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T CELL RECEPTOR (TCR) FOR ANTIGEN: A COMPARATIVE STUDY BETWEEN THE TCRα/β AND TCRγ/δ SUBSETS IN NONINFECTED AND HIV INFECTED INDIVIDUALS

By

France Lacroix

THESIS

Submitted to the School of Graduate Studies in partial fulfillment of the requirement for the degree of

Master of Science

Department of Microbiology and Immunology

Faculty of Medicine

University of Ottawa

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France Lacroix

ABSTRACT

The human immunodeficiency virus (HIV) causes one of the most devastating infections known today. HIV infects principally $CD4^+$ T-lymphocytes and other cell types such as monocytes/macrophages. The infection leads to failure of the immune system, and to the development of a clinical condition known as the acquired immune deficiency syndrome (AIDS). HIV infection is associated with characteristic quantitative changes in T-lymphocyte subsets: progressive depletion of the $CD4^+$ T-lymphocytes and an increased number of the $CD8^+$ T-lymphocyte. The $CD3^+$ T-lymphocyte count remains stable throughout the disease.

In this study, I report the results of a flow cytometric analysis of the expression of CD3, CD4, CD8, TCRα/β, and TCRγ/δ antigens. The results are correlated with the World Health Organization (WHO) AIDS clinical classification of AIDS. The T Cell antigen Receptor (TCR) of the vast majority (95%) of $CD4^+$ and $CD8^+$ T lymphocytes are α/β heterodimers (TCRα/β). To determine what changes may occur in the TCRγ/δ subset I included this receptor in the study. It is known that in HIV infection the TCRα/β T cells are activated, thus we also compared the state of activation of the TCRγ/δ with the TCRα/β T cells.

Lymphocyte subset analysis was performed on a group of 57 HIV seropositive individuals (Class II-IV) and 27 noninfected volunteer donors using multiparametric flow cytometry. My observations confirmed previously reported data on CD3, CD4 and CD8 subpopulations.

I observed that $CD8^+TCR\alpha/\beta^+$ cells increased early in HIV disease ($p<0.01$) whereas the frequency of $CD4^+TCR\alpha/\beta^+$ cells was relatively unchanged. The frequency of $TCR\gamma/\delta^+$ cells remained unchanged. However, the mean fluorescence intensity (MFI), reflecting surface antigenic density, varied and allowed a clear distinction among different stages of infection.

The expression of three activation markers (HLA-DR, CD38, CD57) was clearly increased in HIV infected individuals. CD25 (IL-2R), on the other hand, exhibited a slight decrease as the disease progressed. The TCRα/β subset showed more substantial variation for activation markers. In the TCRγ/δ subset, the CD57 antigen seemed to be the most affected by the state of the disease and showed the greatest increase ($p<0.01$).

The CD5 antigen, another activation marker, was also investigated and the results showed that at stage III and IV of the disease significant increase of the TCRγ/δ^+ cells lacking the CD5 marker was observed ($p<0.01$).

These results confirm that there is a quantitative decrease of $CD4^+$ cells in HIV infected samples. The number of $CD3^+$ lymphocytes remained stable throughout the disease. The number of TCRα/β^+ cells showed an increase in HIV infected samples whereas no differences was observed for the frequency of TCRγ/δ^+ cells. However the changes in the MFI for the TCRγ/δ molecule was associated with the progression of the disease. In samples from infected individuals, both TCR subsets showed changes in activation characteristics.

In conclusion, the absolute count of the TCRγ/δ T-lymphocytes does not change with disease progression. However, these cells show the presence of cell activation markers and heterogeneous expression of TCRγ/δ antigen density. The pathogenic and clinical significance of my observations remains to be determined.

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I dedicate this thesis to my dearest friend Alecos.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
BCS	Bovine Calf Serum
CD	Cluster of Differentiation
CDC	Center for Disease Control
FCS	Fetal Calf Serum
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HTLV-I	Human T cell Leukemia/Lymphoma Virus type 1
HTLV-II	Human T cell Leukemia/Lymphoma Virus type 2
HTLV-III	Human T cell Leukemia/Lymphoma Virus type 3
LAV	Lymphadenopathy Associated Virus
LCDC	Laboratory Center for Disease Control
MMWR	Morbidity and Mortality Weekly Report
PBS	Phosphate Buffered Saline
PBMNC	Peripheral Blood Mononuclear Cells
PCP	Pneumocystis Carinii Pneumonia
TCR	T Cell Receptor
TCRα/β	T Cell Receptor alpha/beta
TCRγ/δ	T Cell Receptor gamma/delta

