DISCUSSION
A. IMMUNOLOGICAL CATEGORIZATION OF LEPROSY PATIENTS AND HEALTHY CONTACTS

In vitro CMI response to unfractionated (WCFE) M. leprae antigens

In vitro CMI response to fractionated (nbCFE) M. leprae antigens

B. GENETIC VARIATIONS AT T CELL RECEPTOR GAMMA (TCRγ) AND DELTA (TCRδ) LOCI IN IMMUNOLOGICALLY CATEGORIZED LEPROSY PATIENTS AND HEALTHY CONTACTS

Germline polymorphism at Jγ locus

Germline polymorphism at VJCγ region

Germline polymorphism at Cδ locus

Rearrangements at TCRγ locus

Rearrangements at TCRδ locus
Even more than 100 years after the discovery of *M. leprae*, leprosy continues to be an enigma. This is despite the fact that there has been no dearth of attempts made in this direction. It has been known for long that the clinical pattern and the ultimate outcome of the *M. leprae* infection is largely dependent on the extent of the host's immune response to the pathogen, which in turn controls bacillary growth within their tissues (Godal, 1978). However, there exists the tremendous range of T cell response in individuals infected with *M. leprae*, reflected either as strong reactivity observed in tuberculoid patients or as the total anergy associated with exuberant growth of bacilli in lepromatous disease. The genetic mechanisms underlying the spectrum of T cell reactivity in patients with different clinical manifestations of leprosy are poorly understood and even controversial.

The present study was carried out to identify the potential genetic variations associated with the heterogeneity of immune response against *M. leprae* antigens in leprosy patients and healthy contacts, categorized on the basis of their immune response.

A. IMMUNOLOGICAL CATEGORIZATION OF LEPROSY PATIENTS AND HEALTHY CONTACTS

The cell mediated immune response of leprosy patients and healthy contacts was evaluated by the lymphocyte transformation test (LTT), an *in vitro* test for cell mediated immunity to foreign antigens (Pearmain *et al.*, 1963). This test is based on the observation that an early event in the immune response is a selective amplification of lymphocytes for a given antigen. The clonal expansion of antigen
reactive cells in response to specific antigen is evaluated by adding radioactively
labelled nucleotides such as, tritiated thymidine ($^3$H Thy) in the final stages of
culture and counting the subsequent incorporation of labelled thymidine into DNA.

**IN VITRO CMI RESPONSE TO UNFRACTIONATED (WCFE) *M. leprae* ANTIGENS**

Our findings for *in vitro* LTT tests with PBMCs of leprosy patients across
the spectrum in response to WCFE antigens of *M. leprae* were in line with the
earlier observations (Han et al., 1971; Saha and Mittal, 1971; Nath et al., 1977;
Smelt et al., 1981; Haregewoin et al., 1983; Adams et al., 1990), demonstrating
positive and negative proliferative responses in tuberculoid and lepromatous
leprosy patients respectively. Even long term treated patients with multibacillary
form of the disease failed to show lymphoproliferation to *M. leprae* antigens. This
confirmed previous observations suggesting that *M. leprae* specific anergy in
patients with lepromatous leprosy is long lasting and unchanging phenomenon
(Nath et al., 1977; Smelt et al., 1981). However, the immunologic defects leading
to reduced cell mediated immunity in lepromatous leprosy patients are not yet fully
understood.

In the present study, majority of healthy contacts of the leprosy cases were
found to be nonresponders to WCFE of *M. leprae*, negating the previous
observations demonstrating positive responses by healthy individuals with known
exposure to leprosy patients (Godal et al., 1972; Converse et al., 1988). Further
*in vitro* anergy to WCFE was observed in healthy contacts irrespective of the
clinical category of the source case. Three contacts of paucibacillary cases were
found as nonresponders, whereas two contacts of lepromatous cases responded to
WCFE. This was in contrast to previous studies showing a lower proportion of responders among contacts of active lepromatous cases (Godal and Negassi, 1973). Thus, no association was found between the type of the disease in source case and \textit{in vitro} immune response in their contacts. As source cases of leprosy patients were undergoing treatment, it was possible that \textit{in vitro} anergy to \textit{M. leprae} in their contacts, probably resulted from the lack of \textit{M. leprae} sensitized lymphocytes at sufficient density in their repertoire.

An interesting observation was the higher frequency of responders in BT/BB group as compared to BT group of leprosy patients. These borderline tuberculoid leprosy patients progressing towards borderline border (BT/BB) leprosy did not mount significant response to BCG and PPD as revealed by other set of experiments on same subjects (carried out as part of another study in our laboratory). This observation suggested that responses of these BT/BB subjects to WCFE were not false positive responses evoked by cross reactive mycobacterial antigens. It is possible that these BT/BB patients may move towards borderline tuberculoid or tuberculoid pole in due course of time.

