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COMPARISON OF THE ACTIVATION OF FUNCTION OF T-CELLS FROM BLOOD AND TISSUE LESIONS OF LEPROSY PATIENTS

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Abbreviations

APC	Antigen presenting cells
BSA	Bovine serum albumin
CPM	Counts per minute
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity reaction
FITC	Fluorescein Isothiocyanate
FACSCAN	Fluorecense activated cell scanner
FCS	Fetal calf serum
3H(Tdr)	Tritiated thymidine
IL-2	Interleukin-2
IL-4	Interleukin-4
IFN-γ	Interferon-gamma
LCM	Lymphocult
LDA	limiting dilution analysis
MHC	Major histocompatibility complex
MDT	Multi-drug therapy
MB	Multibacillary
moAb	monoclonal antibody
NHS	Normal human serum
PB	paucibacillary
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate

PE	**Phycoerythrin**
TH1	**T-helper-1**
TH2	**T-helper-2**
TCR	**T-cell receptor**
Ts	**T-suppressor cell**

ABSTRACT

While characterizing the functional properties of T-cells across the leprosy spectrum, significant differences between proliferation of T-cells isolated from blood and tissue lesions were consistently noted. The differences occurred in more than 80% of cases when comparison was made between blood and skin derived lymphocytes from leprosy patients; in 50-80% cases when comparing lymphocytes isolated from nerves with those from blood of leprosy patients, and > 85% when comparing lymphocytes derived from blood or skin lesions of patients with other skin inflammatory diseases. The differences were seen throughout the leprosy spectrum. Skin derived lymphocytes from patients with multibacillary (MB) or paucibacillary (PB) leprosy proliferated poorly when compared to lymphocytes isolated from blood and lymphocytes derived from nerves of some PB and most MB patients proliferated poorly when compared to lymphocytes from blood. Stimulation with a strong co-mitogen did not restore responsiveness of T-cells isolated from skin lesions. Cells from different tissues also differed from each other in activation requirements. Analysis of molecules known to differ between T-cell subpopulations and to play a role in T -cell activation indicated some differences in the molecules expressed between blood and tissue lesion derived T-cells. However, the differences observed did not appear to be striking enough to account for the poor responsiveness of tissue lesion derived T-cells. Assays for lymphokine production showed that more IL-2 and IL-4 were produced by T-cell cultured from blood compared to cells from skin, IFN-γ production was found to be made in equal amount by cells from both tissues. The absence of marked differences between

blood and lesion derived T-cells, except in the production of IL-2 and IL-4, indicates that a possible explanation for the difference in proliferation is anergy as anergic cells are not able to produce IL-2 or respond to IL-2 from an external source. Other possibilities which may contribute to the difference are discussed.

1. INTRODUCTION

1.1 The Organism

Leprosy is one of man's most puzzling chronic diseases. It is caused by Mycobacterium leprae. Although the disease is infectious, the causative organism is virtually non-toxic and may grow in the skin in large numbers without any direct clinical symptoms. Most symptoms of the disease, in fact, are due to immune reactions of the host against the bacilli. As a result, leprosy may to a great extent be considered as an immunological disease (Closs and Harboe, 1981).

M. leprae was one of the first infectious organisms identified by man (Hansen, 1874; cited by Closs and Harboe, 1981). The organism is an obligate intra-cellular parasite residing inside the host phagocytic cells. Their intracellular location, a lipid-rich cell wall and protective capsule, preclude attack by the humoral immune system (Bryceson and Pfaltgraff, 1990).

The organism multiplies best in cooler parts of the body, hence the skin of the face and limbs and the more superficial nerves are preferentially invaded (Hastings, 1985). Distinctively M. leprae is non-cultivable on any artificial media and it can only multiply outside the human body in certain animals such as the armadillo, mice, rats, and monkeys (Hastings, 1985).

1.2.Transmission.

The cause of leprosy is fully established, but no satisfactory explanation as to how the bacterium is transmitted from one host to another exists. However, the most likely portals of entry are the respiratory tract and the skin, but transmission by arthropods, tattooing needles and breast milk have still not been excluded

(Ridley,1988). Once in the human body, bacilli are taken up by macrophages, mainly in the skin, from where they infiltrate peripheral nerves by infecting the macrophage-like Schwann cells.

1.3.The Disease

Leprosy is essentially a disease of the peripheral nerves but it also affects the skin. In almost all cases the organism invades and destroys the skin and peripheral nerves, and in more severe cases it may infiltrate the eyes, nose, larynx, mouth, hard and soft palate. Organs of the reticuloendothelial systems, namely lymph nodes, liver, spleen, and bone marrow; as well as internal organs such as the testes, adrenal glands and kidneys are also infected (Hastings, 1985).

The incubation period of the disease is difficult to assess. The bacilli have the longest generation time known for any infectious bacterium (11-13) days when compared with about 20 minutes for E. coli (Ridely,1988).

1.4.The History of Leprosy

Leprosy is one of the most ancient diseases known to man. The origin of leprosy, however, is not well established. Two places suspected of being the origin of leprosy are India and Egypt. But whether it was in existence in both countries at the same time can not be decided with certainty (Cochrane, 1959).

The earliest absolute evidence of leprosy is seen in Egyptian skeletons of the 2nd century B.C and in two coptic mummies of the 5th century A.D. (Bryceson and Pfaltzgraff, 1990). The earliest written records describing leprosy, however, come from India, dated about 600 B.C (Hastings, 1985).

From India leprosy appears to have spread to China in about 500 B.C and then to

Japan (Hastings, 1985). Good clinical description of leprosy comes from China, dating 190 B.C. The cause was considered to be overcrowding, promiscuity, lack of hygiene and dirt (Anderson, 1969). The disease was then spread to Greece by soldiers of Alexander the Great, returning from the Indian campaign at about 327-326 B.C. It then spread slowly through the Greek and Roman Empires (Ohman, 1986).

The spread of leprosy to Europe, was from the crusaders returning from the Ottoman Empire. It is not known how leprosy reached the Australian aborigines. The introduction of leprosy to the Americas, however, can be traced to the Spanish and Portuguese settlers, and to the importation of African slaves to the regions (Hastings, 1985).

During the middle ages and until the late 19th century people strongly associated the disease and immorality and the cause was associated with cardinal sin. Moreover, the disease was also considered to be hereditary. In the early 1880s, however, Armauer Hansen, in Norway, for the first time identified leprosy bacillus from skin lesions (Hansen, 1974; cited by Ohman, 1986).

1.5. Distribution and Prevalence

Leprosy has a higher prevalence in the poorer developing countries (Strickland, 1983). More than 1.6 billion people live in countries where the estimated prevalence of leprosy is greater than one case per 1000 population, and it is generally assumed that all are at equal risk (WHO,1988).

The prevalence of leprosy per 10,000 population was estimated in February, 1992 as: 16.34 for the South-eastern Asia region, 0.08 for the European region, 2.31 for

the eastern mediterranean region, 0.72 for the western pacific and 6.58 for the African region (WHO, 1992).

The estimated total number of leprosy cases world wide in 1991 was 5.5 million (WHO, 1992) but the actual number is two or three times this figure as diagnostic criteria are undefined and enumeration of cases is incomplete. Information on leprosy cases registered for treatment is more reliable than that on estimated cases. Hence, information up to February 1992 gives a total of 3,087,788 registered cases as opposed to 3, 737, 375 cases in October 1990, a reduction of 17.4 percent for that period and reduction of 43.0 per cent since 1985. This drastic reduction was attributed to the success of multi-drug therapy (MDT). Based on this success WHO has designated global elimination of leprosy by the year 2000 by reducing the case load to less than 1/10,000 individuals (WHO, 1991).

Of the registered cases nearly 95 percent occur in 25 countries of which India, Brazil, Nigeria, Myanmar and Indonesia (in descending order of magnitude) contribute 83% (WHO, 1991). The prevalence of leprosy in the African regions is second highest to South-East Asia.

1.6. Leprosy in Ethiopia.

The endemic nature of leprosy in Ethiopia has been recorded for a long time. According to Zien and Kloos (1988) an estimated 120,000 cases of leprosy exist in Ethiopia. The peak prevalence was reached in 1982 (26/1000) and had declined sharply by 1990 (0.7/1000). Higher rates of prevalence are consistently observed in the densely populated highlands, particularly in central and northern Ethiopia (Genderson, 1987). The reason for the higher prevalence may include higher

population density, crowding, climatic conditions and low living standard (Kloos and Zein, 1993). The direct relationship between altitude and leprosy prevalence is not, however, found in all areas (Debrezion, et al. , 1990). According to a 1988 report in Ethiopia, the number of registered cases was 31,753, the second highest in Africa being exceeded only by Nigeria (WHO, 1991). At the end of 1990, 15,547 leprosy cases (versus 84,627 in 1982) were registered for treatment (ALERT,1991).

1.8.Leprosy and its social consequence

Leprosy continues to be of major concern in all developing countries because of deformities it causes in a large proportion of patients. Crippling mutilations, facial disfigurement, nerve damage, eye damage and personality disorders are impairments directly resulting from leprosy (WHO, 1993). Of the estimated number of leprosy cases more than 50% have deformities (WHO, 1993). Disabled patients are incapable of supporting themselves or their families.

Besides all these, the attitude of people who consider leprosy as a sign of immorality and the biblical consideration of leprosy as a divine punishment for sinfulness have caused sufferers to be shunned by the society and has forced the patients to live as outcasts in appalling conditions. In general, the impact of the disease on the patient and their families is life long and the disease causes an adverse reaction in the community and much distress and unhappiness (WHO, 1979).

1.8.Clinical and Immunological aspects of Leprosy.

Clinical symptoms and signs in leprosy are extremely varied with respect to both nature and extent. Thus, leprosy is not a single clinical entity; rather the disease

presents as a clinical spectrum (Hastings, 1985). The most useful and detailed system of classification of leprosy is the Ridley-Jopling classification which classifies patients with leprosy into five categories according to immunity (Ridley and Jopling 1966; cited by Hasting, 1985).

The spectrum is composed of two polar forms, tuberculoid leprosy (TT) and lepromatous leprosy (LL) and intermediate borderline forms (BB), borderline tuberculoid (BT), borderline lepromatous (BL). The factor which mainly determines the place of an individual patient disease on the spectrum is the extent to which cell mediated immunity against M. leprae is expressed (Ridely and Waters, 1969). In tuberculoid leprosy cellular immunity and delayed hypersensitivity are well developed whereas in lepromatous leprosy specific cellular immunity to M. leprae is impaired. In addition, the antibody responses at the poles differ although this difference may not directly contribute to other aspects of the disease (Melson, 1983). Between these poles, the majority of patients are not fixed in the spectrum; if treated, they tend to gravitate toward the tuberculoid and if untreated to the lepromatous pole.

Ridley and Jopling's classification is the most scientific one, however, WHO has adopted a new approach to classification for the purpose of treatment (WHO, 1988). The WHO study group classified patients as having multibacillary (all mid-border line (BB), borderline lepromatous (BL) and polar lepromatous (LL)) or paucibacillary (polar tuberculoid (TT) and borderline tuberculoid (BT) leprosy.

T-lymphocytes are instrumental in acquired resistance to infectious agents. In leprosy, the defect in M. leprae specific cell mediated immunity is associated with

uncontrolled growth of the parasite. Beyond a spectrum of immune responses, ranging from well-developed cell-mediated immunity in the PB patient to lack of immunity in MB cases, exaggerated responses also cause inflammation leading to immune pathology.

Despite the fact that M. leprae has a low toxicity, leprosy patients may succumb to attacks of inflammatory reactions. Reactions occur particularly upon treatment when the clinical picture can change rapidly, often resulting in severe tissue damage. There are two types of reactions, type 1 and type 2. Both histological and immunological features suggest that type 1 (reversal reaction) is precipitated by rapid increase in cell mediated immune reaction to M. leprae (Ridley and Waters, 1969). On the other hand, humoral immunity also contributes to the other form of inflammation, the type 2 reaction (Goodless, et al., 1991). Several causes have been put forward for the precipitation of an immune response resulting in reactions, including action by soluble mediators (Nathan, 1986), and quality of antigens (Ridley, 1976).

Several lines of evidence indicate that patients with lepromatous leprosy are characterized by a specific cell-mediated immunological unresponsiveness to antigens of M. leprae (Shepard, 1968; Turk, 1969). The nature of this unresponsiveness has mainly been investigated in vitro on peripheral blood samples. About 60-70% of human blood lymphocytes are T-cells (Froland and Natvig, 1972), and T-cells recirculate in peripheral blood in order to carry out their function (Myrivang, et al., 1972). These and other considerations have made peripheral blood lymphocytes a good source of T-cells for functional studies. Quiescent human

peripheral blood T-lymphocytes can be stimulated to grow by antigens (Myrivang, et al., 1973), monoclonal antibodies (moAb) (Reinhertz, et al., 1983), lectins such as concanavalin A (Con A) and Phaseoius vulgaris phytohaemagglutinin (PHA) (Bullock and Fasal, 1971) and these in vitro responses are thought to resemble processes occurring in vivo.