LIST OF REAGENTS AND MATERIALS

Simulset Leucogate	Becton Dickinson
IgG_1-FITC Isotype control	Becton Dickinson
IgG_1-PE Isotype control	Becton Dickinson
IgG_{2a}-FITC Isotype control	Becton Dickinson
IgG_{2a}-PE Isotype control	Becton Dickinson
Leu4-PE	Becton Dickinson
Leu2a-FITC	Becton Dickinson
Leu2a-PE	Becton Dickinson
Leu3a-PE	Becton Dickinson
Leu16-PE	Becton Dickinson
Leu11c-PE	Becton Dickinson
Leu19-PE	Becton Dickinson
TCR-1-FITC	Becton Dickinson
TCRδ1-FITC	T Cell Sciences
anti-HLA-DR-PE	Becton Dickinson
anti-CD25-PE	Becton Dickinson
Leu17-PE	Becton Dickinson
Leu7-Biotin	Becton Dickinson
Leu1-PE	Becton Dickinson
CaliBRITE beads	Becton Dickinson
Quickcal beads	Flow Cytometry Standards Corporation
QC3 beads	Becton Dickinson
Fetal Bovine Serum	Hyclone
Phosphate Buffered Saline	GIBCO Laboratories

LIST OF REAGENTS AND MATERIALS

(continued)

12 X 75 mm polystyrene round-bottom RIA tube	Falcon
Immunoprep	Coulter Electronics
Paraformaldehyde	BDH
Isoton II	Coulter Electronics

I. INTRODUCTION

I.1 Human Immunodeficiency Virus (HIV)

I.1.1 History of HIV-1

In the 1960s and 1970s rare cases of unexplained immunosuppression accompanied by opportunistic infection were recognized in industrialized societies (Huminer et al, 1987). However, it was only in June 1981, with a short report in the Morbidity and Mortality Weekly Report (MMWR) of the United States Centers for Disease Control (CDC) that the presence of a new syndrome was recognized. That report described five cases of previously healthy, homosexual men treated for multiple episodes of Pneumocystis carinii pneumonia (PCP), extensive mucosal candidiasis, and severe viral infections (Gottlieb et al, 1981a). Eight months later the first Canadian case of AIDS (Acquired ImmunoDeficiency Syndrome) was reported to the Laboratory Center for Disease Control of Health and Welfare Canada (LCDC) (Doherty and King, 1982). By the end of 1982, it was clear that the outbreak of an immunodeficiency syndrome was not limited to a few cities in Canada or to defined populations. The populations afflicted had been expanded to include hemophiliacs, transfusion recipients, sex partners of risk-group members, and children born to mothers at risk. All of these observations pointed to a transmissible agent spread through seminal fluids and blood, and efforts at isolating and identifying an etiologic agent were intensified. In July 1992, Health and Welfare Canada had received reports of 6,560 cases which met the surveillance case definition for AIDS. These included 6,488 adults and 72 pediatric cases (<15 years of age). Up to now, in Canada, a total of 4,112 deaths (63%) has been reported. In the past three years, the higher incidence of cases was found in Ontario with 2,600 cases (40% of the reported Canadian cases) followed by Quebec with 2,015 cases (31%). In British Columbia 1,212 cases have been reported (18%). Alberta reported 6%, the other provinces and territories

reported 1% and less. Health and Welfare Canada estimates that in 1993, nearly a thousand new cases of AIDS will be reported throughout the country. Their predictions have been accurate from 1990 to 1991. In 1992, the same number of new cases was predicted to occur (980), but only 205 cases have been reported up to now (first 6 months of the year). Worldwide, thousands of Europeans and possibly millions of Africans are also infected with HIV (Quinn et al, 1986). For the past 3 years, the highest number of new cases was reported by CDC in the United States of America, however the highest rates of AIDS per capita were reported by four countries of South and Central America (Bahamas, Bermuda, French Guinea and Barbados) and by six countries of Africa (Uganda, Malawi, Congo, Zimbabwe, Tanzania and Rwanda).

I.1.2 Etiology of AIDS

Four types of human retrovirus have been described since 1980. The first, human T-cell leukemia/lymphoma virus type I, known as HTLV-1, is the prototype human retrovirus, as reported by Gallo and colleagues (Poiesz et al, 1980). The second human retrovirus, HTLV-II, also reported by Gallo and colleagues, has not been clearly linked to a particular human disease (Kalyanaraman et al, 1982).