Further, we found that a significant proportion of borderline tuberculoid (BT) leprosy patients did not respond to \textit{M. leprae} \textit{in vitro} conditions. This observation did not correspond to their histological picture in skin biopsies which showed evidences for cell mediated immunity i.e. lymphocyte infiltration and epitheloid cell infiltration. We ruled out the possibility of technical failure as a reason for this discrepancy. Utmost care was taken in preparation of the lymphocyte suspension. Lymphocytes were isolated as soon as possible after blood collection from each donor. Cell viability was also checked for each lymphocyte.
suspension before performing LTT tests. Cells were also cultured with PHA to determine cell viability. Moreover, lymphocytes were cultured in presence of 10% foetal calf serum instead of autologous plasma as there was partial evidence for the presence of inhibitory factors in plasma of leprosy patients, which could suppress the lymphoproliferation (Mehra et al., 1972). Although variations were found in mean c.p.m. for unstimulated cultures from leprosy patients as well as healthy contacts, high c.p.m. in unstimulated cultures did not correlate with high in vitro responses to M. leprae antigens. Some BT/BB patients showed high c.p.m. in unstimulated cultures, still they showed low in vitro responses to M. leprae antigens. This observation ruled out the potential influence of any minor variations in cell viability from donor to donor on in vitro responses in our experimental set up. The high $^3$H background incorporation observed in some patients could be attributed to bacterial load as speculated in one report (Haregewoin et al., 1983).

The low LTT responses of paucibacillary patients in this study could be the result of some transient immunological imbalances in these patients such as trapping of M. leprae sensitized lymphocytes in lesions, as it has been reported in literature that the number of specifically sensitized lymphocytes in peripheral blood represents a balance between their production and their trapping in the lesions (Thestrap Pederson, 1974). Therefore, the number of circulating M. leprae sensitized lymphocytes might fluctuate depending on the stage of infection, effect of treatment or mycobacterial load, thereby leading to differential in vitro response to M. leprae antigens by patients belonging to the same clinical category i.e. tuberculoid leprosy. However, we failed to find any significant correlation between in vitro immune response to M. leprae antigens and treatment period in the patients as both untreated as well as treated cases were found within the
nonresponder group of paucibacillary patients. These observations suggested that the explanation given above probably did not hold true for the low LTT responses in paucibacillary leprosy cases.

Another possibility underlying the low LTT responses by paucibacillary patients could probably be due to the non optimal concentration of *M. leprae* antigen preparation used for stimulating the lymphocytes in these nonresponder patients, as optimal concentration of antigen was supposed to vary for each patient. It was likely that the WCFE concentration (10 µg/ml), used for stimulation, was not optimal for nonresponder paucibacillary patients. However, dose response studies for this antigen preparation carried out as part of another study in our laboratory demonstrated that some paucibacillary patients did not respond to *M. leprae* antigens over a range (2-40 µg/ml) of WCFE concentration. Still, it was possible that these nonresponder patients would have responded at some untested concentrations of *M. leprae* antigen preparation. In light of such arguments, assigning responder/nonresponder status to the patients on the basis of LTT tests seemed unreliable. However, these arguments themselves indirectly vouch for existence of variations in immunological environment in different patients. Further, given the fact that *in vitro* tests such as, LTT are prone to variations arising because of transient changes in reactive cell population in circulation (Nelson and Gatti, 1976), it seemed risky to categorize the patients on the basis of LTT tests. Nevertheless, considering that all other *in vitro* tests available to evaluate the immune response of patients are also not free from such pitfalls, we relied on LTT to assess *in vitro* immune response of PBMCs isolated from clinically and histopathologically classified patients.
Results of *in vitro* tests of PBMCs with unfractionated *M. leprae* antigen preparation i.e. WCFE established a correlation of *in vitro* cell mediated immune response with clinical and histopathological spectrum in the patients with leprosy. However, a significant degree of heterogeneity in immune response was evident in each category. This heterogeneity in immune response against WCFE of *M. leprae*, which did not seem to result from variations in mycobacterial load, treatment profile, duration of the disease and nutritional status etc. compelled us to probe their immune responses with distinct groups of antigenic components of *M. leprae*. This was based on the presumption that even within one category, defined by clinical and histopathological parameters, there could exist subgroups which differ in their immune response with respect to the antigen they recognise. Existence of such subgroups, if confirmed, might explain the heterogeneity of immune response as observed in the present study. Presence of such subgroups within lepromatous leprosy have been documented by some workers (Kaplan *et al.*, 1985a, 1985b; Ottenhoff *et al.*, 1990). These subgroups, hyporesponders and nonresponders of lepromatous leprosy, were presumed to have different immunoregulatory mechanism, since *in vitro* anergy to *M. leprae* could be reversed only in hyporesponder leprosy patients whereas nonresponsiveness was found irreversible in other group of the lepromatous leprosy patients.