Some of the evidence accumulated so far, suggests that PBMC from significant numbers of patients with active lepromatous leprosy are deficient in their response to PHA in vivo (Nath, et al., 1977). The response to PHA was only moderately depressed in patients with tuberculoid leprosy, and in patients whose lepromatous disease had been rendered inactive by long-term therapy (Dierks and Shepard, 1968). However, this view is controversial and both in vivo and in vitro experiments show that the unresponsiveness is M.leprae antigen specific.

Most lepromatous patients respond well to recall antigens and particularly to tuberculin PPD but are completely unable to respond to antigens present in M. leprae (Bloom, et al., 1992). Moreover, studies of several hundred patients using thymidine incorporation in lepromin containing cultures showed that over 85% of lepromatous patients and less than 15% of tuberculoid patients were unresponsive (Mehra, et al., 1980). All these show that the unresponsiveness is remarkably selective and specific, except in advanced-stage disease.

The reason for the defect in leprosy, remains obscure. The presence of serum inhibitory factors (Bullock, 1971) a defective immune system (Dwyer, et al., 1973); immunosuppression (Shepard, 1968; Bloom, et al., 1986) and immunological tolerance (Godal, et al., 1971) are some of the major theories of unresponsiveness.

Immunological tolerance is the lack of ability to mount an immune response to epitopes to which an individual has the potential to respond (Clane, 1990). This occurs in a variety of ways including classic tolerance through clonal deletion or anergy.

Immature T-cells are more sensitive to tolerance than mature T-cells and T-cells are more sensitive to tolerance than B-cells (Shwartz, 1990).

Anergization (i.e., acquisition of antigen nonresponsiveness) of T-lymphocytes may become manifest in different ways, and various phenotypes of anergic, tolerized T-cells have been described. Some of the possible causes of anergization are:1) T-cells that have lost the capacity of transcribing the interleukin-2 (IL-2) gene but respond to exogenous IL-2 (Schwartz, 1990). 2) T-cells that down-regulate the expression of co-receptor molecules (Gajewski, et al., 1989). 3) T-cells that express subnormal densities of the T-cell receptor (TCR) after in vitro tolerization with high antigen doses in the human system (Lamb, et al., 1987).

The focus of leprosy is primarily the skin and nerves in which the battle between the pathogen and the immune system is waged (Modlin, et al., 1986). Protective immunity against intracellular bacteria as well as mycobacteria is a local event which is focussed on granuloma formation. Several studies of tissue sections with monoclonal antibodies and immunohistochemistry (Cathleen, et al., 1989; Bloom, et al., 1992) have shown that granulomas of lepromatous leprosy patients contain an excess of lymphocytes bearing the CD8 marker compared with predominance of CD4 bearing T-lymphocytes in tuberculoid leprosy. Furthermore, there are few lymphocytes within lepromatous granulomas in contradistinction to the granulomas

of the tuberculoid forms of leprosy. Infiltrates in the skin and nerve granulomas of a given type of leprosy have similar characteristics (Kumar, et al, 1989). Reduction of delayed hypersensitivity responses to intradermally administered antigens (Dwyer, et al., 1973) and the observation that the paracortical area of lymph nodes, normally heavily populated with T-cells is largely replaced with macrophages in lepromatous leprosy (Sharma, et al., 1958) and presence of few lymphocytes within lepromatous granulomas all indicate the same picture of unresponsiveness in tissue granulomas.

1.9. T-cell activation and Function

An efficient immune system is characterized by the ability to develop a rapid T-cell proliferative response to eliminate the pathogen which has invaded the body (Mueller, 1989). Specific activation and subsequent proliferation of T-lymphocytes is initiated by recognition of surface-bound antigen and major histocompatibility complex (MHC) molecules by the clonotypically expressed T-cell receptors (TCR) (Knusbeek, 1985). Interaction of the TCR with its antigen results in activation and other effector functions. The TCR is associated with CD3 in a functional complex that transduces signals as a consequence of antigen binding. Hence, the level of CD3 is proportional to that of T-cell receptor There are two types of TCRs, $\alpha/ß$ (alpha-beta) and γ/δ (gamma-delta) (Janeway, 1988). The majority of T-cells bear $\alpha/ß$ TCR and γ/δ TCR in man comprises about 5% of the CD3+ cells in all organized lymphoid organs as well as in the skin and gut associated lymphoid tissues (Bloom, 1986). Virtually all of the known, antigen specific cell-mediated effector and regulatory mechanisms of the immune system are carried out by $\alpha/ß$ TCR. The majority of T-cells identified from leprosy patients also express $\alpha/ß$

TCR. The nature of the specificity and immunological functions of γ/δ cells remains enigmatic. It has been suggested that they are involved in mediating immunological surveillance of epithelia, monitoring the integrity of the cell layer that separates the internal from the external milieu (Janeway, et al, 1988). Moreover, there is a view that they are involved in antibacterial immunity in and stimulating granuloma formation (Modlin, 1988).

Activation of T cells involves a complex cascade of cellular interactions which culminate in lymphoproliferation and differentiation (Mills, et al. 1985). For lymphocyte activation and proliferation two signals are required (Brether and Cohn, 1970). Interaction of antigen-MHC complex with the T-cell receptor complex is the first signal and an accessory cell-derived co-stimulatory signal, the second. The presence of the first signal in absence of the second signal leads to clonal anergy and a defect in primary signal is also a cause for unresponsiveness (Mueller, et al. 1989).

The receptors on T-cells which interact with co-stimulatory stimuli have not been unambiguously identified. However, the human T-cell antigen CD28 is a strong candidate for the second signal which activates T-cells to proliferate and secrete lymphokines (Martin, et al., 1986). The molecule carrying the CD28 antigen is involved in T-cell activation and the level of expression of CD28 may determine whether a positive or a negative signal is generated (Lesslauer, et al., 1986). These molecules are not generally expressed on T-suppressor cells and they are used to separate T-helper from T-suppressor cells (Guangli, et al., 1990).

Recent studies have focused on the lymphokine profile of M. leprae reactive T-

cells. Lymphokines encompass all non-immunoglobulin and non TCR substances that are made by the cells of the immune system upon stimulation, and that by acting on these cells, promote activities aimed at the elimination of invading parasite and the repair of damaged tissue (Clane, 1990). Interleukin-4 (IL-4), interleukin-2 (IL-2) and interferon-gamma (IFN-γ) are the major lymphokines involved in T-cell proliferation and other functions. It has been found that IL-4 antagonizes some of the activities of IFN-γ (Salgame, et al., 1991). IL-4 is the major growth factor for T-helper 2 (TH2) cells , down-regulates IL-2 receptors and blocks IL-2-dependent proliferation and IFN-γ production by human blood mononuclear cells (Salgame, et al, 1991). IFN-γ is a growth factor for T-helper 1 (TH1) cells and these cells are critical in the generation of delayed-type hypersensitivity responses (DTH) as a result of the ability of these cells to secrete lymphokines (including IFN-γ) (Mueller, et al., 1989).

The other group of co-receptors which are involved in aiding signalling are the CD4 and CD8 molecules. The division is based on the expression of different surface molecules and they are expressed in a mutually exclusive fashion on different subpopulations. CD4+ T-cells function as helper/inducer and CD8+ cells encompass cytotoxic and suppressor cells. Both CD4+ and CD8+ cells can be discriminated into subpopulations that differ in their function and patterns of lymphokine secretion. Accordingly, studies with murine T-cell clones suggest that type one or TH1 secrete IFN-γ and IL-2 and type 2 or TH2 secrete IL-4 and IL-5 (Mosmann and Cofman, 1989). Although the majority of human T-cells may produce a mixture of these lymphokines, there is evidence for patterns suggestive

of TH1 and TH2 cells in certain settings (Romagnani, 1992). Type 1 CD8+ cytotoxic T-lymphocytes produce predominantly IFN-γ and IL-2 while CD8 derived from immunologically unresponsive lepromatous leprosy produce predominantly IL-4 (Bloom, et al., 1992).

Analysis of lymphokine mRNA of T-cells isolated from lesions has also shown that in tuberculoid lesions IFN-γ and IL-2 predominate while in lepromatous lesions IL-4 mRNA predominates (Cooper, et al., 1989).

Lymphokines orchestrate the complex processes of inflammatory and immune reactions. IL-2, for example, may exert paradoxical, stimulatory as well as inhibitory effects on in vivo immune responses, such as DTH (Hancock, et al., 1987). Moreover, in many experimental systems, IL-2 may also abrogate immunological tolerance, at least in certain situations (Kroemer, et al, 1991). However, anergization of T-lymphocytes is also caused by T-cells that have lost the capacity of transcribing the IL-2 gene and T-cells that have lost IL-2 responsiveness (Schwartz, 1990 ; Gajewski, et al., 1989).

All these studies support the notion that production of IL-4 and IL-5 leads to suppression and anergy while production of IFN-γ and IL-2 by these cells leads to activation and protection (Britton, 1993).

Unresponsiveness in lepromatous patients might also be mediated by T-suppressor cells (TS) cells (Bloom, et al., 1992). All the suppressor lymphocytes were shown to belong to the CD8 T-cell subset (Mehra, 1980) but both CD4 and CD8 subpopulations in various systems reported are involved in immune response suppression (Bloom, et al., 1992).

from different lymphocytes in different tissues. Lymphokine production pattern of different tissue isolated lymphocytes was also studied.

2. MATERIALS AND METHODS

2.1 Study population

Samples (blood, skin and nerve biopsies) were taken from 73 subjects (mean age 32.2, range 12-50 years). These included 40 leprosy patients 15 multibacillary, 15 paucibacillary, 10 cases with leprosy neuritis, 15 with inflammatory diseases (leishmaniasis (n=5), psoriasis (n=5), folliculitis (n=3), dermatitis (n=2) and impetigo (n=1)) and 18 healthy individuals (control). Lymphocytes isolated from these subjects were cultured and proliferation was measured by thymidine incorporation (Coligan, et al., 1991).

2.2.Laboratory Tests

2.2.1. Extraction of lymphocytes from skin biopsy specimens

Ellipsoid skin biopsy specimens were obtained under local anaesthesia. The skin specimen, stripped of epidermis and sub cutaneous fat was chopped into small pieces by using surgical scalpels and was cultured in RPMI-1640 (Gibco; UK) containing 10% Fetal calf serum (10%FCS) (Sigma; USA) and supplemented with 1% penstrep (1 U/ml penicillin and 1 μg/ml streptomycin (final)) and 1% glutamine (1 U/ml). After an overnight incubation (depending on the number of cells isolated) the sample was incubated, in collagenase (250 μg/ml) (Sigma; USA) for one hour. The pieces were then separated, pressed through a fine mesh, squashed on a screen and washed with 10% FCS in RPMI-1640. Cells released by mechanical disruption and enzymatic digestion were layered on Ficoll-Isopaque (ρ = 1.077, Pharmacia) and separated from debris and other non-lymphoid cells,

and then washed three times with RPMI-1640.

2.2.2.Extraction of Lymphocytes from Nerve Specimens

Nerve biopsies were chopped mechanically and treated enzymatically as described for the skin biopsy specimens.

2.2.3.Isolation of Lymphocytes from Peripheral Blood

Blood obtained from patients was defibrinated with glass-beads and centrifuged at 2000 rpm. Blood cells were resuspended with a 2-3 volume excess of RPMI and layered on Ficoll-Isopaque and centrifuged for 20 minutes at 2000 rpm. The cloudy mononuclear cell rich interface was collected and washed three times in RPMI-1640. Cells were resuspended in RPMI containing 5% normal human serum (5% NHS) supplemented with glutamine and 1% penstrep. The cell suspension was then kept in ice until the time of assay.

2.3. T-Cell Proliferation

To determine the biological response of human lymphocytes to stimuli, proliferation assays were done. The proliferative response was measured by incorporation of tritiated thymidine ([^{3}H] thymidine) into DNA, a process which correlates very well with cell growth as measured by changes in cell number (Coligan, et al.,1991). The approach followed was the "semi-limiting dilution analysis" which combined elements of standard T-cell proliferation assay and limiting dilution analysis. Tissue derived and PBMC isolated cells were serially diluted and added to replicate wells at concentration of 100, 10, and 1 cell/well. To these were added a variety of growth supplements including different concentrations of IL-2 (Roussel UCLAF; USA), 25% Lymphocult (LCM) (soluble factors produced by PHA-stimulated

PBMC) (Biotest, W.Germany), and different concentrations of PHA (SIGMA; USA), and irradiated (3000 rad) feeder cells ($1.5x10^5$), in RPMI and 5% NHS. Experiments were carried out under 9 different conditions: PHA-Hi IL-2- Hi (high PHA and high IL-2); PHA-Lo IL-2-Hi (low PHA and high IL-2); PHA-Hi IL-2-Lo (high PHA and low IL-2); PHA-Lo IL-2-Lo (low PHA low IL-2); No PHA Hi-IL-2(no PHA High IL-2); No PHA Lo-IL-2 (No PHA low IL-2); Hi-PHA H-IL-2 25% LCM (High PHA high lL-2 and lymphocult); Lo-PHA Hi-IL-2 and 25% LCM (Low PHA High IL-2 and LCM); 25% LCM and H-IL-2 (lymphcult and high IL-2).

