* adult soluble antigens ($P<0.01$).

The fraction-6 (14- to 20-kDa) of *S.digitata* adult soluble antigen was used for further study as these antigens were uniquely recognised by the IgG4 isotype antibodies present in the sera of asymptomatic microfilaremic (ASM) individuals and not by any EN individuals' sera. This was not found to be so for fractions-1, -3, and -4. The IgG4 isotype switch in human is under the control of Th2 cytokines [215-217]. Therefore, the differential IgG4 expression must be arising due to different levels of Th2 cytokines induced in these individuals by parasite antigens. By using 14-20 kDa antigens we wanted to know whether these antigens could induce differential Th2 cytokine responses in different individuals. For this we have stimulated the lymphocytes from peripheral blood of antigen negative EN, antigen positive ASM individuals in the presence of SDS-PAGE-fractionated and nitro-cellulose paper transferred 14-20 kDa *S.digitata* adult soluble antigens. These antigens induced differential IL-4 and IFN-γ secretion in the lymphocyte of ASM and EN individuals (Table 5.10). There was a detectable level of IL-4 secretion (35-120 pg/ml) within 24 hours of culture and there was very little IFN-γ secretion (70-300 pg/ml) even after 72 hr. of culture by the lymphocytes of ASM individuals. Under identical conditions there was secretion of higher levels of IFN-γ (250-900 pg/ml) and lower levels of IL-4 (30-

50 pg/ml) in the lymphocytes of EN individuals. Thus 14- to 20- kDa antigens were inducing a reasonable quantity of IL-4 secretion and very little IFN-γ secretion in lymphocytes of ASM individuals, a typical Th2 type response, while in the case of lymphocytes of EN individuals IL-4 secretion was lower and the IFN-γ secretion was much higher, indicative of a preferential Th1 type response.

When the ability of 14- to 20- kDa antigens of adult *S.digitata* to stimulate IL-10 secretion in lymphocytes of EN and ASM individuals was measured (Table 5.11) and the values were plotted for each individual against the IFN-γ secreted by the same individual, very interesting plots were obtained (Figs.-5.22 & 5.25). The quantity of IL-10 secreted by EN individuals was found to be much lower (160-440 pg/ml) than that secreted by ASM individuals (300-1200 pg/ml). Production of IL-10 by T-helper cells has been shown to correlate with down regulation of Th1 cytokine synthesis in human and experimental helminth infection [218]. IL-10 has been shown to act on antigen presenting cells to inhibit production of cytokines even in mouse system [219, 220]. It has also been shown to inhibit T-cell proliferation induced by mitogens by selectively inhibiting macrophage co-stimulatory function [221]. Therefore, EN individuals, who show a polarised Th1 cytokine response, would be expected to produce a lower quantity of IL-10. Quantitation of IL-5 secreted by EN and ASM individuals showed that the former category produced lower levels of IL-5 (18-42 pg/ml) than the later (40-160 pg/ml) (Table 5.11). The synthesis and secretion of IL-4 and IL-5 by T-cells of individuals infected with different helminths appear to follow a similar pattern [222]. In humans it has been shown that IL-4 and IL-5 secreting lymphocyte populations are stimulated preferentially by parasite-derived antigens [223]. Therefore, ASM individuals, who produced more IL- 4, should also produce more IL-5, and EN individuals should be just the opposite.

The IFN-γ vs IL-10 and IFN-γ vs IL-5 plots for EN individuals showed similar patterns, which were different from those of ASM individuals(Figs. 5.21, 5.22 5.24 & 5.25). This establishes further the ability of the 14-20 kDa antigen of *S.digitata* to induce a differential Th1 and Th2 response in the lymphocytes of EN and ASM individuals. Similar type of studies have been done using fractionated antigens of *Mycobacterium leprae* to stimulate peripheral blood lymphocytes of leprosy patients [178], but no differential Th1/Th2 response was observed. However, when 20 mycobacterial antigens were tested for their ability to induce differential IFN-γ secretion, they were found to do so in the lymphocytes of tuberculoid leprosy patients [179].