**IN VITRO CMI RESPONSE TO FRACTIONATED (nBCFE) ANTIGENS**

To probe responses of PBMCs from patients with leprosy across the whole spectrum and healthy contacts to distinct *M. leprae* antigens, T cell western blot technique (Young and Lamb, 1986) was used in the present study. PBMCs from patients and contacts were screened for recognition of distinct group of *M. leprae*
antigenic components separated on the basis of their relative molecular weight (Mw) by SDS-PAGE electrophoresis and electroblotted onto nitrocellulose. We preferred to employ this technique despite the availability of other techniques for separation of *M. leprae* antigens into individual components. Conventional chromatographic procedures require relatively large amounts of *M. leprae* antigens which are often scarce (Abou-Zeid *et al.*, 1987). Although advent of recombinant DNA technology has made it possible to clone mycobacterial genome in an expression vector and thereby obtain large amount of mycobacterial antigens secreted by recombinant clones, the selection of recombinant clones secreting relevant antigens is normally done with murine monoclonal antibodies (Young *et al.*, 1985b). Therefore, selected antigens are not necessarily the immunogenic ones in humans (Converse *et al.*, 1988). Moreover, selected antigens could carry B cell epitopes. Although some of the recombinant antigens have been found to stimulate T cells, the number of T cell antigens identified in this way remains small as compared to large number of proteins, *M. leprae* is composed of (Gulle *et al.*, 1992). Another alternative of probing *M. leprae* gene bank with human T cell lines/clones instead of antibodies requires practical maintenance of T cell lines/clones and cryopreservation a highly time consuming task. Further, efforts to probe *M. leprae* gene bank with human T cells, in the past, have been frustrated by the presence of other non *M. leprae* products in the preparation which affect PBMCs responses (Salgame *et al.*, 1986).

Therefore, we used nitrocellulose bound *M. leprae* antigens to screen PBMCs responses from leprosy patients and healthy contacts to the groups of distinct antigenic components of *M. leprae*. This allowed high resolution, low antigen requirement and identification of T cell stimulating antigens, independent
of the expression of B cell epitope, by these antigens. However, this technique has some limitations. First, some T cell epitopes may be modified or destroyed during electrophoresis. Second, differential transfer and binding to nitrocellulose may influence the assay. Third limitation with the T cell western blot technique is the inability to do dose response studies with each antigen. The antigenic components in each fraction may be present in suboptimal concentrations. However, in the present study which compared the relative strength of in vitro proliferation of PBMCs from leprosy patients and contacts, we did not find any fraction which was only stimulatory or only inhibitory to lymphocyte proliferation for all the subjects tested. Further, the concentrations of *M. leprae* antigens present in each fraction reflected the relative proportion of a given antigen in the whole cell free extract of *M. leprae*. Therefore, potential influences of such limitations on validity of data from in vitro LTT tests with nb CFE fractions of *M. leprae* would be less than significant.

It was found in the present study that a significant number of nonresponder subjects responded to *M. leprae* antigens, when their PBMCs were challenged with fractionated *M. leprae* antigens. The enhanced in vitro immune response was observed in both leprosy patients (paucibacillary as well as multibacillary) and healthy contacts. However, this enhancement was not a nonspecific phenomenon since a sizeable number of leprosy patients and healthy contacts remained nonresponsive to fractionated antigens. In experimental model in which nonresponder animals were turned into responders upon stimulation with modified or amputated fractions, it was assumed that such modified or amputated fractions lacked the epitopes which trigger suppressor T cells (Yowell *et al.*, 1979). However, there is conflict on role of suppressor T cells in leprosy. Some have
*M. leprae* induced suppressor cells in patients with lepromatous leprosy and borderline lepromatous leprosy (Mehra *et al.*, 1980; 1982). While, others have shown higher suppressor activity in tuberculoid leprosy patients as compared to lepromatous leprosy patients (Nath *et al.*, 1980b; Sasiain *et al.*, 1989).

Enhancement of *in vitro* lymphoproliferative responses in patients with multibacillary leprosy on challenge with fractionated antigens in the present study, confirmed that there is no lack of *M. leprae* reactive lymphocytes in the peripheral circulation of lepromatous leprosy patients (Nath *et al.*, 1984a). However inability of such cells to recognise *M. leprae* in *in vivo* and *in vitro* conditions when these were presented in mixture of proteins suggested, that some antigens present in WCFE might prevent proliferation of these *M. leprae* reactive cells to other immunogenic antigens of *M. leprae*. We presumed that these antigens might interfere with the activity of *M. leprae* reactive helper T cells either by acting directly on such helper cells or indirectly by activating possibly a suppressor cell like population (which may or may not be a conventional suppressor cell population). This suppressor cell like population in lepromatous patients might fail to function in absence of the specific antigens under *in vitro* conditions, when their PBMCs were stimulated with fractionated antigens. However, reversal of *in vitro* anergy to *M. leprae* could depend upon the abundance or functional activity of such cell population in the peripheral circulation. Patients with activated suppressor cell like population in circulation might not respond to fractionated antigens in *in vitro* conditions; whereas patients with such cell population in inactivated state are likely to respond to fractionated antigens. However, further studies are required to support this hypothesis.

127
Interestingly, it was found that few patients responded to WCFE but not to fractionated antigens. There could exist two situations to explain these unexpected observations: (a) the response seen against WCFE might be a cumulative result of recognition of number of immunogenic proteins in WCFE, varying in their immunogenic potential in such patients. And, challenge with nitrocellulose bound fractionated antigens could have reduced the additive effect of such proteins. b) In another situation, the in vitro immune response to WCFE but not to fractionated antigens might be mediated by \(\gamma\delta\) T cells in these patients. It is likely that ligands for \(\gamma\delta\) could be present in WCFE but not in separated fractions of nb CFE. Recently, there have been few reports which suggest that \(\gamma\delta\) T cells recognise protease resistant ligands (Pfeffer et al., 1990).

There were few patients who did not respond to both WCFE and fractionated antigens. It was possible that in vivo immune response of these patients could be directed to only those antigens which were exclusively present in whole \(M.\ leprae\) bacilli or their secreted products and not in WCFE and fractionated antigens of \(M.\ leprae\).