T-cells were cultured at various doses together with $1x10^5$ irradiated feeder cells and different supplements defined above in a total volume of 200 μl and incubated at 37°c in a humidified 5%CO_2 incubator (Gelaire; Flowlab; Italy). On the 7th day cultures at 100 cell/well were resuspended and half of the cells were substituted with 25 U/ml IL-2 and fresh medium and were pooled for phenotypic and functional analysis. To the remaining 100 μl 25U/ml IL-2 and 1 μ/Ci (3H) thymidine (Amersham; UK) was added and incubated for 18 hours. Cells were then harvested with a Skatron harvester onto filter paper, allowed to dry and then prepared for liquid scintillation counting in an LKB Rack beta counter (LKB, Instrument Inc; Gaithersburg, Md). Mean of 6 replicates wells was determined, following subtraction of mean count per minute(CPM) of control wells which contained irradiated feeder cells, growth supplements and with no responding cells.

2.4. Analysis of Cell Surface Markers

Cell surface phenotype was analyzed for each sample before culturing. Depending

on the tissue source 5000-100,000 cells were washed with PBS containing 0.1% sodium azide and 1%Bovine Serum Albumin (1%BSA) and aliquot added into flat-bottom microtiter plate. Cells were then incubated with the following combination of monoclonal antibodies(moAb) conjugated with Phycoerythrin (PE) and Fluorecense Isothiocyanate (FITC) (Becton Dickenson; USA): 5μl CD3 FITC and 5μl CD28PE; 5μl CD3PE and 5μl CD45RA FITC; 5μl CD4FITC and 5μl CD8PE 5μl α/ß PE and 5μl γ/δ FITC ; 5μ CD14 FITC and 5μ CD40 PE for one hour at 4^0c. Isotope control was also set up by adding 5μ of IgGγ1 FITC and 5μ IgGγ2a PE.

After one hour of incubation at 4^0c stained cells were washed three times in PBS/BSA/Azide and were analyzed on FACSCAN flow cytometer machine (Becton Dickenson, USA) equipped with LYSSIS2 software.

2.5. Induction of Lymphokines

Lymphokine induction by immobilized anti-CD3 moAb was performed after culturing the lymphocytes with different culture conditions. To 96-well microtiter plates 100 μl of 1:1000 diluted monoclonal anti-CD3 antibody was added and incubated for an overnight at 37°C in 5% CO_2 incubator.

Plates were washed three times with PBS-azide and to each well 10^5 cells were added and incubated for 24 hours at 37 ^{0}C in a 5%CO_2 incubator, centrifuged for 5 minutes at 2000 rpm and supernatants collected and stored at -70 °C until the day of the assay.

2.5.1.Lymphokine Assay

Lymphokine assays were carried out using commercially available kits according to

the manufacturers specifications. These kits employ quantitative "sandwich" enzyme immunoassay technique. A monoclonal antibody specific to a lymphokine is coated onto wells of a microtiter plate and serially diluted standards and T-cell culture supernatants are then added. Unbound material is washed off, and an enzyme-linked polyclonal antibody (rabbit polyclonal anti-human IL-2, IL-4 and goat polyclonal antibody for IFN-γ) is added to the wells. After repeated washing, a third antibody (biotin labelled goat anti-rabbit immunoglobulin for IFN-γ) is added. Finally, following a wash an enzyme reagent, (Streptoavidin-peroxidase), a substrate solution Peroxide reagent and OPD (chromogen) are added to the wells and the color developed is proportional to the amount of lymphokine bound in the initial step. The color development is stopped by adding 100 μl of 2N H_2SO_4 and the intensity of the color is measured at 492nm . For IL-2 and IL-4 ELISA, the substrate was added directly to the enzyme-conjugated 2nd antibody, the reaction stopped and the color developed was measured at 450nm.

A curve was prepared, plotting the optical density versus the concentration of lymphokine in the standard wells. By comparing the optical density of samples to this standard curve, the concentration of the lymphokine in the unknown sample was then determined.

The lower level of sensitivity of the methods was 31.3pg/ml for IL-2 and IL-4 and 100pg/ml for IFN-γ.

2.6. Optimization of reagents

Higher and lower doses of PHA and IL-2 were determined by taking blood from healthy individuals. Lymphocytes isolated as detailed previously were cultured at

different concentrations (100, 10 and 1 cell/well) by varying PHA (0-500 μg/ml) and holding constant the number of IL-2 and feeder cells. The same procedure was repeated for IL-2 except that the variable component was IL-2 (0-500U/ml) and PHA and feeder cells were kept constant.

Determination of maximal peak of 3H (Tdr) incorporation was also carried out by culturing cells at 100, 10, and 1 cell/well from healthy individuals and pulsing with 3H(Tdr) on different days for each concentration.

As with other variables of the proliferation assay, titration for the number of feeder cells required was done by varying the number of irradiated feeder cells (50000-200000 cell/well) and PHA (5 and 100μg/ml) and keeping other culture conditions constant.

2.7.Statistical Analysis

Data was entered into a commercially available all purpose Excel program and statistical analysis was performed. The mean and standard deviation was calculated and comparison of blood and tissues was done by calculating t-test and comparison between methods was done by calculating chi-square. All statistical analyses were carried out assuming the population was distributed normally.

3. RESULTS

3.1.0. Determination of Optimal Dose of Culture Conditions.

The first goal of this study was to select optimal culture conditions titrating different activation agents (PHA, IL-2 and feeder cells) by using blood from healthy individual The optimal length of culture prior to addition of (3H)thymidine was also determined.

The results for PHA (Fig. 1) show that the proliferation at different doses of PHA was not the same. Maximum proliferation was observed at 100μg/ml (final) and least proliferation was observed at 10μg/ml (final). Based on this, high and low dose was determined to be 100 and 10μg/ml (final). The same result was also obtained for IL-2 and 100 and 10U/ml was found to be the high and low doses respectively. The peak day of 3H(Tdr) incorporation was not the same for the different concentrations (Fig.2.). Peak day was found to be 6th, 10th and 12th day for 100, 10 and 1cell/well, respectively.

Titration of required feeder cells was done and (Fig. 3) shows that there is an increase in proliferation as the number of feeder cells is increased. But as peripheral blood mononuclear cells (PBMC) were in short supply, we selected a dose of 150000cells/well, which was not optimal, but still sufficient to support good proliferation.

Once the minimum and maximum concentrations of some of the variables (PHA,IL-2 and feeder cells) was established experiments were carried out under 9 activation conditions and comparisons were based on the results at 100 cells/well.

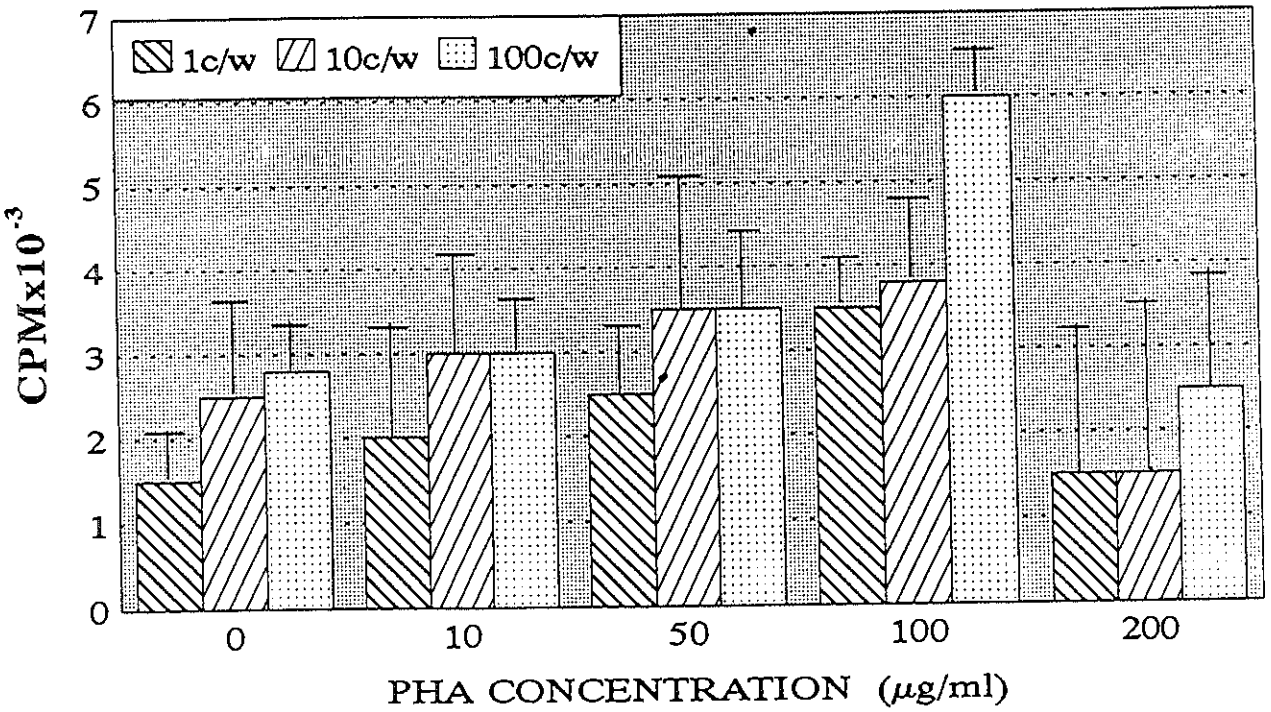

Fig. 1. PHA Optimization Curve. Samples were obtained from three healthy individuals. Cells at a concentration of 100, 10 and 1cell/well were cultured with culture medium (5%NHS), IL-2 (100U/ml final) and variable concentrations (0-500ug/ml) PHA. The cells were pulsed with 3h (Tdr) (on 12th, 10th and 6th) days for 100,10 and 1cell/well, respectively, harvested after 18 hrs and counted with a Scintillation counter. Mean back ground absorbance of six replicate wells was subtracted from the count and the result was expressed as mean plus or minus standard error of mean. The result showed maximum and least proliferation at 100 and 10µg/ml.