The third human retrovirus, human immunodeficiency virus-1 (HIV-1), formerly known as HTLV-III or lymphadenopathy-associated virus (LAV), was discovered by Montagnier and Gallo and is the etiologic agent of AIDS (Barre-Sinoussi et al, 1983; Popovic et al, 1984). This virus is linked to a broad spectrum of disease conditions, most of which reflect the indirect pathogenic effects of the virus resulting from its immunosuppressive properties. Other direct effects, most notably on the central nervous system, have also been recognized and are under investigation. The fourth human retrovirus, HIV-2 (formerly HTLV-IV and LAV-2), reported by Clavel (1986)

and Kanki et al (1986) has been linked in some cases to patients of African origin with AIDS-like illness.

I.1.3 Biological Characteristics of HIV-1

I.1.3.1 Genomic organization of HIV-1

Most retroviruses that replicate contain 3 genes : *gag*, *pol*, and *env*. The lentiviruses differ from other retroviruses by their genomic complexity. HIV-1 contains in its 9kb RNA genome the three essential genes common to all replication-competent retroviruses plus at least six additional genes: *vif*, *vpu*, *vpr*, *tat*, *rev*, and *nef* involved in regulation of the virion replication, maturation and morphogenesis. The initial genome-lenght RNA to be transcribed encode the HIV-1 regulatory proteins. In a later phase, the unspliced and singly spliced mRNAs, that encode the viral structural proteins, are transcribed (Kim et al, 1989).

The initial population of genomic-length viral messenger RNA (mRNA) encodes the various regulatory proteins of HIV-1, including the products of *tat* and *nef* genes (Kim et al, 1989). The product of the *tat* gene transactivates the expression of all viral genes and is essential for the replication of HIV-1 (Dayton et al, 1986; Fischer et al, 1986; Sodroski et al, 1985; Arya et al, 1985). The product of the *nef* gene is expressed early in the course of HIV-1 infection. Although considered, at first, as an inhibitor of HIV-1 replication (Ahmad and Venkatesan, 1988; Niederman et al, 1989) its authentic *in vivo* function has yet to be identified. Like the product of the *nef* gene, the product of the *vpr* gene is not essential for viral replication. It functions as a weak, albeit promiscuous, transcriptional activator (Cohen et al, 1990) but its precise functional role remains enigmatic.

The transition between the synthesis of early regulatory genes and late structural-gene products appears critically dependent on the HIV-1 *rev* protein. Like the

product of the *tat* gene, the product of the *rev* gene is essential for HIV-1 replication. It appears to regulate viral reproduction at a post-transcriptional level by activating the cytoplasmic expression of the unspliced and singly spliced forms of HIV-1 RNA that encode the products of the *gag*, *pol* and *env* genes (Malim et al, 1988).

Recent studies have also revealed the production of a unique chimeric HIV-1 protein termed *tev* (Benko et al, 1990) or *tnv* (Salfeld et al, 1990). This protein results from an unusual splicing pattern of three different HIV-1 genes, and appears to retain the biologic activities of the products of both the *tat* and the *rev* genes. However, the full range of functional activities of *tev* or *tnv* and its relative effect on the replicative life cycle of HIV-1 are not known.

The protein products of two additional HIV-1 genes, *vif* and *vpu*, appear to have important functional roles during the late stages of virion morphogenesis. The product of the *vpu* gene promotes the efficient release of the budding virions from the surface of the cell (Klimkait et al, 1990). The product of the *vif* gene also appears to be necessary for full infectivity of the released HIV-1 virions (Sodroski et al, 1987; Strebel et al, 1987).

I.1.3.2 Morphogenesis of HIV-1

The products of the HIV-1 *gag* and *pol* genes form the core of the mature HIV-1 virion, whereas the products of the *env* genes are the principal exterior-coat proteins. HIV-1 has an icosahedral structure (Gelderblom et al, 1987) containing 72 external spikes. These spikes are formed by the two major viral envelope proteins, gp120 and gp41. Various host proteins such as Class I and II histocompatibility antigens which are acquired during budding of the virus are also found at the surface of the virus. The core of the virus contains four nucleocapsid proteins, p24, p17, p9, and p7 which originate from a 53kd *gag* precursor proteolytically cleaved by the HIV-1 protease.

