DISCUSSION
It is generally accepted that the outcome of infection with \textit{M. leprae} depends on the integrity of the host's specific CMI response, but the reason(s) for diminished cell mediated immunity to \textit{M. leprae}, resulting in overt disease, are far from clear. It has not been possible to pinpoint the specific defect which directs the immune response to opposite poles. Alterations observed in almost every parameter of immune function may just be the reflective symptoms and not cause(s) of leprosy (Rojas-Epsinosa 1994). A variety of possible factors have been postulated by immunologists only to be contradicted by others. These point towards: abnormal antigen presentation (Birdi 1989), primary fault in the macrophage and/or T cells (Ramos et.al. 1989; Sengupta 1993), inappropriate suppressor cell activity (Mehra et. al 1980; Nath et. al 1984), imbalance of cytokines (Sieling et. al. 1993), and genetic constitution (de Vries et. al. 1980; Jazwinska and Serjeantson 1988 and Skamene 1989). These contradictions may have arisen due to a number of influencing factors, such as heterogeneity in patient population, variation in treatment profiles, and the state of activation of the patient's macrophages.

A. LEPROSY AND CELL ME-DIATED IMMUNITY

The cellular immune response is a two-edged sword: it protects, by limiting bacillary growth, yet it also can harm. If turned on improperly, it can induce severe pathology like in tuberculoid leprosy and leprosy reactions. And, if it is turned off improperly, bacteria can reach massive numbers and cause diffuse lepromatous pathology (Ottenhoff 1994).

The form and persistence of leprosy in a person depends on the ability of his body to develop effective cell mediated immunity. In case of leprosy, since protection appears to be mediated predominantly by cell mediated
immunity (Shinde et al. 1993), it was imperative to analyse T cell mediated recognition of the mycobacterial antigens, in light of the genetic makeup of the leprosy patients and their familial contacts.

The healthy household contacts and the normal controls were evaluated as a single category in the present study since the normals were presumed to be exposed to *M. leprae* and therefore were considered close to the healthy contacts. Emphasis was made in this study to include the analysis of responsiveness of healthy familial contacts since they appeared to be infected but mounted a protective and non pathogenic immune response so that the infection remained sub-clinical. It was suggested that due to host factors, 'negative' healthy contacts (negative for bacilli and lepromin test) could exhibit lesion-bacillary positivity in the skin and gradually become 'positive' for lepromin test (Koticha, 1990). Further, the healthy contacts (HCs) studied showed variable responses to mycobacterial antigens, PPD, BCG, WCFE and a few responded more strongly than the other HCs or the diseased familial cases. These variations could probably be explained by both genetic and non genetic factors. However, the variations in response due to the genetic makeup of the individual (de Vries et al. 1980; Vidal et al. 1993) or the repertoire of crossreactive/and specific antigenic determinants (Kaplan and Cohn, 1986) or the other immunoregulatory mechanisms (Sieling et al. 1993), remain to be ascertained.

The predominant finding of the present investigation drew attention to the dichotomy in CMI perturbations that occur in paucibacillary and multibacillary forms of leprosy (Sengupta 1993). The polyclonal T cell responses showed a distinct profile when probed with various mycobacterial antigens in comparison to the general mitogen PHA. The stable BT subjects displayed the expected integrity of T cell proliferative response to PHA as well.
as other mycobacterial antigens (BCG, PPD and WCFE) in contrast to the hyporesponsive/anergic lepromatous leprosy (BL, LL) patients. The present study supported previous findings concerning the lack of cellular immune response to \textit{M. leprae} in LL and BL patients (Sengupta 1993). However, the multibacillary patients included in this study showed a generalised low response to other mycobacterial antigens, BCG and PPD as well, thereby indicating that their anergy was not specific to \textit{M. leprae} but even extended to other crossreactive mycobacteria.