Irrespective of the underlying reasons for differential patterns of in vitro immune response to WCFE and fractionated \(M.\ leprae\) antigens in leprosy patients, the results in this study suggested that the differences within each group i.e. healthy contacts and patients with paucibacillary as well as multibacillary form of the disease, existed with respect to the antigen recognition and immuno regulatory mechanisms.

When analysed for response to different groups of \(M.\ leprae\) antigens, presented in nitrocellulose bound form, it was observed that antigens in the M.w.
range 66-150 kDa (FI) were recognised by leprosy patients as well as contacts. This was in partial confirmation with the observation indicating that high M.w. antigens of \textit{M. leprae} were recognised only by multibacillary patients (Ottenhoff \textit{et al.}, 1989). Though the frequency of responders to FI was apparently higher in multibacillary group as compared to paucibacillary patients, response to FI antigens was not confined to multibacillary patients only. Paucibacillary patients also recognised FI antigens. This was also shown by another report indicating that borderline tuberculoid patients tended to respond to the high M.w. (100 kDa or more) (Filley \textit{et al.}, 1989). Probably, within FI fraction, there might exist some antigens which discriminate immune responses of paucibacillary and multibacillary patients.

We observed that the magnitude of FI induced \textit{in vitro} lymphoproliferation was significantly higher as compared to WCFE induced response in multibacillary cases. This finding negated the previous observations which could not detect any enhancement of response by fractionated antigens (Samperio \textit{et al.}, 1989; Filley \textit{et al.}, 1989).

Antigens in the M.w. range 66-45 kDa (FII) were recognised more frequently by healthy contacts. Even in FII nonresponders, FII induced response was comparable to WCFE induced response. FII induced response was always found stronger as compared to FI and FIV stimulated response. These findings are in conformation with previous report indicating that PBMCs of healthy contacts of patients in a leprosy endemic country showed prominent response to \textit{M. leprae} in the higher ranges of 45-66 kDa (Converse \textit{et al.}, 1988). Similar observations were made in 2D separated antigens in another study where responses of contacts
were directed to protein fractions of M.w. 50-80 kDa (Gulle et al., 1992). Capability to induce response in contacts suggested the presence of immunogenic proteins of *M. leprae* in FII.

In significant number of paucibacillary patients, the FII fraction failed to induce immunogenic response. Such a low response to 45-66 kDa *M. leprae* antigens in paucibacillary patients has not been reported so far. Inability of FII to elicit lymphoproliferation in significant number of paucibacillary patients suggests the presence of suppressor cell like population in circulation of these patients which probably interfere with proliferation of cells reactive to immunogenic antigens of FII. It is possible that recognition of these immunogenic antigens may not confer protection, since paucibacillary patients do not respond to these antigens in *in vitro* conditions, but still show less number of bacilli in their tissues. It is likely that inability to respond to FII antigens might be encoded by certain genetic factors which modulate the immune response of such patients. These factors may not predispose individuals to leprosy but act as an additional risk factor by acting in conjunction with the factors primarily predisposing individuals to leprosy. It is likely that in FII responders, these genetic factors remain silent.

It was found in the present study that antigens in M.w. range of 45-36 kDa (FIII) were also recognised more frequently by healthy contacts. This was in contrast to earlier report indicating that frequency of responders to 35 to 40 kDa was similar in leprosy patients and healthy contacts (Samperio et al., 1989). We found that within leprosy patients, these were more frequently recognised by paucibacillary as compared to multibacillary patients, which confirmed the previous observation of paucibacillary patients responding more frequently to 36 kDa (Filley et al., 1989).
Another interesting observation was of stronger FIII induced responses in WCFS nonresponders as compared to WCFS responders within all categories. This probably could be explained if 45-36 kDa \textit{M. lepra}e antigens were more immunogenic when presented in fractionated form to WCFS nonresponders, however, it did not evoke the response of same magnitude in WCFS responders suggesting that the FIII fractionated \textit{M. lepra}e antigens might not have all the immunogenic proteins. It has been reported that lipoarabinomannan, a component of mycobacteria that migrates on the gel at 30 to 40 kDa range acts as a nonspecific inhibitor of T cell proliferation (Kaplan \textit{et al}., 1987). However, inability of FIII (45-29 kDa) to elicit immunogenicity could not be explained by presence of lipoarabinomannan, since FIII fraction induced significant responses in contacts.

Further, it was observed that \textit{M. lepra}e proteins with M.w. range of 36-29 kDa (FIV) were recognised at similar frequency in \textit{in vitro} conditions by leprosy patients and healthy contacts.

Few patients were also analysed for their response to \textit{M. lepra}e antigens with low M.w. antigens (14-3 kDa). These antigens were found to be recognised more frequently by PBMCs from multibacillary patients as also observed by others (Ottenhoff \textit{et al}., 1989).