The phosphorylated p24 polypeptide forms the main component of the inner shelf of the nucleocapsid. The myristylated p17 protein is associated with the inner surface of the lipid bilayer. The p7 protein binds directly to the genomic RNA and together with p9 forms the nucleoid core. The retroviral core contains two copies of the single-stranded HIV-1 genomic RNA which are associated with various viral enzymes including the reverse transcriptase, integrase, and protease.

I.1.3.3 The life cycle of HIV-1

The life cycle of HIV-1 is characterized by a period of proviral latency followed by active replication. The replication cycle is summarized below.

The CD4 molecule is the receptor for HIV-1 and the $CD4^+$ human T lymphocytes are the well recognized cellular targets for the HIV-1 virion (Fauci, 1988). Other $CD4^+$ cells such as B lymphocytes and monocytes/macrophages are also infected with HIV-1 but at a lower frequency (Nicholson et al, 1986; Gartner et al, 1986; Ho et al, 1986; Salahuddin et al, 1986, Koyanagi et al, 1987; Castro et al, 1988; Folks et al, 1988a; Gendelman et al, 1990; Innocenti and Seigneurin, 1990; Potts et al, 1990; McElrath et al, 1989; McElrath et al, 1991). When monocytes/macrophages are infected, they present fewer cytopathic effects by the HIV than the $CD4^+$ T lymphocytes do. They serve as viral targets and viral reservoirs (Ho et al, 1986; Gartner et al, 1986; Koenig et al, 1990; McElrath et al, 1989; Gendelman et al, 1990; McElrath et al, 1991). When infected, monocytes/macrophages also act as regulators that seem to control viral replication and the progression of disease by dysregulation of their own cytokine synthesis and microbicidal activity (Matsuyama et al, 1989; Okamoto et al, 1989; Osborn et al, 1989; Duh et al, 1989). Recent work done by Spiegel's group suggests that follicular dendritic cells in lymph nodes and T cells of memory phenotype may also be reservoirs for HIV (Spiegel et al, 1992). HIV-1 appears to be able to infect normal and neoplastic glial cells

(Gabudza et al, 1986; Koeing et al, 1986; Vazeux et al, 1987; Koyanagi et al, 1987), gut epithelial cells (Castro et al, 1988), bone marrow progenitors (Dohanue et al, 1987), and several other non lymphoid cells (Belsito et al, 1984; Salahuddin et al, 1986). This capability of infecting such a broad array of cells may be a partial explanation for symptoms frequently observed in HIV-1 infected patients such as dementia, diarrhea-wasting syndromes, and hematological abnormalities.

HIV-1 specifically infects $CD4^+$ cells because the CD4 membrane antigen is the cellular receptor for the viral envelope glycoprotein gp 120 (Dalgleish et al, 1984). Whether HIV-1 infection of the diverse cell types that it can infect is mediated exclusively through CD4 or involves yet another cellular receptor remains unknown and is the source of active investigation.

The receptor-bound HIV-1 virions are brought inside the cell by either classic receptor-mediated endocytosis or virus-mediated membrane fusion (Maddon, 1986; Stein, 1987). Membrane fusion is the most supported mechanism for virion entry and involves the gp41 envelope protein. The gp41, noncovalently associated with the gp120, contains a domain that anchors both envelope proteins in the lipid bilayer, the fusogenic domain. Activation of this domain leads to fusion of the viral and host-cell membranes, which then promotes the internalization of the virion.

After internalization, the HIV-1 virion is rapidly uncoated in preparation for the replicative phase of its life cycle (Varmus, 1988). The HIV-1 life cycle continues by conversion of the RNA genome into a DNA duplex. This process is critically dependent upon the action of the retroviral reverse transcriptase as well as on various ill-defined cellular factors (Varmus, 1988).

After entry into the $CD4^+$ cell, HIV-1 may establish a latent or persistent form of infection. Using PCR technique it was found that 1 in 1,000 peripheral blood $CD4^+$ T cells from patients with AIDS express HIV-1 RNA (Schnittman, 1989). It was also found that 1 in 100 of these $CD4^+$ cells contains detectable HIV-1 DNA. Therefore

for every cell actively producing virus, nine other T cells contain latent virus (Greene, 1990). The biochemical basis of this apparent viral latency is poorly understood but may be determined by the overall state of cellular activation. HIV-1 does not replicate in resting T cells, presumably because critical host factors are absent (Cullen, 1989). Another latent form of HIV-1 has been detected in resting T cells that corresponds to an incompletely reverse-transcribed core of HIV-1 RNA (Zack, 1990).