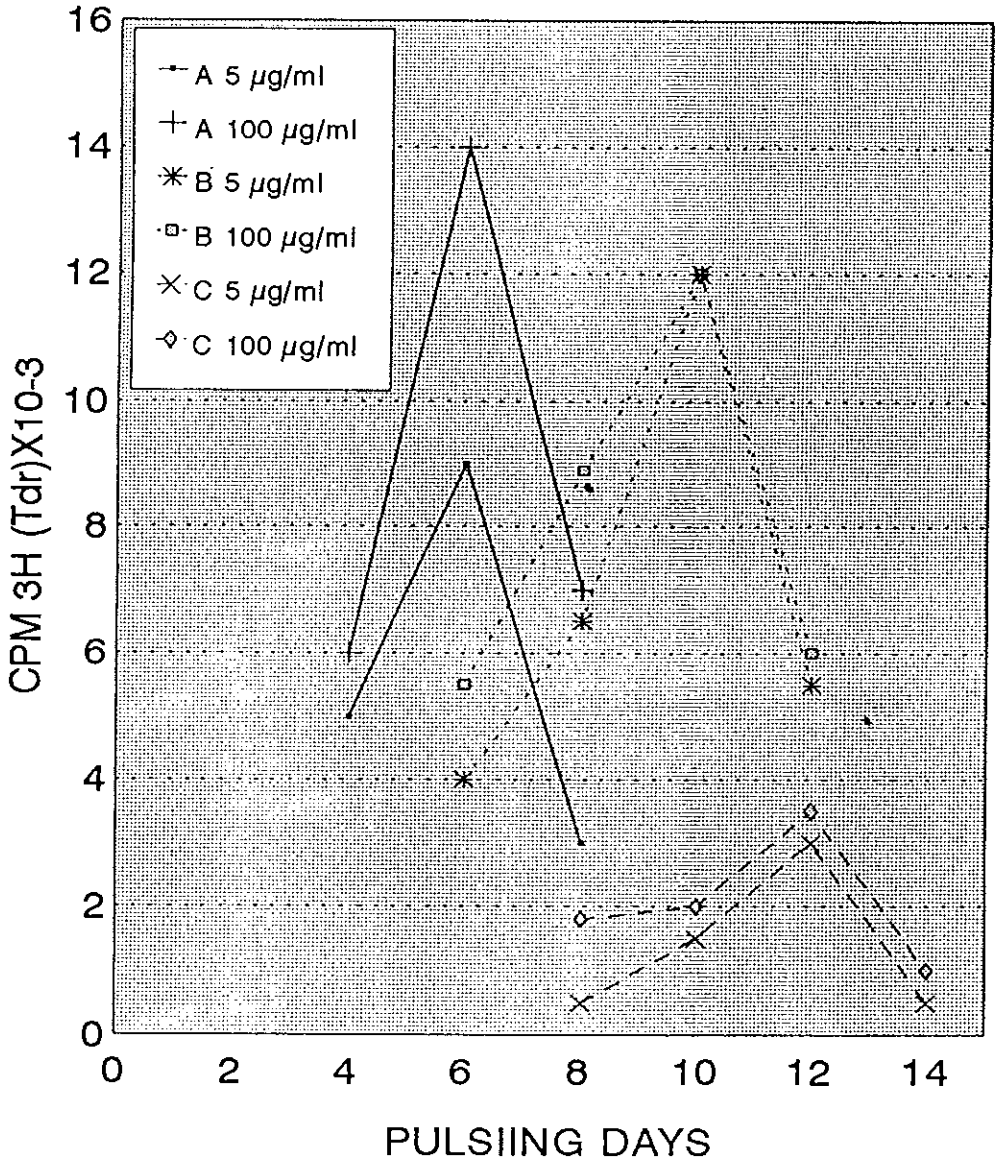

Fig.2. Maximum proliferation peak determination curve. Peripheral blood was obtained from three healthy individuals. Lymphocytes were isolated and were cultured at a concentration of 100(A) 10(B) and 1(C) with PHA (5 and 100μg/ml final), the cells were pulsed with 3H(Tdr) on 4th, 6th and 8th days for 100cell/well; 6th, 8th and 10th days for 10cell/well and 8th, 10th and 12th days for 1cell/well and were harvested, counted after 18 hrs. Mean back ground absorbance of control wells was subtracted and the result was expressed as mean plus or minus standard error of mean. The peak activation day was found to be 6th, 10th and 12th days for 100, 10 and 1cell/well, respectively.

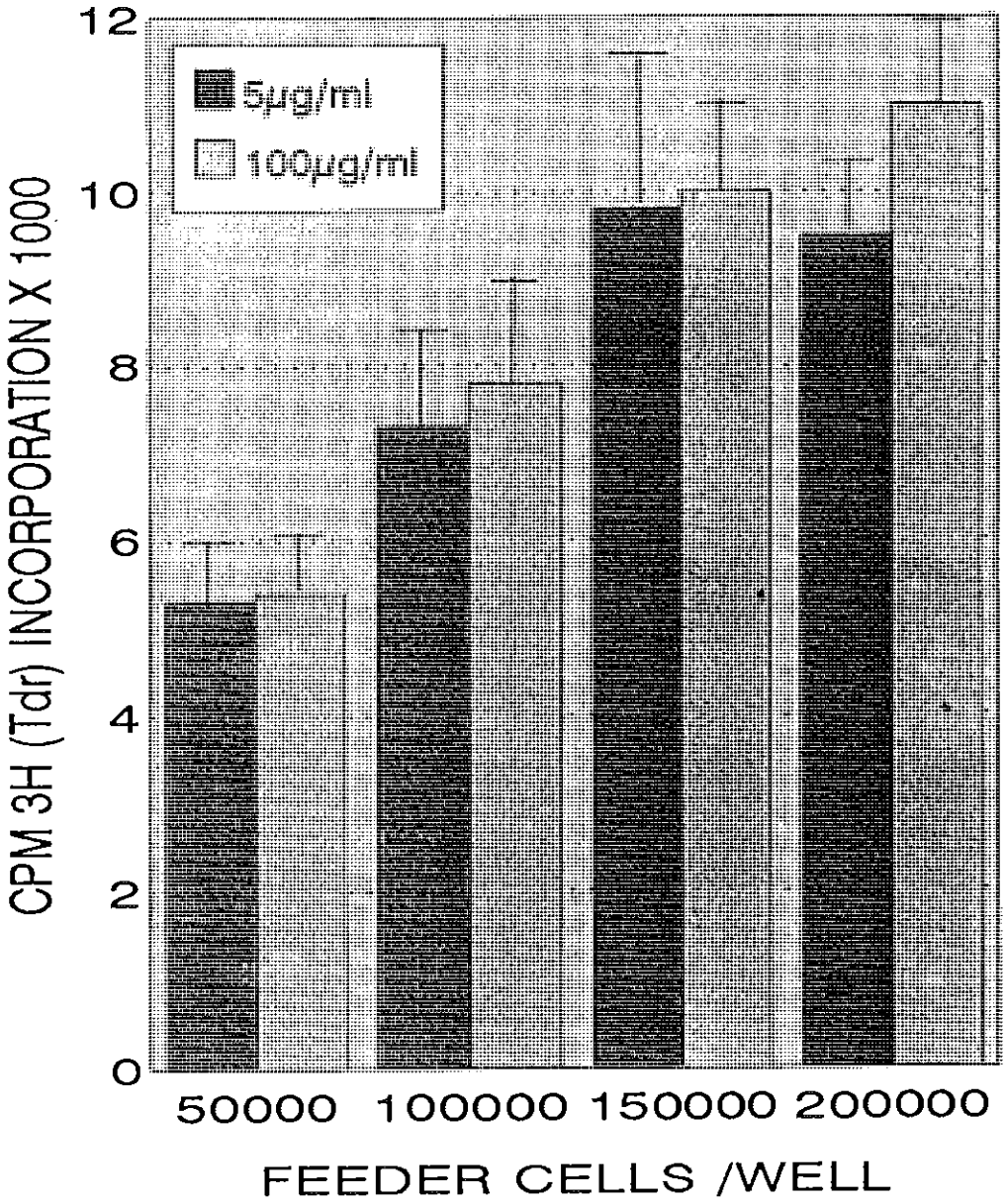

Fig.3. Feeder cell optimization curve. PBMC from three healthy individuals at a concentration of 100cell/well was cultured with medium, IL2(100U/ml), PHA (5 and 100µg/ml) and variable number of irradiated feeder cells (50000-200000). Cultures were pulsed with 3H(Tdr) on the 6th day, harvested and counted after 18 hrs. The result was expressed as mean plus or minus standard error of mean and shows that there is an increase of proliferation as the concentration of feeder cells was increased.

3.2. Comparison of Blood and Skin Isolated Lymphocytes (Leprosy).

The proliferation of cells isolated from peripheral blood and granulomatous skin lesion of leprosy patients were compared over a wide range of activation conditions. Significant differences between blood and skin-isolated lymphocytes were observed based on thymidine incorporation (Fig.4.). The difference in proliferation between blood and skin isolated lymphocytes were clear cut and many fold. In condition 2, for example, the proliferation of blood isolated lymphocytes was more than 10 fold greater than skin derived lymphocytes (1500 vs 15000 CPM).

In more than 80% of the cases there were significant differences between proliferation of blood and skin cells as assessed by paired t-test of 6 replicate samples for blood and skin for each activation condition (Fig.5). For most of the leprosy cases the difference between blood and skin isolated lymphocytes in proliferation was very highly significant ($p < .001$). Regardless of clinical status there was significant difference between blood and skin isolated lymphocytes (Fig.6) in leprosy through the whole spectrum. That is, in all conditions under which the experiments were carried out there was poor responsiveness of skin isolated lymphocytes when compared with blood lymphocytes regardless of the clinical status of the patient. This was apparent when comparing proliferation of blood and skin derived lymphocytes from MB and PB (Fig.6) as well as a comparison of the percentage of patients where proliferation of PBMC was statistically significant by paired t-test (Fig.5).

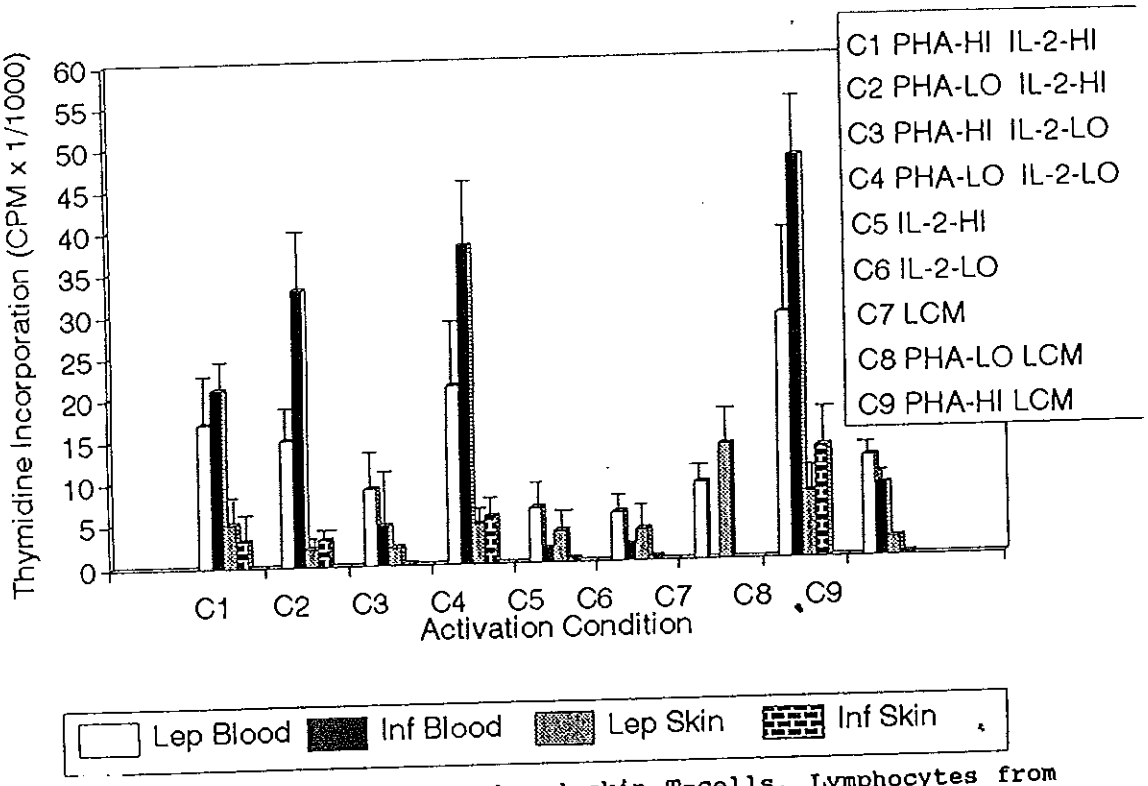

Fig.4 Proliferation of blood and skin T-cells. Lymphocytes from leprosy (n=27) and other inflammatory skin diseases (n=15), were isolated by Ficoll-density centrifugation from blood (PBMC) or by mechanical and enzymatical treatment from skin lesions. Cells were cultured with medium, high and low concentrations of IL2 and PHA under 9 different conditions (C1-C9) as indicated under materials and methods. Cultures were set up in six replicate wells for each condition. Control wells used for background CPM contained all supplements without responding cells. Cells were pulsed with 3H(Tdr)on the 6th day, harvested and CPM assessed using scintillation counter after 18 hrs. The result was expressed as mean plus or minus standard error minus back ground CPM. Comparison between blood and tissue lesion infiltrating lymphocytes were made by the paired t-test. Significant differences existed for C1,C2,C4 and C8 ($p<.001$), C3($P<.05$) but not for C5, C6 and C7.