The long-lasting unresponsiveness observed in most of the lepromatous leprosy patients (even after chemotherapy) probably supported the hypothesis which attributes the development of immune tolerance to presence of high concentration of antigenic load (Esquenazi 1990). Probably, the continuous presence of mycobacterium contributed to the depression of the multibacillary patients' cellular immunity. Depressed skin testing responses to cross-reactive antigens such as PPD, impaired contact sensitivity, prolonged skin allograft survival, and impaired leucotaxis at abraded skin sites in lepromatous patients have all been reported to be associated with very high bacillary loads (Kaplan et. al. 1987). Other factors that probably contributed to the inter-individual variations in CMI within a clinical category in the present study could be due to the occurrence of reactional states, irregularity in treatment, extent of exposure to \textit{M. leprae}, nutritional status, etc. Our findings turned out to be in accordance with the early reviews of the defects in CMI of LL that have indicated a generalised defect in CMI. These data were derived from skin sensitization tests to dinitrochlorobenzene (DNCB) and picryl chloride and in-vitro proliferation assays of patient lymphocytes to various antigens and mitogens. (Sansonetti and Lagrange 1981).
The nonspecific suppression effect leading to anergy was also suggested to be mediated by the macrophages of the cutaneous lesions or by the relatively high numbers of CD8$^+$ T lymphocytes (Modlin et. al. 1983a). However, it is not clear whether *M. leprae* induced suppression that actually occurs in vivo is the cause or result of lepromatous disease.

Lack of the long term follow up of clinical histories of patients, their response to lepromin skin tests, repeated evaluation of their bacillary and morphological indices during chemotherapy in order to clinically qualify the defects in CMI in nonresponsive patients was, no doubt, a limiting factor in interpreting the CMI results in this study. Nevertheless the approach adopted was sufficient to categorise the patients as responders or non-responders for further genetic studies which was our major objective.

In this study, an attempt was made to determine the phenotype of lymphocyte populations prevalent in vivo in circulation together with functional studies in order to grade CMI across the spectrum in leprosy.

Since most lymphoid and nonlymphoid organs are included in the migration routes of lymphocytes (roughly 500 billion travel through the blood each day with a mean transit time of around thirty minutes, Bain 1989), alterations due to a variety of conditions including disease, in the lymphocyte composition within those organs might get detected by studying lymphocyte subsets in blood, representing 2% of the lymphocyte pool in the normal adult human body. When extrapolated from adult normal ranges, the T cell enumeration plays a vital role in the diagnosis and management of the disease.

One of the objectives, therefore, of this study was to measure T lymphocyte subsets in leprosy patients, compare the findings with normal
controls, and suggest a possible role for these cells and their cytokine products in infections in these patients. In this study the freshly isolated PBLs were immunostained with monoclonal antibodies OKT4, OKT8, 2H4 and UCHL1 and enumerated flow cytometrically. The study indicated that the imbalances of T cell subsets may be important in many aspects of the disease. Differences were apparent in the proportion of CD4+ and CD8+ cells in the tuberculoid and lepromatous ends of the leprosy spectrum. In accordance to other reports (Narayanan et al. 1983), the helper: suppressor ratios in the peripheral blood apparently declined from healthy contact and tuberculoid to lepromatous forms. These data are in conformity with the suggestion that CD8+ Tc cells may contribute over a prolonged period of time in the chronic LL disease, to the functional depletion of M.leprae reactive CD4+ cells from the immunologic repertoire of LL patients (Modlin 1988).

In this study the CD4:CD8 ratio was apparently high in HC responders to WCFE but not to PPD. Conversely, the proportion was high in LL responders to PPD as compared to responders to WCFE, possibly resulting from the predominance of CD4 (T_h) cells in the responders and the predominance of CD8 (T_s) in the non responders. But this explanation appeared contradictory in the HC and LL cases who responded to PPD and WCFE, respectively despite low CD4:CD8 proportions. The high prevalence of CD4+ T cells in the LL nonresponders to WCFE, and HC nonresponders to PPD can be explained on the assumption that T_H2 type cells which probably provide help to humoral response in-vivo (commonly observed in LL) clonally proliferate in-vitro and recognise crossreactive and/or specific mycobacterial antigenic determinants in different clinical categories. Further, the present study showed that the ratio of memory (UCHL1+) to naive (2H4+) cells were high in the immunologically more active, tuberculoid form of leprosy and HC, responsive to both WCFE and
PPD. The same was observed in LL responders to PPD. However, the memory to naive ratio was observed high in LL non-responders to WCFE but low in LL responders. Since we had also noticed low CD4:CD8 ratio in circulation of LL responders to WCFE, we assume that these less prevalent CD4 cells, which possibly were naive and $T_{H1}$ type, got selected and probably manifested in-vitro cell mediated immune response. This, however, could not be confirmed since in-vitro stimulated lymphocytes could not be analysed by FACS in this study. The perturbation in the balance of these reciprocal CD45R subsets in LL could also be explained by the study (Scott et al. 1991) where instead of normal reciprocal pattern of CD45RA/CD45RO expression, the acquisition and coexpression of CD45RO on the membrane of CD45RA$^+$ lymphocytes could also occur and consequently affect both CD4$^+$ and CD8$^+$ lymphocytes. Nevertheless, the cellular events which control and modulate the differential expression of CD45R isoforms in leprosy patients and their familial contacts need more work in this direction.