Linear forms of the DNA duplex are subsequently integrated into the host cell genome by the viral integrase protein, thus establishing the proviral form of HIV-1. Viral RNA is replicated by transcriptional activation of the HIV-1 provirus mediated through its 5' long terminal repeat (LTR). Initiation of this process appears to require an "activated" T-cell environment and the presence of various inducible host transcription factors (Nabel, 1987). The initial low level of viral gene transcription mediated by T-cell activation of the LTR is markedly amplified by the action of the products of the HIV-1 *tat* (transactivator) gene (Cullen, 1989; Peterlin and Luciw, 1988). At this point, the infected cell virtually becomes a virus-producing factory with cessation of many normal functions.

A second viral transregulator, *rev*, promotes expression of the structural proteins required for the assembly of infectious viral progeny. Assembly of the virion initially involves aggregation of the ribonucleoprotein core in the cytoplasm. These cores are composed of the HIV-1 RNA, the products of the *gag* gene, and the various enzymes encoded by the *pol* gene.

Finally, the newly formed cores move to the inner surface of the cell and bud through the plasma membrane, where they acquire their lipid membranes and the two protein products of the HIV-1 *env* gene. The protein products of two additional HIV-1 genes, *vif* and *vpu*, appear to have important functional roles during these late stages of virion morphogenesis. The product of *vpu* gene promotes the efficient release of the budding virions and the product of *vif* gene appears to be necessary for full

infectivity of the released HIV-1 virions (Sodroski, 1986; Strebel, 1987). Budding of these virions from the cellular membrane promotes dissemination of the virus to other $CD4^+$ cells and also contributes to the death of the infected cell.

I.1.4 Pathogenesis of HIV-1 infection

The hallmark of the immunodeficiency in AIDS is a depletion of $CD4^+$ T cells (Gottlieb et al, 1981b; Bowen et al, 1985). HIV has specific tropism for $CD4^+$ cells, which include lymphocytes, monocytes and macrophages (Klatzmann, 1984). The CD4 antigen composes at least part of the cell surface receptor for HIV (Haseltine and Wong-Staal, 1988). HIV binds to the cell through this receptor, is uncoated, and the viral core is introduced into the cell (Haseltine and Wong-Staal, 1988; Ho et al, 1987). After transcription, much of the DNA of HIV remains unintegrated in the cytoplasm. The HIV replication cycle is restricted at this stage until the infected cell is activated. Activation may be achieved by other pathogens, such as cytomegalovirus, hepatitis B virus, and herpes simplex virus and allogeneic stimulation such as exposure to semen, blood, or allografts (Ho et al, 1987). Upon activation, integration, transcription, protein synthesis and processing occurs. Viral proteins and genomic RNA are assembled at the cell surface generating mature virions by budding.

HIV persists in infected individuals; much of the virus appears to be latent or restricted and not susceptible to immune clearance. Several investigators have shown that HIV-neutralizing antibodies can be detected in the serum of most infected individuals (Ho et al, 1985; Robert-Guroff et al, 1985; Weiss et al, 1985). The presence of antibody does not always indicate a high degree of infectivity (Burger et al, 1986). Not all persons with antibodies to HIV transmit the virus to their regular sexual partners, even over long periods. Susceptibility of the host to HIV may depend on a number of factors, including genetic predisposition such as the presence of HLA-DR5, (Friedman-

Kien et al 1982, Metroka et al, 1983), the size of the inoculum, and the route of exposure. The recipient's immune status at the time of exposure is an important factor that influences the progression of infection (Anderson et al, 1986). This may be compromised by a combination of factors in individuals at risk. These factors include effects of illicit drugs such as heroin and volatile nitrites (Newell et al, 1985), malnutrition, and chronic infection with viruses such as cytomegalovirus, Epstein-Barr virus, and hepatitis.

Circulating HIV is present in the plasma of infected individuals (Ho et al, 1989), and patients with more advanced disease have higher titers of circulating virus. In the early or acute phase, which lasts for weeks, the level of virus production is high. The middle or chronic phase is characterized by smoldering low levels of HIV expression that can last for several years. Finally a crisis phase occurs with recrudescence of viral replication, resulting in the clinical syndrome of AIDS-related complex or AIDS, which lasts for months to years depending at least partly on the efficacy and availability of treatment. All stages involve active replication of the virus, and the infection has no totally latent phase when circulating virus is not present. Some investigators beleive that the high degree of HIV viremia suggests that direct cytopathic effects of the virus are largely responsible for the pathogenesis of AIDS (Ho et al, 1989). Other researchers beleive that cytotoxic T lymphocytes are responsible for $CD4^+$ T cell clearance (Shearer et al, 1988).