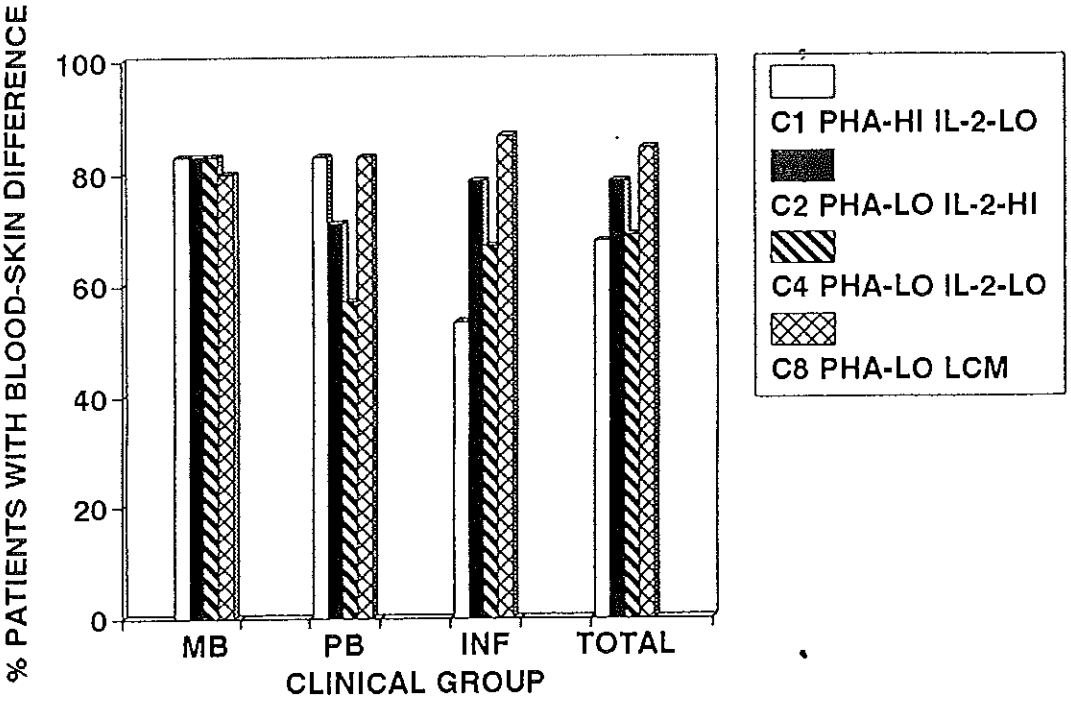

Fig. 5. Percentage of total significance levels between blood and skin. 6 Replicate wells from each condition were compared for blood and skin derived lymphocytes by the paired student t- test. Percentage of individuals from which blood skin pair were significantly different ($p<.05$) is illustrated. The data were obtained from the patients described in Fig.4.

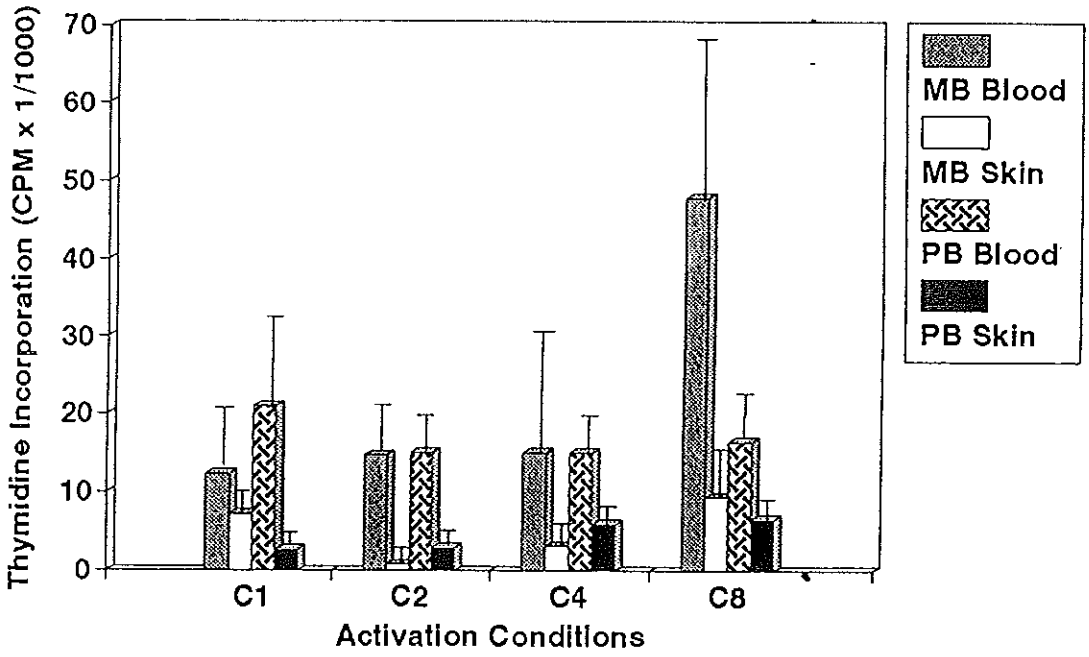

Fig.6. Proliferation of T-cells from multibacillary versus paucibacillary leprosy patients. Results from Fig.4. were reexpressed to show the difference in proliferation between different clinical groups of blood and skin derived lymphocytes. The difference in proliferation was not specific to a certain clinical status in leprosy. Both MB and PB patients showed reduced proliferative responses by skin derived lymphocytes.

3.3.Best Condition Selection.

None of the conditions were ideal for cell proliferation for all patients:- a condition which is optimum for one patient may not be optimum for the other patients. This was particularly true when comparisons were made with high and low doses of PHA. Thus, PHA at 100μg/ml (C1) was for some optimal, and for other patients highly inhibitory.

Similarly, PHA at 10μg/ml (C3) was optimal for some and inhibitory for others. However, some conditions were clearly superior to others as observed for each set of replicate conditions.

To determine the best overall ranking activation condition, proliferative responses for each of the activation conditions were examined. The condition producing the highest proliferative response was scored positive, and all the other conditions negative. The scoring was repeated for each of the 6 replicate wells established at each of 3 responder cell concentrations. Thus, theoretically a given condition would be given top rank a maximum of 18 times/patient/tissue sample. Table 1 shows the number of times each condition received top rank for either blood, skin or nerve. Once top values were assigned by the ranking method, comparison between top ranking conditions was carried out by calculating chi- square(from 2x2 contingency table). Table 2 shows p-values calculated between methods by chi-square.

Top ranking and chi-square results indicated (Table 1 and 2) for both skin derived and blood derived T-cells, condition-8 (C8), a condition with highly activated T-cell product (lymphocult) ranks first followed by C4, C2 and C1.

TABLE 1. Table showing the number of times each set of culture conditions produced maximum proliferation of PBMC from each source.

Condition	Blood Total	Blood First Rank	Blood Non-first Rank	Skin Total	Skin First Rank	Skin Non-first Rank	Nerve Total	Nerve First Rank	Nerve Non-first Rank
C1	828	100	728	559	24	535	234	23	211
C2	828	174	654	559	50	509	252	44	208
C3	180	25	155	145	4	141	18	1	17
C4	827	171	656	559	79	480	252	27	225
C5	180	3	177	145	3	142	18	3	15
C6	180	1	179	144	4	140	18	3	15
C7	72	0	72	37	4	33	18	0	18
C8	750	243	507	498	110	388	243	28	215
C9	108	28	80	108	15	93	0	0	0

Table 1. Shows each set of condition the total number of times th experiment was carried out for each tissue, i.e., the number of replicate wells times the cell doses and times the number of samples was determined and the number of times it showed highest proliferation for all conditions was determined as first rank and the number of times it failed to be top rank was indicated as non-first rank. Visual inspection of tissues showed the four conditions (C8, C2, C4 and C1) for blood and skin, conditions (C2, C8,C4 and C1) were the best conditions for nerve.

Table 2. P-values calculated from chi-square analysis of (Table 1) between different activation conditions.

		C2	C4	C8
Blood	C1	p<.001	p<.001	p<.001
	C2		NS	P<.001
	C4			p,.001
Skin	C1	p<.01	p<.001	p<.001
	C2		P<.05	p<.001
	C4			p<.001
Nerve	C1	p<.02	NS	NS
	C2		p<.05	NS
	C4			NS

NS= non significant
> = p<.02
>> = p<.001

Blood C8>>C4=C2>>C1
Skin C8>>C4>C2>C1
Nerve C2>C1=C4=C8

The values for each activation condition within each clinical group were compared by a 2x2 contingency table with values for each condition with the same clinical group. The result of the comparison between conditions was indicated by p<or >signs. All values were significant except between C2 and C4 blood, C1 and C4, C2 and C8 ,C4 and C8 nerve.

3.4. Comparison of Blood and Nerve Isolated Lymphocytes.

Once the difference in proliferation between blood and skin derived T-cells was clearly identified, we also wanted to ask whether this unresponsiveness was unique to skin or if it occurred in other tissues, i.e., we wanted to know if cells from nerve cases (Neuritis) were also responsive. Following the same procedures, proliferation of T-cells isolated from nerve tissue was compared with blood isolated lymphocytes from the same subject. The result appearing in (Fig. 7) shows that there were significant differences between blood and nerve derived lymphocytes. As in the case of the skin, the difference in proliferation of blood derived lymphocytes was many fold higher than nerve derived lymphocytes. The percentage significance level is lower, i.e., there are more non-significant results in neuritis cases when compared with skin (Fig.8). Proliferation of nerve derived lymphocytes was clearly reduced in all MB cases and in some PB cases of neuritis (Fig.9). Thus, unresponsiveness depends on the clinical status of the patients in neuritis cases. Nerve derived T-cells were more inhibited by high doses of PHA (C1) when compared with that of the skin. A Lymphocult containing condition (C8) was not the condition under which good growth could be observed for nerve derived lymphocyte proliferation. C2 (a condition with low PHA and IL-2) was the best condition followed by C8, C4 and C1 (Table 1 and 2). The overall results showed that there A is significant difference in proliferation between blood and nerve derived T-cells in leprosy.

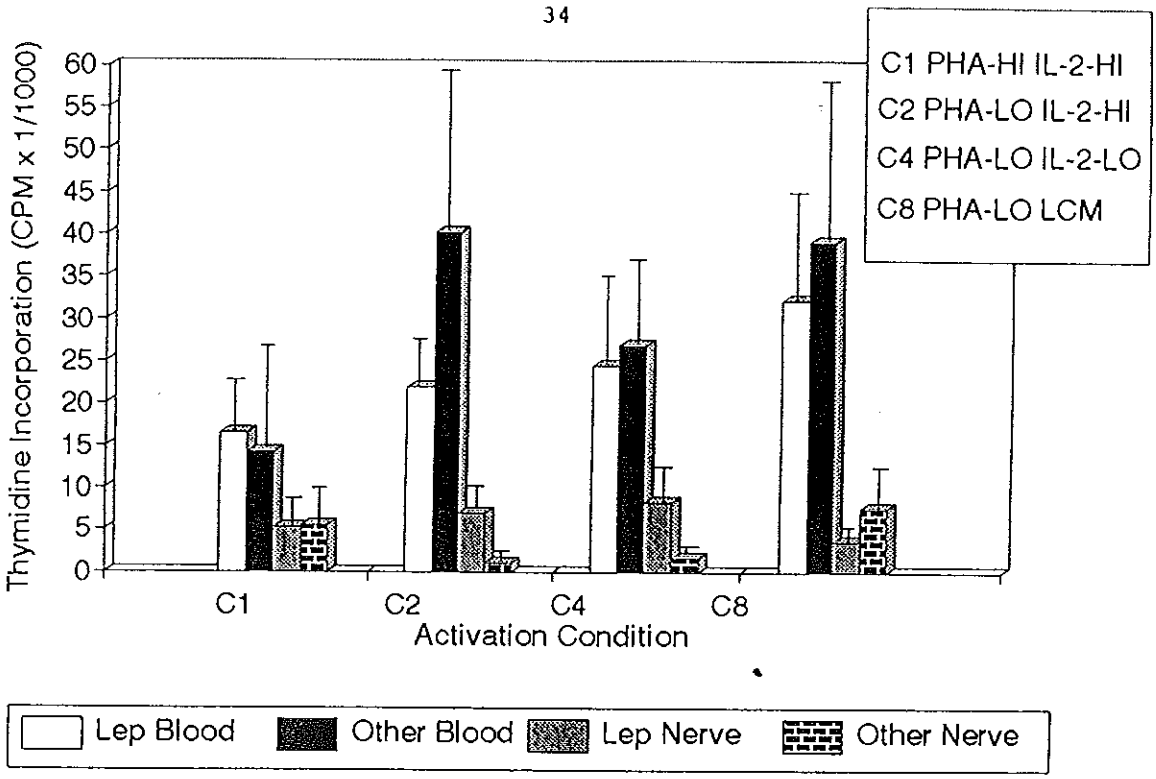

Fig.7. Proliferation of Blood and Nerve T-cells. Nerve samples were obtained from 10 patients and 4 control subjects. The experiment was set up as indicated in Fig.4. except that it was carried out under only four selected conditions. Differences activation of T-cells from between blood and nerve were significant ($p<.05$) as assessed by the paired T-test under all activation conditions except C1.