It has been postulated that cytokines might tilt the balance in favour of either the TH1 or the TH2 subpopulations (Romagnani 1994). Some genetic factors (not necessarily HLA-related) might also participate in the turning on/off of the TH1 or TH2 cells (Rojas-Espinosa 1994). Realising the need to combine phenotypic determinations with analysis of function and activation states eg., the cytokine profiles, an attempt was made in this study to examine the same by RT-PCR. However, the study could concentrate on expression of r-IFN only in a few representative cases and did not cover analysis of other cytokines. Apparently, the amount of r-IFN mRNA derived from same number of WCFE stimulated lymphocytes was monitored in the form of RT-PCR product intensity. The intensity appeared to be slightly higher in tuberculoid patients than in lepromatous individuals. Moreover an extra band of 515 bp for
7-IFN and an extra >400 bp band for IL4 was also detected, which could have arisen from "illegitimate" alternate splicing events. Similar observations have been reported for G-CSF, IL4, IL2 and IL5 in human bladder carcinoma cell lines and the biological role of these alternative mRNAs has yet to be determined (Sorg et al. 1991).

The present study attempted to categorise the various subjects on the basis of their CMI response. The study showed prevalence of generalised anergy, low CD4:CD8 proportions, and apparently low 7-IFN synthesis in multibacillary forms of leprosy in comparison to paucibacillary forms of leprosy. These diverse observations were further correlated with the inherent heterogeneity in the genetic constitution.

B. LEPROSY AND THE GENETIC PREDISPOSITION

The genetic differences at the individual level are known to contribute to modulate the nature of the specific immune response, as suggested by following observations: (1) all subjects are exposed to common environmental mycobacterium, but the pathogen preferentially activates and/or expands Th1 like cells only in predisposed populations (Modlin et al. 1988) and (2) the response to mycobacterium that is protective for the majority of people, however proves less protective in others and can even evoke polar chronic immunopathology. The understanding of fine tuning of events occuring at the recognition triad level as a consequence of compatibility between antigen profile and host genetic background is of basic importance to test specific strategies not only to control the disease but also for manipulation of complicated immunopathological reactions. The intensity of CMI responses to infections have been shown to coincide with a scale of severity of infections.
from subclinical infections, through the highly resistant tuberculoid form of the disease to, finally, the completely nonresistant lepromatous form of leprosy. The lepromatous form of leprosy appears to represent the threshold of this continuum of resistance, beyond which immune responses to the bacterium are totally lacking. Thus, many genes that affect the immunologic defense system might determine one's position on this scale after exposure to \textit{M. leprae} (Smith 1979).

Leprosy is a complex trait and, thus, genetic analysis of the disease is complicated by many factors including reduced penetrance, genetic heterogeneity of the trait, gene interactions, and presence of phenocopes (Levee et al. 1994). Numerous genetic marker studies were conducted to identify pleiotropic effects of hypothetical genes like ABO, Rh, G6-PD deficiency etc. (Abel 1989; Smith 1979), but only the HLA II associations (de Vries et al. 1980, Ottenhoff and de Vries 1987) proved to be convincing.