In 1983, Spivak et al first reported pancytopenia associated with AIDS. Quantitative markers of blood T lymphocyte subsets appear to be useful markers of immunologic status. The depressed absolute $CD4^+$ and elevated $CD8^+$ lymphocyte counts have prognostic significance in the evolution of the disease (Goedert et al, 1987; Melbye et al, 1986; Polk et al, 1987). Many mechanisms for CD4 T cell depletion have been proposed, such as replication and budding of the virus from infected cells damaging the cell membrane, and syncytium formation or cell fusion. In the latter, the viral

envelope protein (gp120) expressed on the infected cell binds to the CD4 molecule on a noninfected cell, resulting in membrane fusion and virus core entry into the new cell. Binding of free gp 120 to the CD4 molecule of noninfected cells, making them appear infected, elicits an immune response contributing to this T cell subset's destruction.

The $CD4^+$ T helper lymphocyte is central to the immune response, interacting with monocytes, macrophages, cytotoxic T-cells, natural killer cells, and B-cells, so the selective CD4 depletion can result in a multitude of immunologic defects. The profound depression of immune function in AIDS patients and subsequent susceptibility to opportunistic infections, oncogenic viruses, and malignant neoplasms may therefore arise from quantitative deficiency of CD4 cells. This deficiency would be caused by cytopathic effects of the virus or impaired function of viable T lymphocytes that have proviral sequences incorporated into their genomes. The integration of the viral genome results in a lack of inductive function for monocytes, macrophages, T cells, including $CD8^+$ suppressor/cytotoxic cells, NK cells, and impairs helper function of B cells. B cell hyperplasia results from loss of the normal T cell surveillance, and polyclonal B cell activation by HIV infection. Gurney et al reported that a segment of the HIV envelope is similar to lymphokines that induce B cell activation and immunoglobulin synthesis (Gurney et al, 1986a; 1986b). Enhanced release of monokines such as interleukin-2, tumor necrosis factor, and cachectin has been used to explain the chronic fevers and wasting of AIDS patients. Many of the immune defects noted in AIDS may therefore be traced to depletion and functional defects in the $CD4^+$ helper/inducer subset of lymphoctes following infection with HIV.

Monocytes and macrophages may be directly infected with HIV, causing a defect in chemotaxis. Involvement of alveolar macrophages may explain the high incidence of Pneumocystis infections in patients with AIDS (Ho et al, 1987). Infected monocytes may serve as the vehicle for transport of HIV to the central nervous system.

The reduction and final depletion of $CD4^+$ T lymphocytes, which

parallels the development of immunodeficiency, does not adequately explain all the immunopathological effects observed during HIV infection (Ennen et al, 1990). Several models for the pathogenesis of AIDS have been proposed. Dalgleish's group postulated that HIV induces an autoreactive process in susceptible individuals by its ability to mimic MHC class II molecules and stimulate alloreactive lymphocytes (Dalgleish and Colizzi, 1992). There are structural homologies between MHC class II (and I) molecules and the viral gp120 (Golding et al, 1988; Young et al, 1988). Hounsell and her colleagues (1991) demonstrated that a large alpha helix is conserved through all known HIV isolates, and has structural identity with the alpha helix of MHC class I and, by extrapolation, with MHC class II molecules. Dalgleish and Colizzi (1992) suggested that since this area is close to the CD4 binding loop on the carboxy-terminal, it may be seen by the T cell receptor (TCR) after CD4/gp120 binding has taken place as the CD4 molecule is crosslinked to the TCR during antigen presentation. Their hypothesis was supported by *in vivo* studies demonstrating that uninfected allogeneic human lymphocytes were able to protect rhesus macaque monkeys from infection following an SIV challenge, and that allogeneic cells were able to induce a humoral response in certain breeds of mice which cross-reacted with the gp120 and the p24 proteins of HIV (Stott, 1991; Kion et al, 1991).