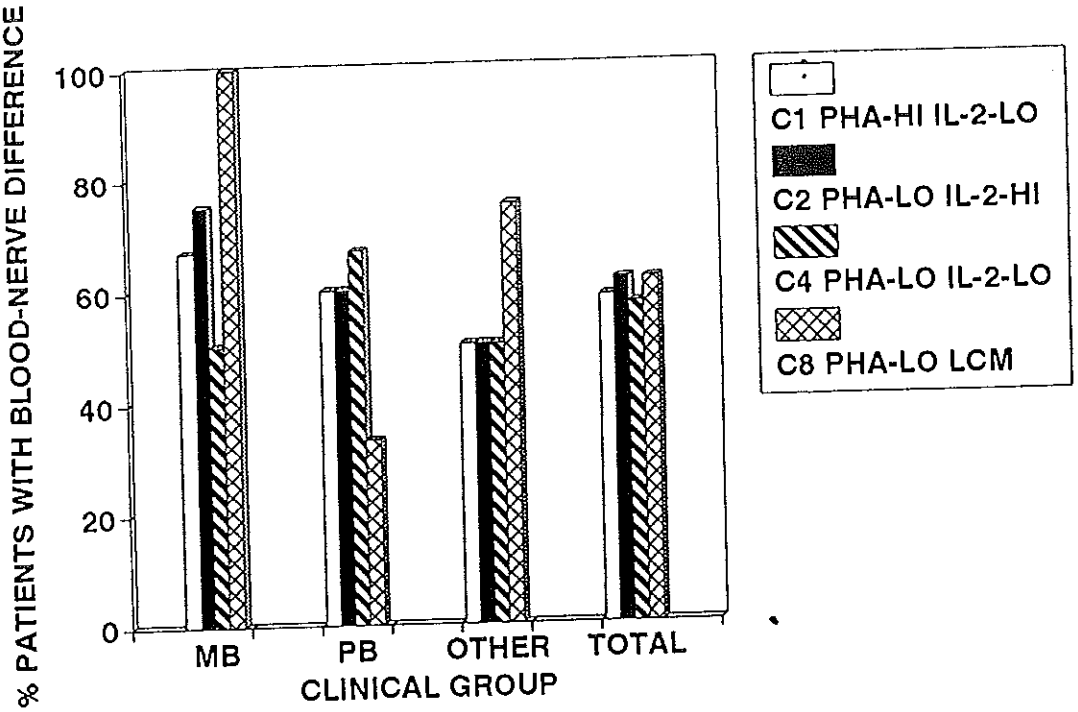

Fig.8. Percentage of total significance levels between blood and nerve. The average of six replicate wells of blood and nerve derived lymphocyte proliferation was compared by calculating p-value. The percentage of all p-values (total $p<.05$) was plotted to show the percentage of non-significant cases. Others are control from healthy individuals.

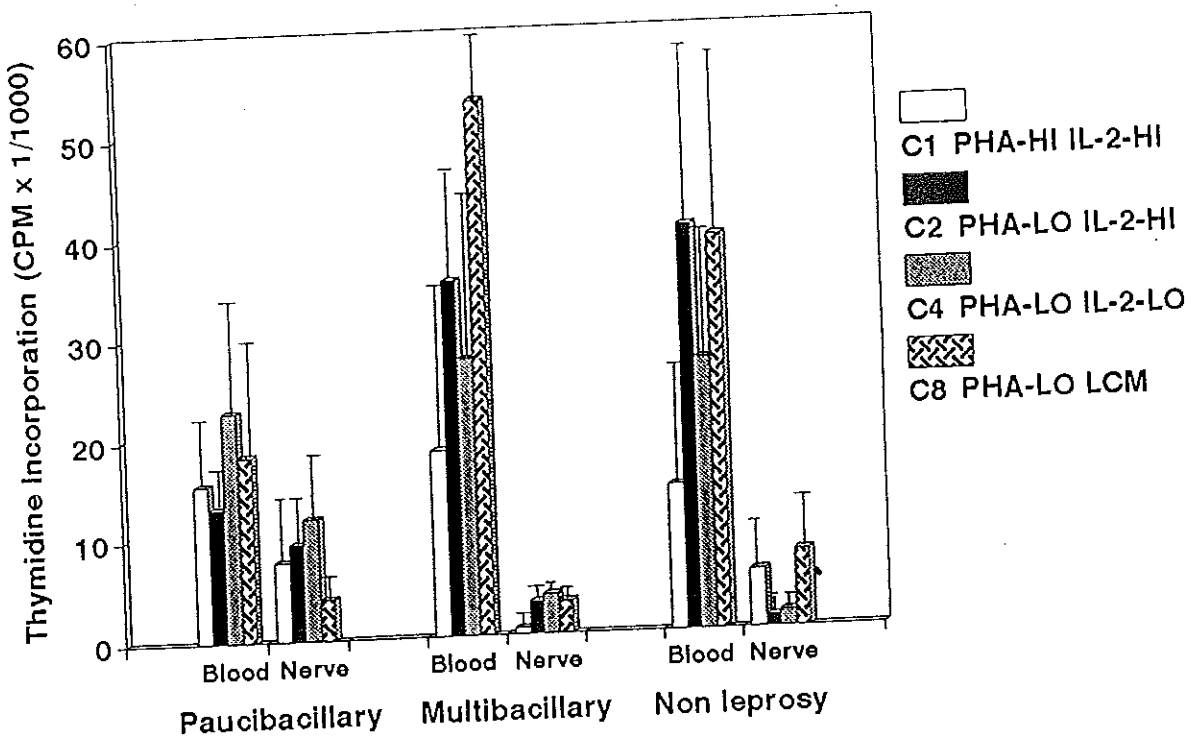

Fig.9.Proliferation of blood and nerve derived T-cells PB vs MB. Results from and fig.7. were replotted to show the difference in proliferation between blood and nerve derived lymphocytes in relation to clinical status. Note that the difference in proliferation between blood and nerve derived T-cells is less striking among PB than MB patients.

3.5. Comparison of Blood and other Inflammatory Skin Diseases Isolated Lymphocytes.

Granulomatous lesion and inflammation are not unique to leprosy, as a result we also wanted to know if differences in proliferation between blood and lesion derived cells occur in other inflammatory and granulomatous lesions. Lymphocytes were isolated from blood and lesion of granulomatous diseases such Leishmaniasis, Psoriasis and some other diseases which accumulate lymphocytes in the skin. The result (Fig.4) was comparable to the difference seen between skin and blood cells in leprosy. Their activation requirement of cell from other skin inflammatory diseases is the same as leprosy, C8 was the best condition.

3.5.1.Proliferation of T-cells with a more potent Co-mitogen

Following recognition of the difference between blood and lesion derived T-cells using PHA as a non-specific mitogen, we wanted to know if the proliferative response of tissue derived T-cells could be restored with a strong co-mitogen. Phorbol Myristate Acetate (PMA) a pharmacological mitogen was selected. PMA and PHA are highly co-mitogenic and potentiate significant lymphokine induced proliferation of long term mixed lymphocyte culture cells (MLS), cloned cytotoxic T-cells and IL-2 dependent T-cell lines (Orosz and Roopenian, 1984) Cells from skin and blood of 14 leprosy patients and 4 controls were cultured by using condition-8 (low PHA, LCM, IL-2) with high and low doses of PMA. The results (fig.10.) showed that the proliferative response of lesion-derived T-cells was not restored. Infact, PMA significantly reduced the proliferative responses to levels below that of C8 in most of the cases.

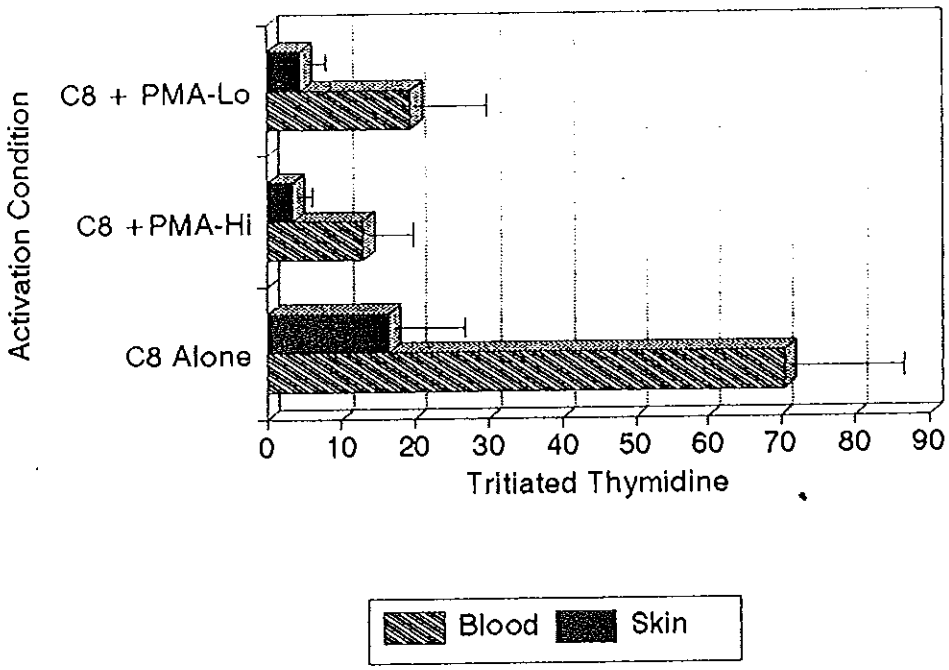

Fig.10. Effect of PMA on the proliferation of blood and lesion T-cells. Samples were obtained from 14 patients and three control subjects. Lymphocytes were isolated from blood (PBMC) and tissue lesions as indicated in Fig.4. Cells were cultured at a concentration of 100cells/well under activation condition C8. In addition, PMA was added at 2.5 or 5ng/ml to the wells. The cells were cultured for 6 days, pulsed with 3H(Tdr)on the same day (6th), harvested and counted after 18 hrs. The result was expressed as mean plus or minus standard error of mean after subtracting background absorbance.The result show the proliferative response was not restored after stimulation by PMA.

3.6.Analysis of Cell Receptors and Cell Surface Markers.

It was of interest to determine whether the poor proliferative response of skin and nerve derived lymphocytes correlated with the presence or absence of molecules to be expressed on T-cells. To rule out the possibility that differences between blood and lesion derived T-cells could not be due to absence of T-cells or TCR the number of CD3+ cells was analyzed for all tissues and for each patient before culture by using monoclonal antibodies to CD3. The results (Table 3) show that there was no significant difference ($p > .05$) between the numbers of cells in blood and skin from leprosy patients or those with nerve and blood and other inflammatory skin diseases. In addition, there was no observable difference in the density of CD3 molecules on the cell surface (Table.3).

The results for anti-CD3 suggested that the lack of response observed in skin or nerve derived cells was not due to lack of T-cells or TCR/CD3 complex. Thus, we stained for other molecules to see if there were gross difference in the molecules expressed by blood and lesion derived cells. We were particularly interested in the expression of CD45RA and CD28 molecules, as these have been implicated in T-cell activation. There were more CD28+ cells in tissue lesions than in blood (Fig.11.). CD28- cells were more in blood when compared with tissue lesions. The differences were, however, not statistically significant.

CD3+CD45RA+ cells were observed in greater numbers in blood than in the skin or nerve lesions (Fig. 11.). In skin lesion more CD45RA- cells were seen than CD45RA+ cells. Overall, CD45RA+ cells were found in equal proportions with different clinical status in both blood and lesion tissues.

Table 3. Percent (mean ± SEM) of CD3+ cells in diffrenet blood and tissue sources.

Comparisons	% CD3+ cells Blood	Tissue	Paired-t p value
Blood-Skin (Leprosy),n=9	55.8±19.3	62.6±21.3	0.49
Blood-Nerve(Leprosy),n=9	49.0±20.5	56.2±19.2	0.47
Blood-Skin(Inflamm),n=8	53.1±19.3	58.5±24.9	0.64

Table 3 shows tissue isolated lymphocytes from patients with leprosy and other inflammtory diseases. The tissues were stained with monoclonal antibodies specific for CD3, and percetage CD3+ were determined following subtraction of results with isotope control antibodies. The result shows that the difference between tisssue isolated lympocytes were not significant ($p>.05$).