We concluded that almost every fraction had the potential to elicit positive response in \textit{in vitro} conditions. This was suggestive of the fact that immunogenic proteins of \textit{M. lepra}e were spread over a broad range. These results also indicated that there existed a heterogeneity in immune response which seemed to be independent from effect of variations due to mycobacterial load, treatment profile.
and duration of the disease. We did not find any group of *M. leprae* antigens which was recognised exclusively by patients with particular clinical form of leprosy. It was not surprising since each fraction of *M. leprae* used for LTT tests contained a broad range of *M. leprae* proteins and each might have suppressor as well as helper epitopes. However, it did not dissuade us from assuming that patients with same clinical manifestations, but responding to different fractions (i.e. different antigens) of *M. leprae*, might differ at certain genetic loci. It was also expected that such differences, if found, could give a clue about the probable reason for discrepant observations on almost every immunological aspect such as, immunological tolerance, suppressor cell activity, patterns of cytokine secretion etc., studied in leprosy patients. Further, we hypothesised that such genetic differences may or may not predispose individuals to leprosy per se, but by acting in conjunction with other genetic loci or some unknown environmental factors, may influence the course of infection with *M. leprae* in these patients, ultimately resulting in heterogeneous immune response.

### B. GENETIC VARIATIONS AT T CELL RECEPTOR \( \gamma \) AND \( \delta \) LOCII IN IMMUNOLOGICALLY CATEGORIZED LEPROSY PATIENTS AND HEALTHY CONTACTS

Among the non HLA genes, playing a role in immune response to mycobacterial antigens, the ones that encode T cell receptor (TCR) are perhaps the most plausible candidates (Jazwinska and Serjeanston, 1985). Further, a multitude of studies implicating a role for T cell bearing \( \gamma \delta \) T cell receptor in
initiating an immune response against mycobacterial antigens (Modlin et al., 1989; Janis et al., 1989; Born et al., 1990; Tsuyuguchi et al., 1991), directed us to analyse genetic variations at TCR \( \gamma \) and \( \delta \) loci. This was based on the notion, that such variations if found, could be associated with variations in immune response in leprosy patients.

TCR \( \gamma \) and \( \delta \) loci in leprosy patients and healthy contacts were screened for germline variations using T cell receptor specific probes. Since T cell receptor \( \gamma \) and \( \delta \), like TCR \( \alpha \) and \( \beta \) gene segments assemble by somatic rearrangements of germline V-(D)-J segments (Davis and Bjorkman, 1988), the risk of assigning any somatically rearranged fragment as germline polymorphic fragment was minimized by determining the relative percentages of \( \gamma \delta \) T cells in circulation of leprosy patients. Immunophenotypic analysis revealed no significant alteration in relative percentage of \( \gamma \delta \) T cells in peripheral circulation of leprosy patients as compared to healthy contacts. These results were in good agreement with previous observations (Barnes et al., 1992b). Moreover, Southern hybridization of DNA from total PBMCs was reported not to be sensitive enough to detect a rearrangement, occurring in less than 10\% of total cell population (Savill et al., 1987), there was no possibility of assigning a rearranged fragment in \( \gamma \delta \) T cells as a "polymorphic" band. Further to rule out a mix between the germline pattern and rearranged configurations, a germline restriction pattern at \( \gamma \) and \( \delta \) loci was established in DNA from B lymphoblastoid cell line, expectedly with no rearranged \( \gamma \) and \( \delta \) gene segments.
GERMLINE POLYMORPHISM AT J\(\gamma\) LOCUS

Allelic variants of \(J_\gamma\) gene segment were studied in leprosy patients as well as healthy contacts. The frequency of the variation at \(J_\gamma\) was found apparently higher in multibacillary as compared to paucibacillary patients, probably suggesting it to act as an additional risk factor and not as a predisposing determinant for contracting the disease in \(M.\) leprae infected individuals. Exploring larger sample size for the presence of RFLP at \(J_\gamma\) in Indian population may substantiate this presumption in future. Further, it may probably also explain the higher incidence of leprosy cases among Indian population possessing \(J_\gamma\) allelic variants. A study carried out on healthy subjects from different populations revealed that in the sample of Black Africans, frequencies of allelic restriction fragments of \(J_\gamma\), identified by EcoRI and Hind III were significantly different from those found in French, Lebanese and Tunisian (Buresi et al., 1989). However, we did not detect such allelic polymorphism at EcoRI sites in any of the subjects.

It was further observed that leprosy patients with different allelic forms of \(J_\gamma\) differed in their in vitro immune response to \(M.\) leprae antigens. An apparent association was found between \(J_\gamma\) polymorphism and anergy to 66-45 kDa(FII) \(M.\) leprae proteins. A sizeable number (46.7%) of FII nonresponders belonging to paucibacillary group were found polymorphic for \(J_\gamma\) gene segment. Results from family studies were also suggestive of a probable association of \(J_\gamma\) RFLP with in vitro anergy to 66-45 kDa (FII) \(M.\) leprae antigens. It was intriguing that the frequency of \(J_\gamma\) RFLP did not vary in responders and nonresponders within
healthy contacts. This could probably be explained by hypothesising that RFLP at Jγ may be more influential in presence of a hypothetical primary susceptibility gene(s), expected to be absent in healthy contacts.