However, a working hypothesis was suggested for a two-locus model of genetic susceptibility to leprosy (Abel et al. 1989). The major gene controlling susceptibility to leprosy per-se was suggested to be an analogue of the mouse natural resistance associated macrophage protein gene ("Nramp") or the Bcg/Ity/Ish gene located on the long arm of human chromosome 2. Whereas, the severity or progression of the disease across the spectrum was suggested to be regulated by secondary genes like HLA or HLA linked locus. Moreover, the gene controlling susceptibility to leprosy per-se and that controlling susceptibility to nonlepromatous were indicated to be different, acting at successive stages of the immune response to infection with \textit{M. leprae} (Abel et al. 1989). The present study was performed to investigate whether there was a genetic marker on chromosome 2q for susceptibility to leprosy or a
particular type of leprosy and to test the segregation of the allelic variants of these markers with the CMI response status. The study focussed upon the probable intra- and inter-allelic influences of COL3A and CTLA4, both located at chromosome 2q31-33 region.

The present study initially attempted to analyse genetic variation in CRYG1, located at 2q33-35 but did not continue these studies since the gene did not exhibit sufficient allelic variations when analysed in a few representative cases. This observation of cryg1 being an uninformative locus was also reported by Shaw et al. (1993) who suggested an absence of linkage disequilibrium at the population level since the \( \tau \)-crystallin (CRYG) gene family mapped approximately 8 cM proximal to `Bcg' in mouse.

The COL3A amplified products when analysed by PAGE showed 5 allelotypes, ranging from 236 to 312 bp size. A few extra, low molecular size amplicons were also observed. However, these did not interfere with allelic detection, and acted as internal controls for the technical success for amplification as shown in PASA (PCR amplification of specific alleles) (Sommner et.al. 1992). The additional amplicons so obtained probably resulted either because of the presence of homologous polymorphic sequences at different sites in human genome or the impurities (truncated oligos) of the synthesised primers. Interestingly, some of these additional amplicons were noticed in all the subjects and some of these bands were unique and polymorphic. Their further analysis in future could help in unveiling the consequential phenotypic influences of heterogeneity in the chromosome 2q 31-35 region.

The CTLA4 amplified products of 84 to 120 bp size representing 8 alleles when analyzed on denaturing PAGE, displayed simple sequence length polymorphism (SSLP). The allelic sizes differed as a multiple of 2 bp and
suggested that these differences were because of the dinucleotide repeat units nested within the amplicon.

One of the observations made in the present investigation was the high predominance of 250 bp allelomorph of COL3A and 84 bp allelomorph (not reported earlier) of CTLA4 in both normal plus healthy contact category as well as total leprosy patients. Further, the 236, 284 and 312 bp alleles of COL3A and the 98, 114 and 120 bp allelomorphs of CTLA4 were not observed in homozygosity both in normal and diseased subjects studied.

A comparison of status of the allele(s) in responsive/nonresponsive healthy contacts and leprosy patients showed that the 270 bp COL3A homozygotes (270/270 bp) responded to WCFE. Whereas, the 312 bp COL3A heterozygotes were nonresponsive to both PPD and WCFE, irrespective of the clinical status of the cases analysed, suggesting a possible association between heterozygosity for 312 bp allele of COL3A and the nonresponder status to mycobacterial antigens in general. Further, the study revealed the probable association of N+HC, paucibacillary and multibacillary forms of leprosy with 104 bp, 98 bp and 106 bp CTLA4 alleles, respectively. An absence of, COL3A allelomorphs of 236 bp in homozygous/heterozygous conditions in multibacillary leprosy patients and, 270 bp in homozygous state in N+HC category was also observed. However, the association of COL3A and CTLA4 allelomorph heterogenity with the disease did not yield unequivocal results.

Nevertheless, in this study a probable demarcation between the type of the disease and the responder/nonresponder status of the diseased subject was made apparent, where the latter showed an association with some of the genetic markers used in the present study.
It is suggested that further study on similar lines, involving the amplification of interlocus regions i.e, regions between COL3A, CTLA4 and CRYG on 2q31-35; and exploring the possibility of as yet unidentified gene in this region in linkage disequilibrium with the susceptibility to leprosy, will be required to unravel the enigmas hidden in the disease.