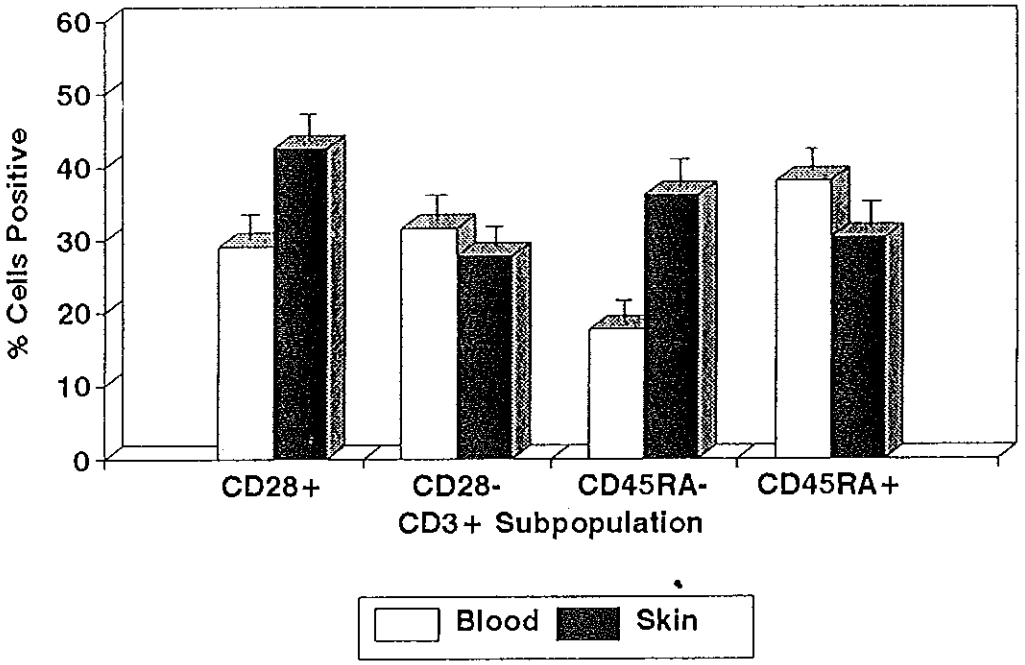

Fig.11. CD3+ Subsets in blood and skin of leprosy patients. Samples were obtained from 26 patients and lymphocytes isolated from blood and skin prior to culture were double stained with moAb reagent specific for CD3 and CD28 or for CD3 and CD45RA and analyzed by flow cytometry. Matched blood-skin samples were analyzed by paired t-test the difference in percentage of CD3+CD28+, CD3+CD45RA- and CD3+ CD45RA+ cells between different tissues were statistically significant($p<.05$).

The difference in the numbers of CD45RA+ and CD45RA-cells between blood and tissue lesions was significant ($p < .05$).

CD4+ and CD8+ subpopulations were also analyzed before and after culture. In blood before culture almost equal number of CD4 and CD8 bearing cells were present. In tissue lesions, however, more CD4 bearing cells were present before culture (Fig.12). In tissue lesions, in both PB and MB cases more CD4 cells were present when compared with CD8 bearing cells (Fig.13). The difference between CD4+ and CD8+ cells in PB cases was highly significant ($p < .01$) while the difference between CD4+ and CD8+ in MB cells was not significant. There was also a statistically significant difference in the percentage of CD3+CD8+ cells between PB and MB lesions ($p < .05$) (Fig.13).

All subpopulations were increased in number after culture and the increment was equivalent in each case although the difference between CD8+ cells before and after culture was significant ($p < .05$) (Fig.12).

Similar analysis for $\alpha/ß$ and γ/δ before culture (Fig.12.) showed that the majority of T-cells present were $\alpha/ß$ bearing TCR. The proportion of γ/δ in both blood and tissue lesions was less than 10%. But there was a slight decrease γ/δ relative to $\alpha/ß$ in tissues and this was also significant ($p < .05$).

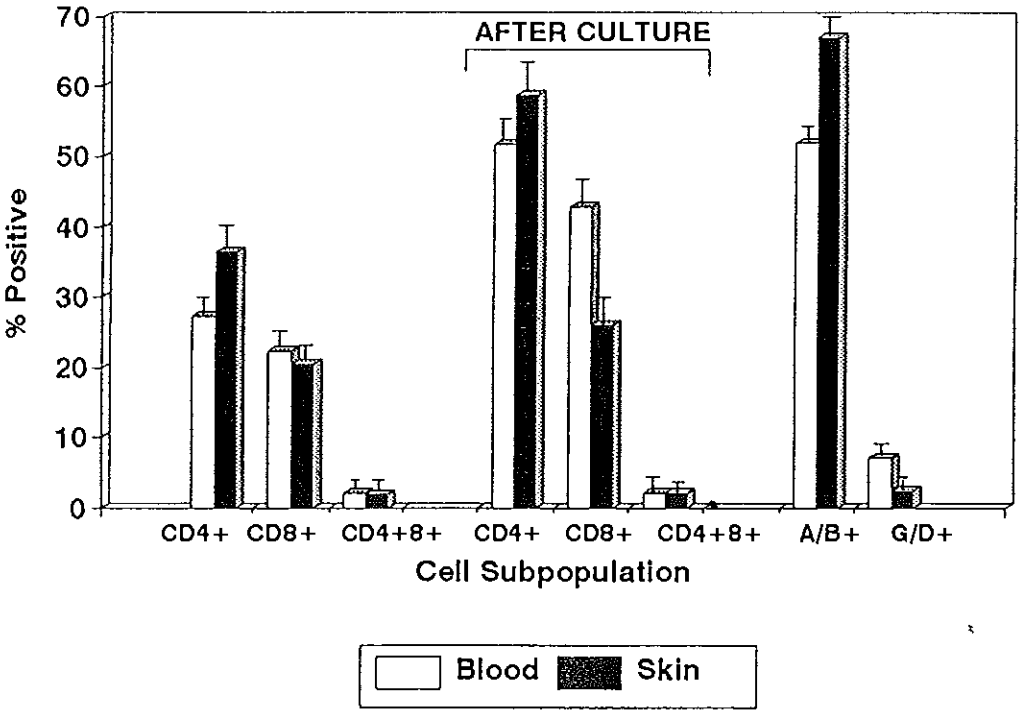

Fig.12. T-cell subpopulations in blood and skin of leprosy patients. Samples were obtained from 27 patients and lymphocytes isolated from blood and skin were stained before, and in some cases, after culture with specific monoclonal antibody conjugated with fluorochrome (PE and FITC) were analyzed with FACSCAN. Background staining with isotope was subtracted, and results expressed as mean plus or minus standard error of mean. Of all the subpopulations indicated only the percentage of CD8+ after culture was statistically significant ($P<.05$) between blood and skin. Similar staining for α/β and γ/δ from 25 patients show that the majority of T-cells activated were α/β TCR The difference between tissues in both subpopulations were not significant.

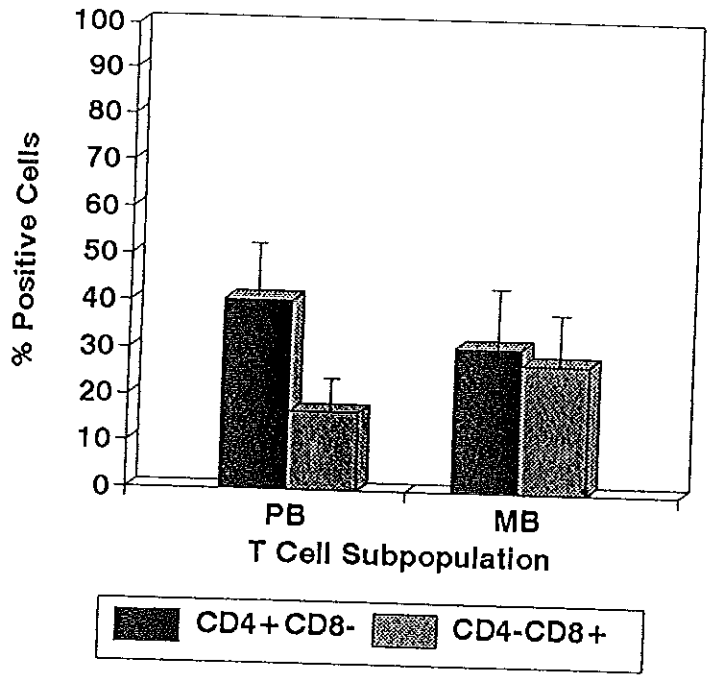

Fig.13. CD4+ and CD8+ subsets in leprosy PB vs MB in Skin. The result obtained from Fig.10. was replotted only for CD4 and CD8 subpopulation of skin cells. The result show that within PB skin sample significantly more CD4+CD8- than CD4- CD8+ ($p<.05$), where as within MB lesion the level of these 2 subpopulations were the same.

3.7. Lymphokine Production by Lymphocytes from Tissues.

In light of the differences observed in lymphocyte proliferation between blood and tissue lesions, we decided to study if there were parallel differences in the patterns of lymphokines produced. For IL-2 from blood 23 out of 27 samples were positive (Fig. 14A) while in the skin only 15 out of 26 were positive. The difference between skin and blood was statistically significant ($p < .05$). The results for IL-4 (Fig.14B) was similar except that fewer cultures in both blood (12 out of 20) and skin (6 out of 21) produced IL-4. This difference was also significant ($p < .05$) and in addition the level of IL-4 produced by cultured blood derived cells was higher than those derived from skin. In contrast, the difference in the amount of IFN-γ produced by T-cells derived from blood or tissue lesions was not significant and IFN-γ was produced in almost equal amount in both blood and tissue derived T-cells (fig.14C).

Because IFN-γ and IL-4 are known to have mutually exclusive expression on TH1 and TH2 cells we replotted the data from figure 14 B and C with IFN-γ production on the x-axis and IL-4 on the Y-axis. The results, shown in figure 15 A and B indicate many samples produced only one or the other lymphokine, some produced both, and some neither. Thus, these data are consistent with known features of TH1-like and TH2-like cells. More TH1-like were observed, as our cultures were heterogenous one can not make definitive conclusions. Besides, based on this small sample size, there was no clear correlation between clinical groups and secretion pattern.

The most significant finding of our lymphokine induction analysis is the reduced

capacity of lesion-derived cells to produce lymphokines. This has been clearly observed for both IL-2 and IL-4. Whereas, IFN-γ level was identical in both blood and skin isolated lymphocytes.

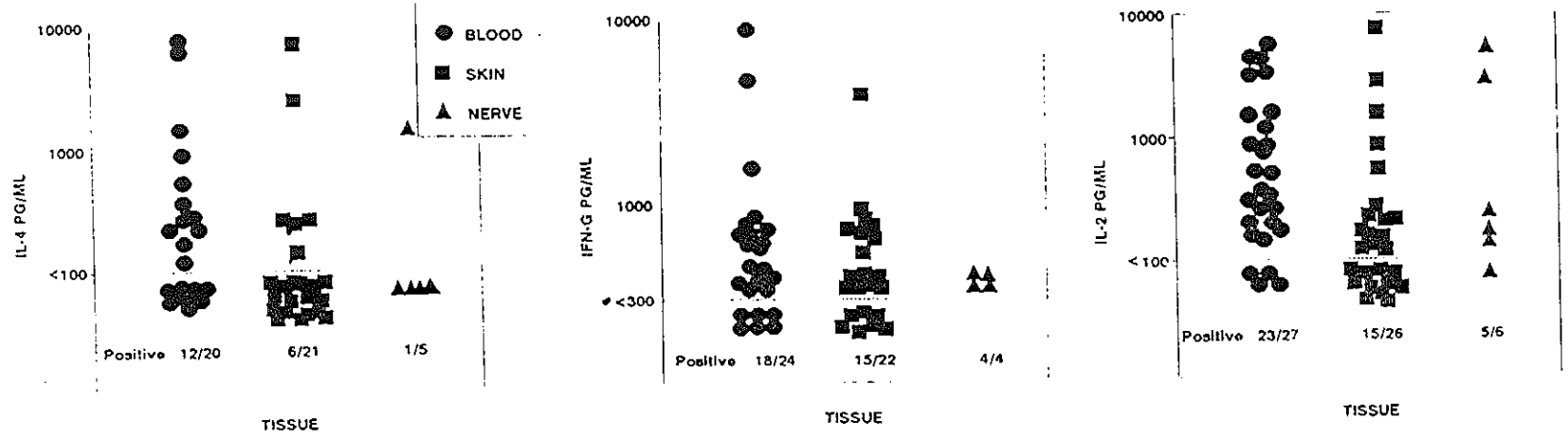

Fig.14.IL-2 (A) ,IL-4 (B) INF-γ (C) production by blood and tissue lesion derived lymphocytes. Lymphocytes isolated from blood (circle), nerve (square) Skin (box) and tissue lesions from 27 individuals were cultured under various culture conditions at 100cell/well with PHA, IL2 and LCM. Cells from culture were pooled, thoroughly washed and cultured for 24 hrs with plastic immobilized ant-CD3. Supernatant was collected after 24 hrs and stored at -70^{0}C. The lymphokine was detected by an ELISA method. The dotted lines show level of sensitivity multiplied by dilution factor. Each symbol represents the level produced by cultured cells from one patient. The number of supernatant containing lymphokines above the limit of detection is indicated.