Genomic heterogeneity is a hallmark of HIV, the molecular events responsible for the virus variability can be summarized as: either mutation of the virus or as a consequence of the inability of the host to limit virus replication "*ab initio*" (Siliciano et al, 1988). HIV evolves at a rate approximately a million times as great as that of eukaryotic DNA genomes (Hahn et al, 1986). Infidelity of reverse transcription is responsible for the DNA sequence diversity among the different HIV variants. This hypermutability could be central to the pathogenesis of HIV (Preston et al, 1988; Roberts et al, 1988). Loss of memory T cells has been observed in asymptomatic HIV-infected individuals with normal numbers of $CD4^+$T cells (De Martini et al, 1988; Giorgi and

Roger, 1989; Fletcher et al, 1989; De Paoli et al, 1988). The continuous generation of more virulent HIV variants with altered antigenic make up, although only transiently expressed, may be responsible for memory T-cell turnover. In addition to the memory T-cell depletion, loss of antigen-presenting cell functions has been reported and may contribute to the breakdown of the immune system, leading to the emergence and overt replication of highly virulent HIV variants (Ennen et al, 1990; Macatonia et al, 1992; Knight and Macatonia, 1991; Chirmule et al, 1990).

Once rapidly replicating virulent HIV variants are developed, the induction of $CD4^+$ T cell decline may be accelerated by several mechanisms: more rapid lysis of infected $CD4^+$ T cells (Asjo et al, 1986), impediment of T cell replacement by infection of T cell precursors (compatible with the broadened cytotropism of syncytium-inducing variants) (Folks et al, 1988b), and depletion of uninfected $CD4^+$ T cells by syncytium formation (Lifson et al, 1986; Tersmette et al, 1989a, b; Cheng-Mayer et al, 1988). As the disease progresses, enhanced expression of viral antigen results in increased efficiency of $CD4^+$ T cell clearance by HIV-specific $CD8^+$ CTL which may be induced via a $CD4^+$ helper T-cell-independent pathway (Shearer et al, 1988). As a result of the $CD4^+$ T cell depletion, AIDS develops in 6-24 months, depending on the virulence of the effector HIV variant (Tersmette et al, 1989a, b). Therefore, the pathogenesis of HIV infection can be explained by the tendency of the virus to evolve *in vivo*, generating subpopulations of increasing cytopathic potential (Wain-Hobsan, 1989).

Major factors contributing to pathogenesis, including tropism, reproduction, latency and persistence, appear to be largely determined by the interaction of a single major virus component, the envelope glycoprotein gp160, with receptors on helper T cells, monocytes/macrophages and specialized dendritic cells. The CD4 molecule is recognized as the receptor for the viral envelope protein gp120 (Dalgleish et al, 1984). The initial interaction between gp160 and its ligands may account for most of the features of HIV disease (Habeshaw and Dalgleish, 1989). As previously mentioned,

those ligands include CD4, major histocompatibility complex (MHC) class II molecules and also the T-cell receptor. The TCR subsets are the object of this study, and particular attention will be given to the comparison between different TCRs in noninfected subjects and HIV seropositive patients.

I.2 T Cell Antigen Receptor (TCR)

I.2.1 Antigen specific receptor

The immune system provides the primary mechanism of specific recognition and protection from foreign pathogens. This focal point in immunity is mediated by molecules on B lymphocytes and T lymphocytes called immunological antigen receptors.

The B lymphocyte receptor is the immunoglobulin molecule. A large number of antibody (Ab) molecules form a unique site that is involved in recognition of antigen (Ag). Also, secreted Ab molecules may directly bind to the cognate Ag, marking it for removal or destruction.

T cells differ from B cells in Ag recognition since they often recognize different determinants, and generally do not react with soluble or free Ag. Also, instead of an antibody at the surface of the cell, the T cell has a molecular complex called the T cell antigen receptor (TCR). This TCR recognizes Ag on the cell surface in conjunction with products encoded by self-major histocompatibility complex (MHC) genes (Shearer and Schmitt-Verhulst, 1977; Zinkernagel and Doherty, 1979).