Our study has shown that the 14-20 kDa antigens of adult *S.digitata* can induce differential expression of IL-4, IL-5, IL-10, and IFN-γ in the lymphocytes of ASM and EN individuals. ASM individuals show preferential polarisation towards a Th2 type of response, while EN show a preference toward a Th1 type response. This may be the reason why ASM individuals produce IgG4 antibodies capable of binding to 14- to 20- kDa antigens, while EN do not produce any IgG4 antibodies reacting with these antigens. To the best of our knowledge, no one has demonstrated earlier that the same filaria parasite antigens can induce a preferential Th2 type of response in one category of individuals (ASM individuals) while inducing a preferential Th1 type of response in the other group (EN). Since we used EN who have been followed-up clinically and parasitologically for 3 years and ASM individuals who have been observed for three months to ensure that they continue in the same state before inclusion in the study, our observations assume added significance. If we adopt this approach of careful selection of truly endemic normal individuals and compare them with other clinical categories and use well characterised parasite antigen

for stimulation of their lymphocyte, it should be possible to define the cytokine profile associated with the protective immune response to the *W.bancrofti* parasite [224].

We have shown before, *W.bancrofti* microfilaria (mf)-excretory secretory (ES) antigens behave like 14- to 20 kDa antigens of *S.digitata* adult soluble antigens. Since these antigens are derived from homologous parasites we made an attempt to study the cytokine profiles associated with protective immune response using these antigens to stimulate *in vitro* the peripheral blood lymphocytes from Ag(-)ve EN, and Ag(+)ve ASM individuals. PPD was used as the control antigen. When the proliferative responses of individuals belonging to different groups were compared it was found that the mean proliferation (S.I.) of PBMC from Ag(-)ve EN individuals in response to *W.bancrofti* mf-ES was significantly ($P<0.025$) higher than that of Ag(+)ve ASM individuals. Upon determination of levels of IL-2 and IFN-$\gamma$ secretion by PBMC it was found that these two cytokines are differentially expressed in different groups of individuals in response to mf-ES antigens, whereas there were no significant differences in the expression of IL-2 and IFN-$\gamma$ in response to PPD. These observations indicate that there exist differential immune response to parasite antigens among individuals belonging to EN and ASM groups.

As with proliferation, mf-ES antigens induced significantly ($P<0.02$) higher levels of IL-2 secretion in the lymphocytes of Ag-ve EN individuals than that of Ag(+)ve ASM individuals. These results indicate that in the absence of circulating filarial antigen as well as microfilaria, Th1 like responses dominate. The presence of circulating antigens may be associated with a modulation of these responses. Given the counter-regulatory nature of some Th1 and Th2

cytokines [225, 226] it would be reasonable to hypothesize that this modulation would be associated with Th2 like responses. This hypothesis was well supported by our data.

We have treated 122 ASM individuals from our study population belonging to both the sexes and a wide range of ages with diethylcarbamizine (DEC) to see its effect on clearance of circulating microfilariae and their parasite antigen specific IgG4 titres (Table 5.12). When clearance of circulating microfilariae was monitored by staining slides containing smears of blood collected after 10 p.m . It was found that 72% of them became free of circulating microfilariae after DEC therapy . But measurement of circulating parasite antigens in the blood showed that only 65% of them became free of circulating parasite antigens.

Measurement of parasite antigen specific IgG4 titres in the blood of the same individuals before and after DEC therapy, in the categories who cleared parasites, as indicated by no parasite antigens circulating in their blood and those who did not clear parasite showed that, when parasite got cleared the parasite antigen specific IgG4 titres also got reduced significantly (table 5.13) But when DEC therapy had no effect on circulating parasite, the parasite antigen specific IgG4 titres was also not reduced very much. This observation is interesting and is in line with what had been observed by others (196). Our study population also behaved like those observed by Kwan-Lim in Papua New Guinea. It also strengthened our argument that longitudinally observed EN individuals, who had no clinical symptoms and showed lower levels of parasite antigen specific IgG4 titres as well as no parasite antigens circulating in their blood are probably free of circulating parasites and therefore may be protected.

When the lymphocytes of ASM individuals who cleared microfilariae from as a result of DEC therapy, were stimulated with S.digitata adult 14-20 kDa antigen (Fr-6) as well as W.bancrofti mf ES antigen, before DEC therapy and after DEC therapy, there was no change in the levels of IL-2,IL-4,IL-5,IL-10 and IFN-γ secreted by them (Table 5.14 & 5.15) . This is very interesting and has not been reported earlier.

DEC therapy is known to kill microfilariae. But its effect on the adult parasite is not very clear. While clearance of microfilariae causes reduction of parasite antigen specific IgG4 titres, the antigen specific T-cells, which secrete different cytokines when stimulated by the parasite antigen may be circulating for a longer time, particularly when the adult parasite is still present in the lymphatics. This needs to be established by further study.