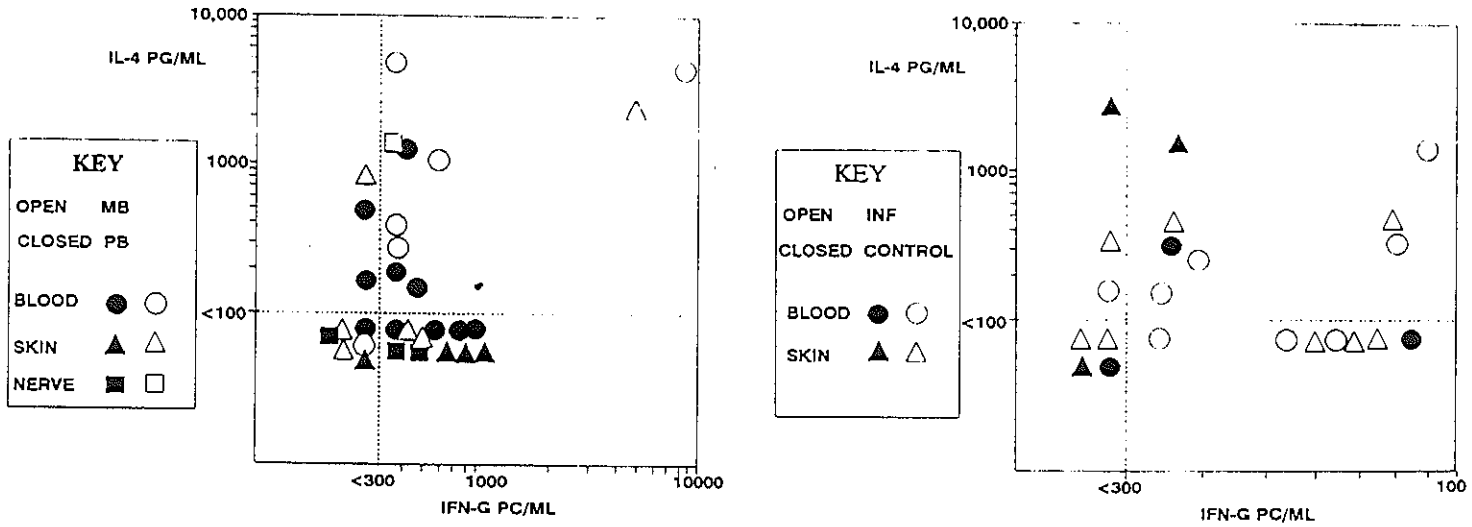

Fig.15. (A and B) IL-4 (A) and INF-γ (B) production pattern Panel A shows results from (blood and nerve leprosy patients) and (blood and skin patients) with non-leprosy skin. Panel B shows results from skin and blood of MB and PB leprosy patients.Data illustrated in Fig.14 was replotted with INF-γ on the X-axis and IL-4 of the same sample on the Y-axis. The dotted line shows the level of sensitivity multiplied by dilution factor. The circle represent blood, square represents nerve and box represents skin.

4. Discussion

The overall effectiveness of T-cells depend on an efficient activation of their functional properties as well as an extended clonal expansion. As a result, lymphocyte proliferation is one of the key elements of an adaptive immune response. Characterization of functional properties of T-cells across the leprosy spectrum from blood and tissue lesions, showed that there was a significant difference in proliferation to polyclonal stimuli between blood and skin derived T-cells. This difference was significant in more than 80% of the cases. The differences were clear cut and many fold in most of the cases. A difference was also observed between nerve infiltrating lymphocytes and blood lymphocytes in proliferation in which case around 50-80% of the cases were significant. Similar observations to that of T-cells of leprosy cases was observed for the blood and skin pairs from patients suffering from other inflammatory skin diseases such as leishmaniasis and psoriasis. Activation with a co-mitogen thought to be a strong stimulator did not restore responsiveness of lesion derived T-cells. Our data from lymphokine studies supports the data from the studies of lymphocyte proliferation in that there is an increase in proliferation with an increase in IL-2 and IL-4 or decrease in these lympokines in reduction of proliferation.

In leprosy skin lymphocyte nonresponsiveness was irrespective of the clinical status of the patients. There was unresponsiveness in both poles of the spectrum. In neuritis cases, however, some of the PB tissue derived lymphocytes were able to proliferate and responded as well as blood derived lymphocytes.

The cells from different sources were also different from each other in activation

requirements. Skin and blood derived lymphocytes from all sources proliferated very well under a condition containing PHA activated T-cell product, LCM (C8). Nerve infiltrating lymphocyte, however, proliferated well under the condition C2 in which low doses of PHA and IL-2 was present. The difference was only in the requirement for LCM as this was the only difference between C8 and C2. Thus, skin and blood lymphocytes need additional growth substances or factors present in the LCM but the growth substance or factor present in the LCM was not required or present for nerve derived lymphocytes.

Analysis of the percentage of cell-surface molecules showed that the majority of cells isolated from blood, skin and nerve expressed the α/βTCR in both blood and tissue lesions and there was no difference in percentage of CD3+ cells in the tissues, i.e., the percentage CD3+ cells was approximately the same in blood and lesions. Thus, expression of molecules at the T-cell/TCR was normal and apart from a slight reduction of γ/δTCR lymphocytes in tissue lesions no observable difference was noted in any of the tissues analyzed.

CD45RA markers are expressed on the surface of naive T-cells and memory T-cells are negative for CD45RA (Janeway and Golstein,1992). Our result showed that T-cell subpopulations in tissue lesions were enriched with memory T-cells and this is also consistent with previous results (Cathleen, et al., 1989). There are known differences in activation requirement of CD45RA+ and CD45RA- T-cells but both subpopulations respond well to PHA (Mackay, 1993). Thus, the difference in the percentage of CD45RA+ and CD45RA- cells does not account for poor responsiveness observed in tissue lesions.

The CD28 receptor in response to its ligand, can provide T-cells with an important signal necessary for proliferation. It has been demonstrated that loss of the CD28 cell surface molecule render cells more susceptible to anergic signals and less responsive to certain antigens and mitogens (June, et al., 1987). From our study on CD28 expression, however, the enrichment of CD28+ in tissue lesion suggests that CD28 molecules are unrelated to poor responsiveness in this study.

The distribution of CD4 and CD8 bearing T-cells was statistically different between blood and tissue lesions. Although distribution of CD4 and CD8 cells in tissue lesions particularly in PB cases was demonstrated to be statistically significant from blood, the differences were not dramatic enough to account for the many fold differences observed.

After culture there was an increase in both subpopulations (CD4+ and CD8+) and this was possible as PHA stimulates T-cells selectively. Overall, more CD4+ cells were present both before and after culture but there was significant increase of CD8+ cells in blood after culture. The reason for the significant increment of CD8+ cells in blood was not clear from results obtained from this experiment.

Over all, the results for surface markers, show that there is difference in the molecules expressed by blood versus tissue lesion derived T-cells. While these findings illustrate different cell composition in blood as opposed to tissue lesion, no results were clear cut enough to account for or explain the poor responsiveness of tissue lesion derived T-cells.

Our result of surface marker analysis partly agrees with others (Mehra and Modlin, 1989; Modlin, 1989). The predominance of CD4+ cells in PB lesions is similar to

the finding of others(Mehra and Modlin,1989) while the presence in equal numbers of CD8 cells in MB is unusual. The possible reason may be the use of more sensitive method (FACSAN) for the analysis of cell surface markers or variability among patients.

Previous studies on CD28 molecule on cells expressing CD8 markers show that there is a decrease in CD28 molecule in MB and an increase in PB (Modlin, 1989). Our analysis, however, was not only on CD8+ cells but on total CD3+ cells. As a result, an increase of CD28+ cells in our case may be due to total expression on both subpopulations of CD4+ and CD8+ cells.

It is true that cells expressing certain cell surface markers may predominate in one tissue rather than the other, our result of CD28 and CD45RA expression, however, is not only different in predominance but also shows an inverse correlation with strong proliferation. It is thus possible that their function may be different from what currently is known or perhaps more likely is a failure to respond because of the presence of other as yet unidentified molecules, or the absence of respective ligands for these molecules in tissue lesions.

We don't yet have any data which can account for why T-cells from tissue lesions respond less to stimulation by mitogen. However, it can be hypothesized that the poor responsiveness could be due to either presence of negative signals or absence of positive signal and one possibility is that tissue lesion derived T-cells are anergized or tolerized and blood isolated lymphocytes are not. Clonal paralysis or anergy was described previously by others, and was induced by the presentation of antigen in the absence of a putative co-stimulatory signal (Kromer, et al., 1991).

This may also be due to the presence of some lymphokines having an inhibitory effect by down regulating some ligands for accessory molecules. The source of lesion infiltrating T-cells is not clearly known but it is doubtless that they originate from the bone marrow, mature in the thymus and migrate to the lymphoid organs(Clane, 1990). Thus, anergy may be induced by tissue-specific antigens expressed in a specific area or by a specific factor released from tissues and in this case, presumably could be induced by M. leprae antigens present in the tissue. It is known that APC differs in blood and lesions as the antigen presenting capabilities vary depending on the cell type and anatomical localization (Kromer, et al., 1991). This functional difference may determine the specialization of each type of APC with respect to specific area and function. The difference between blood and lesion lymphocytes in proliferative capacity may be due to difference in APC requirements or APC which gives positive signal in the blood and hence induce proliferation which may give a negative signal in the skin.

Evidence from several sources (Schwartz, 1990; Gagewski, et al., 1989; Kroemer, et al., 1991) suggest that impairment of IL-2 production is a critical element in the mechanism of nonresponsiveness. In T-cell clonal anergy, the capacity to produce IL-2 is repressed and the cells are prevented from proliferation in response to an antigenic challenge and from helping other immune cells to proliferate and differentiate. Moreover, the inability of such anergic T-lymphocytes to transcribe the IL-2 receptor gene prevents them from utilizing IL-2 from an external source. As a result, they are poorly responsive to IL-2 (Kroemer, et al., 1991).

The tissue lesion derived lymphocytes were deficient in IL-2 production and also

were less-responsive to IL-2 added from external source. Hence, the results of our experiment are in agreement with the previous studies (Schwartz, 1990 ; Kroemer, et al.,1991).

The role of IL-4 in anergy induction is not known. In this study, however, significant amounts of IL-4 were produced in the T-cells from blood when compared with those in skin.

IL-2 is not the only lympokine whose activity is modulated in anergic-cells since IFN-γ is also known to drop by 33% (Schwartz, 1990). In this study, however, the amount of IFN-γ produced by cells in all tissue cultures was the same and this is not consistent with the previous work (Schwartz, 1990) . However, it is important to point out that we are assessing lymphokine produced by cells which grew in cultures. It is possible that there exists cells with the starting population which grew poorly and were therefore not assessed. Thus, it will be important to develop a sensitive assay to detect lympokine production by freshly isolated cells.

Anergy is a possibility, but it does not mean that other mechanisms such as suppression by other cells (T-suppressor cells) are completely ruled out. However, if T-suppressor cells play a role in giving a negative signal they most likely influence cell proliferation prior to culture. Otherwise we should have expected increased proliferation at lower cell doses when T-suppressor cells are diluted out. Unresponsiveness may also be due to lack of positive signal or inability to receive positive signal.It is also possible that the presence or absence of tissue specific factor may affect the ability to accept a positive signal.It is known that within skin many lymphocytes are imbedded in an extracellular matrix complexed with

molecules such as collagen or fibronectin. These molecules have been shown to give positive co-stimulatory signals to T-cells and it is probable that in lesions T-cells have a much greater dependency on these molecules (Nojima, *et al.*, 1990).

From the results obtained in this study, the following conclusions can be drawn and and some recommendations may be put forward:

5. CONCLUSION AND RECOMMENDATIONS

The proliferation of blood and tissue lesion derived lymphocytes is not the same and are significantly different from each other. Their activation requirements are also not the same. Unresponsiveness is found in all spectrum of leprosy patients and in some PB and in all MB neuritis cases.

Analysis of all surface receptors associated with T-cell activation and differentiating subpupulations did not account for the difference in proliferation between blood and tissue lesion isolated lymphocytes. The presence of IL-2 and IL-4 in sufficient quantity in blood when compared with tissue lesions, however, is a marked difference between blood and tissue lesion derived lymphocytes. As a result, the possible reason for the unresponsiveness is anergy, as anergic cells are not able to produce IL-2 and respond to IL-2 from external source. Other unidentified possibilities may also account for the difference between the two tissues isolated lymphocytes

This study has identified a problem, tried to find the reason but has only touched on some of the possible events taking place in tissue lesions. As a result, further studies specifically on the role of special requirements of tissue lesion derived lymphocytes and other possible causes are highly recommended.

Moreover, studies at molecular level using the micro system to asses the effect of other co-factors such as: the role of extracellular matrix, role of antigen presenting cells and role of other possible lymphokines must be carried out to support the forwarded hypothesis or to disprove it and if so propose other hypotheses which would explain the existing situation.

This project has contributed towards the understanding of immunology of cells in leprosy and other inflammatory diseases. Although it is premature to conclude that the results of this investigation will have in use clinical diagnosis and prognosis its modest contribution towards the knowledge of cellular immunology is of importance.

The of knowledge gained from this study may also be useful in understanding other similar disease processes.

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