The exact mechanism by which Jγ polymorphism might influence the response to 66-45 kDa (FII) antigens in leprosy patients remains obscure. This was further intricated by our observation of most of the polymorphic paucibacillary leprosy patients not responding in vitro to FII but still retaining bacilli free status as revealed by their histopathological diagnosis. It has been proposed that distinct antigens of M.leprae are correlated with or even responsible for the different disease stages (Bloom and Mehra, 1984). This led to the supposition that initial immune response to 66-45 kDa antigens could be critical in deciding the future course of M.leprae infection and the response to these antigens might be governed by concerted action of primary susceptibility gene(s) and additional risk factors (i.e. RFLP at Jγ). While clearance of bacilli in vivo could be dependent on recognition of antigens other than 66-45 KDa which in turn may be governed by some other genetic factors. However, to corroborate this conjecture, an extensive analysis is required to identify the antigen, response to which confers protection. This would probably also explain multifactorial nature of the disease.

Interestingly, RFLP at Jγ was detected in all multibacillary cases, who responded to ≥ 66 kDa (Fl) M.leprae antigens, suggesting that the presence of polymorphism at Jγ might not be associated with anergy to ≥ 66 kDa M.leprae proteins. This further implicated that polymorphism at Jγ might be associated with anergy to only specific group of M.leprae antigens. Assuming, TCR polymorphic
at Jγ could be compatible for recognition of some and non permissive for other antigens, it would be of interest to analyse (a) HLA haplotypes of responder and nonresponder leprosy patients in the context of polymorphic Jγ and (b) the \textit{in vitro} immune response of γδ and αβ T cells respectively in responders and nonresponders with Jγ polymorphism.

\textbf{GERMLINE POLYMORPHISM AT VJCγ REGION}

The VJC region of TCR γ locus was analysed in leprosy patients and healthy contacts, using a probe specific to Vγ9-Jγ2-Cγ2 region, to address the question of existence of germline variations at this region, since most of the γδ T cells in peripheral circulation have been reported to preferentially bear Vγ9 encoded TCR (Triebel and Hercend, 1989). Further, antimycobacterial response was shown to be exclusively present in the TCR-Vγ9/Vδ2 expressing subset of γδ T cells (Panchamoorthy et al, 1991).

The polymorphism at VJC region was detected in leprosy patients as well as healthy contacts. However, within leprosy patients, this was encountered more frequently in multibacillary patients. It seemed feasible that polymorphism at VJC region might be an additional risk factor for progressing towards the more severe form of the disease. However, like Jγ variations at VJC region alone, might not be the factor, which predispose \textit{M. leprae} infected hosts to leprosy or leprosy type.
Three polymorphic fragments of 18 Kb, 8.5 Kb and 5.8 Kb were detected in Hind III digested DNA samples screened with VJC specific probe. Interestingly, in polymorphic healthy contacts, only 18 Kb fragment was detected whereas in polymorphic leprosy patients, all three, 18 Kb, 8.5 Kb and 5.8 Kb, fragments were detected. Within polymorphic leprosy patients, paucibacillary subjects revealed all three fragments whereas in multibacillary subjects, 8.5 Kb fragment was not seen. Thus presence and absence of 8.5 Kb appeared to be associated with paucibacillary and multibacillary leprosy, respectively. Extending such analyses on larger sample size may corroborate these observations in future.

When responders and nonresponders within each group were compared for the frequency of variations at VJCγ, no association was found between polymorphism at VJCγ and in vitro anergy to any fractionated antigens in leprosy patients as well as healthy contacts. Further, responders and nonresponders to FII within paucibacillary group did not reveal any noticeable difference viz a viz occurrence of VJC polymorphism. Thus in vitro anergy to 66-45 kDa (FII) M. leprae antigens in paucibacillary patients could not be associated with variations at VJC region of TCRγ locus.

There have been some reports which revealed the presence of 5.4 and 8.0 Kb polymorphic fragments when Hind III digested DNA samples were probed with a sequence containing the triplicated exon (Cγ2) (Buresi et al., 1989). The probe used in the present study for detecting polymorphism at VJC region of TCRγ locus represented in it in part the triplicated exon (Cγ2). It was likely that 8.5 and 5.8 Kb fragments detected by us were the same fragments, reported by
others (Buresi et al. 1989). However, the polymorphic fragment of 18 Kb observed in addition to other two bands in this study, could originate because of the difference in length of the probe used. Further, rehybridization of DNA samples presenting these polymorphic fragments when probed with Jγ region specific probe failed to reveal these polymorphic fragments. It was safe to assume that the polymorphic fragments at VJCγ region originated because of allelic polymorphism displayed by Cγ2 (Buresi et al., 1989).

Absence of any association between VJCγ polymorphism and in vitro immune response to fractionated *M. leprae* proteins might be explained in light of the fact that TCR on a major population of γδ cells in circulating PBMCs is encoded by Cγ1 and not Cγ2 (Triebel and Hercend, 1989). Therefore, polymorphism at Cγ2 could be of less consequence in its effect on in vitro immune response to *M. leprae* antigens. However, this explanation might be partially negated by higher incidence of this polymorphism in multibacillary patients. This disparity could be resolved by presuming that either VJCγ polymorphism and immune response to *M. leprae* antigens are independent phenomenon; or by proving that the γδ TCR on circulating γδ cells of polymorphic subjects is encoded by Cγ2 instead of Cγ1.

T cell receptor γ locus was also screened for potential variations in the methylation pattern in leprosy patients as well as healthy contacts, considering the fact that the methylation of DNA plays an important role in controlling gene expression in a cell or tissue specific manner. The methylation pattern was expected to vary in leprosy patients if there existed a polymorphism at Msp I sites.
Most of the CCGG sequences of TCRγ in DNA from PBMCs were found to be methylated in the present study. However, the degree of methylation at germline level did not vary in leprosy patients and healthy contacts. No polymorphism was detected at Msp I sites in the TCRγ locus in leprosy patients.

Since the DNA used in the present study was derived from all types of cells including αβ, γδ, B, monocytes as well as other cells, change in the methylation could arise not only due to polymorphism at Msp I sites but also because of significant variations in number of specific cell population with undermethylated γ locus. This assumption was based on the reports implying undermethylation of genes in the tissues, in which they were expressed and heavy methylation in the tissues in which they remained silent (Sakamoto et al., 1988). Also there have been some reports suggesting inheritance of the methylation pattern of a specific gene from cell cycle to cell cycle except in case of cellular differentiation (Pollack et al., 1980; Stein et al., 1982). Therefore, the methylation pattern of a specific gene may reflect the lineage of a cell. Accordingly, γδ T cells circulating in blood would have unmethylated γ locus. Isoschizomeric analysis of TCRγ locus demonstrating no variation in the observed methylation pattern suggested no significant increase in number of circulating γδ T cells in leprosy patients. This observation confirmed results of our immunophenotypic analysis of TCRγδ in leprosy patients.

GERMLINE POLYMORPHISM OF Cδ LOCUS

The differences between leprosy patients and contacts with respect to TCR RFLP frequencies were most striking when compared for RFLP at TCR δ locus.
Polymorphism at TCR Cδ was observed in 17.6% of leprosy patients. Although sample size of control subjects was small, it was not observed in any healthy contact/control. Since polymorphism at δ locus was observed in patients within all categories, it might not predispose individuals to develop the tuberculoid or lepromatous leprosy. However, response of polymorphic subjects to fractionated antigens as well as WCFE was not significant. Interestingly, frequency of this polymorphism was found higher in nonresponder multibacillary as compared to responder multibacillary patients. It is likely that presence of this polymorphism could be partially associated with irreversible anergy. It would have been of interest to follow up these cases to observe their response to treatment.

Frequency of Cδ polymorphism did not vary in paucibacillary subjects categorized as responders and nonresponders on the basis of their response to 66-45 kDa (FII) *M. leprae* proteins, suggesting that polymorphism at Cδ might not be associated with anergy to FII fraction which was observed to be less immunogenic in paucibacillary subjects. Thus polymorphism at Cδ did not seem to affect the response to FII fraction.

Leprosy patients and healthy contacts were analysed for alterations in the methylation pattern at TCRδ locus. Although most of the CCGG sequences at Cδ were found to be methylated as revealed by detection of higher molecular weight fragment in HpaII digested samples, it was not surprising since DNA used in the present study was derived from unfractionated PBMCs including αβ, B cells, monocytes with heavily methylated δ loci. The methylation pattern did not differ in leprosy patients and healthy contacts and no germline polymorphism could be
detected at Msp I sites. These observations demonstrating no variation in the methylation pattern suggested that probably there was no significant increase in γδ cells in circulation of these patients. Since in that situation variations were expected in the observed methylation pattern due to undermethylation of δ locus in these cells.

REARRANGEMENTS AT TCRγ LOCUS

Analysis for variations at γ locus in the expressed T cells' repertoire revealed that frequencies of rearrangements involving different Vγ segments were apparently lesser in leprosy patients including paucibacillary as well as multibacillary subjects whereas these rearrangements were detected in all healthy contacts. This was suggestive of the assumption that these rearrangements might be associated with protection against *M. leprae*. It was also indicative of genetic difference in repertoire generation in leprosy patients. It was possible that in these leprosy patients, cells with γ rearrangements involving Vγ9 and Vγ7/8 segment were less in circulation. It was likely that cells with V7/V8 rearrangements or Vγ9 might be more immunopotent with respect to their reactivity to *M. leprae in vivo* conditions.

No association was found between response to fractions and frequencies of these rearrangements in leprosy patients and healthy contacts, when analysed for their response to different fractions. Moreover, these rearrangements were detected in all healthy contacts irrespective of their response to fractions. Within leprosy patients also, no significant decrease in frequency of detectable
rearrangements was noticed in nonresponders to 66-45 kDa (FII), 45-36 kDa (FIII) and 36-29 kDa (FIV) of *M. leprae*. Although, frequencies of Vγ7/8 and Vγ9+ rearrangement seemed apparently higher in patients responding to *M. leprae* proteins with ≥ 66 kDa (FI), these observations did not appear significant as frequency of response to FI *in vitro* was found to be higher in multibacillary patients while, frequency of detectable rearrangements in circulating PBMCs was found to be low in these patients. Therefore it was concluded that probably there was no association between specific rearrangement and response to fractions. It is paradoxical, that despite having an apparent association with "protection" (i.e. frequency of rearrangements highest in healthy contacts, least in multibacillary patients) no association could be found with response to any fraction. It was likely that presence of any specific rearrangement at γ locus was reflection of "*in vivo*" immune status, while response to fractions seen under *in vitro* conditions reflected only part of this *in vivo* immune status. Interestingly, most noticeable variations were observed for rearrangements at Vγ7/Vγ8, Vγ9 gene segments. This was not surprising in light of the observations that the most frequent rearrangement involves Vγ7/8 in αβ T cells, while within γδ T cells, rearrangement occurs mostly at Vγ9 (Alexandre and Lefranc, 1992). The other γδ+ T cells had been more diverse and expressed either a VγI subgroup gene (or the V9 gene) joined to JP1 or J1 or a VγI subgroup gene joined to JP2 or J2. In the α/β+ T cell, the rearrangements have been shown to mostly involve the VγI subgroup genes (that is the most 5' V genes) and JP2 and J2, that is the most 3' J segments (Moisan et al., 1989).
Since DNA was obtained from unfractionated PBMCs including \( \gamma \delta \) and \( \alpha \beta \) T cells, we were not certain whether the specific rearrangement detected by Southern hybridization was contributed by \( \gamma \delta \) or \( \alpha \beta \) cells. As our immunophenotypic analyses for TCR \( \gamma \delta \) did not reveal any major increase in relative frequency of \( \gamma \delta \) cells in circulation, it was assumed that rearrangement profile at \( \gamma \) locus detected by Southern hybridization, was most probably contributed by DNA from \( \alpha \beta \) cells, considering the fact that Southern hybridization would not be able to detect the rearrangement of cells occurring in less than 10% of total PBMCs. (Savill et al., 1987). Although the \( \alpha/\beta^+ \) T cells never express the gamma chain at the cell surface, these cells have been shown to rearrange \( \gamma \) locus on both alleles (Triebel et al., 1988b). These rearrangements at \( \gamma \) locus in \( \alpha \beta \) T cells even if not functionally relevant, could be used as marker for the clonality of \( \alpha \beta \) T cells. It is likely that \( \alpha/\beta \) or \( \gamma \delta \) cells with different rearrangements at \( \gamma \) locus might vary in their immunoreactivity to \textit{M. leprae} antigens.

Higher intensity of \( V_{\gamma7}/V_{\gamma8}^+ \) and \( V_{\gamma9}^+ \) rearranged restriction fragments, observed in DNA from some subjects (including leprosy patients as well as healthy contacts) could not be attributed to either higher frequency of cells with \( V_{\gamma7}/V_{\gamma8} \) rearrangement in circulation or homozygosity at rearranged loci, since digestion with other enzymes did not generate \( V_{\gamma7}/V_{\gamma8} \) or \( V_{\gamma9} \) restriction fragment with similar intensity. Further, no association was found with \textit{in vitro} immune response to the fractions and the altered intensity at \( V_{\gamma7}/V_{\gamma8} \) or \( V_{\gamma9} \), in these subjects.
In addition to rearranged restriction fragments, two unassigned fragments of 7.8 and 6.8 Kb were identified in Hind III digested samples. Interestingly, these fragments were detected in only leprosy patients. It needs further experiments to determine whether these fragments originated due to unidentified rearrangements or due to junctional diversity in rearrangements already reported by others. These fragments could also be germline polymorphic fragments. When these subjects were analysed for their response to fractionated nbCFE, they were found as nonresponders to FII, FIII, FIV and moderate responders to FI.

**REARRANGEMENTS AT TCRδ LOCUS**

Analysis of the TCR δ gene diversity of circulating γδ T cells derived from leprosy patients and healthy contacts revealed Vδ2-Jδ1 amplicons of different lengths. In addition to the expected product of 320 bp, corresponding to Vδ2-Jδ1 rearranged sequences, additional products of 310 bp, 180 bp and 140 bp were detected. These variations in Vδ2-Jδ2 amplicon lengths most probably could arise due to extensive junctional diversity in the δ chain of the γδ T cell receptor (Hata et al, 1988; Loh et al, 1988).

Interestingly, Vδ2-Jδ1 amplicon of 320 bp was detected less frequently in leprosy patients, especially multibacillary patients as compared to healthy contacts. The only multibacillary patient, who showed this rearrangement in circulating γδ cells was ENL patient. Further, Vδ2-Jδ1 amplicon corresponding to 140 bp was detected only in paucibacillary patients and most of these subjects were undergoing type I reactions. These results could be explained in light of the observations suggesting the influence of junctional sequences in the specificity of TCR γδ
(Rellahan et al. 1991). It has also been shown that V-J junctions were critical for antigen recognition (Uuemura et al., 1991). It was probable that cells with Vδ2-Jδ1 rearrangements generating amplicons of varying size differ in their reactivity to *M. leprae in vivo* conditions. This could also be inferred from results of our preliminary *in vitro* experiments. Leprosy patients showing detectable Vδ2-Jδ1 rearrangements corresponding to 320 bp were found to respond to 36-29 kDa (FIV) *M. leprae* proteins, whereas patients with Vδ2-Jδ1 rearrangement generating 140 bp product responded to ≥ 66 kDa *M. leprae* proteins. However to corroborate these findings, further experiments are required on larger sample size to establish a repertoire of Vδ2-Jδ1 rearrangements in circulating γδ cells and their selection by specific antigens. Unexpectedly, a Vδ2-Jδ2 rearrangement was detected in peripheral circulation of two leprosy patients. It requires further analysis to confirm the possibility of circulating cells with Vδ2-Jδ2 rearrangement playing any role in the pathogenesis of leprosy.