Thymus-dependent T lymphocytes can be subdivided based on the cell surface expression of polypeptide chains involved in immune recognition. All T lymphocytes express the CD3 polypeptides (γ, δ, ε, and ζ). On the predominant subset of T cells the CD3 complex is noncovalently associated with an α/β chain receptor heterodimer termed the TCRα/β (Allison et al, 1982; Meuer et al, 1983; Haskins et al,

1983; Borst et al, 1983; Samelson et al, 1985). This subset of T cells can be once again subdivided into class II MHC-specific T cells, expressing CD4 accessory molecules, and class I MHC-specific T cells, expressing CD8 accessory molecules (Swain, 1981; Spits et al, 1982; Meuer et al, 1982; Marrack et al, 1983; Greenstein et al, 1984). Virtually all of the known, antigen-specific cell-mediated effector and regulatory mechanisms of the immune system are carried out by these two subsets of T lymphocytes. Another subset of T lymphocytes expresses the CD3 polypeptide chains in association with a γ/δ chain receptor heterodimer, and most of these cells do not express either the CD4 or CD8 molecules (Brenner et al, 1986; Bank et al, 1986; Moingeon et al, 1986; Lanier et al, 1986d). Existing literature shows that 62-70% of the TCRγ/δ^+ cells are $CD4^-CD8^-$,while a subset of TCRγ/δ^+ cells which range from 28 to 33% are $CD4^-CD8^+$ and a minor subset of TCRγ/δ^+ cells ranging from 2 to 5% would be $CD4^+CD8^-$ cells (Scott et al, 1990; Groh et al, 1989; Kozbor et al, 1989; Borst et al, 1988; Jitsukawa et al, 1987).

I.2.2 Identification of TCRs

I.2.2.1 Identification of TCRα/β

The development of monoclonal antibodies (mAb) (Kohler and Milstein, 1975) and the establishment of T cell clones and hybrids (Moller, 1981; 1983) rendered identification of the T cell antigen- receptor complex possible. Based on the concept that a T cell receptor, like surface immunoglobulin on B cells, might express an antigenic determinant (epitope) unique to one clone of T cells, mAbs were generated that recognized clone-specific cell surface molecules on T cell tumors. The molecules recognized by clone-specific mAb were heterodimers composed of a 40 to 50 kDa acidic TCRα glycoprotein that was covalently associated by disulfide linkage to a 40 to 45 kDa basic or neutral TCRβ glycoprotein. The TCRα and β genes encode transmembrane glycoproteins containing two extracellular domains, variable (V) and constant (C) whose

sequences are homologous to those of immunoglobulins, as well as a connecting segment, a transmembrane hydrophobic segment, and a short intracytoplasmic tail. In addition to intrachain loops in the V and C domains, each chain carries a cysteine residue in the membrane proximal connector that is thought to participate in interchain disulfide linkage.

I.2.2.2 Identification of TCRγ/δ

Recently, it was observed that some $CD3^+$ human lymphocytes lack reactivity with mAbs βF1 (Brenner et al, 1986, 1987) and WT31 (Tax et al, 1983; Oettgen et al, 1984; Spits et al, 1985) which detect framework determinants present on all lymphocytes bearing α/β T cell receptors. Yet, these cells expressed two cell surface polypeptides, a 55 and a 40 kDa, which could be chemically cross-linked to CD3. Following the cloning of TCRβ, Saito et al (1984a), in analyzing T cell specific cDNA clones obtained from the murine CTL clone 2C, identified clones encoding a distinct, but related protein. Like TCRβ, these new clones detected genes that were rearranged and expressed specifically in T cells and were composed of elements displaying homology to immunoglobulin variable (V), joining (J), and constant (C) gene segments. At first, they were mistakenly concluded to encode the α subunit of the TCR. Later, the work of several investigators demonstrated and confirmed that these clones defined a distinct TCR-like molecule which has been renamed TCRγ (Hannum et al, 1984; Fabbi et al, 1984; Jones et al, 1985; Chien et al, 1984; Saito et al, 1984b; Sim et al, 1984).

Using probes derived from murine TCRγ sequences, human TCRγ genes were shown to constitute a T cell rearranging family of genes located on the short arm of chromosome 7 (Lefranc and Rabbitts, 1985; Murre et al, 1985), distinct from the TCR β locus (long arm of chromosome 7) and the TCRα locus (chromosome 14). The locus is now well characterized, consisting of up to 14 V segments, 5 J segments and 2 C

segments (Lefranc and Rabbitts, 1985; Murre et al, 1985; Quertermous et al, 1986a; 1986b; 1987; Lefranc et al, 1986a; 1986b; 1986c; Dialynas et al, 1986; Forster et al, 1987; Huck et al, 1988). The 55 kDa species could be immunoprecipitated using antiserum made against synthetic peptides corresponding to the reduced amino acid sequence of a human TCRγ cDNA clone (Murre et al, 1985; Dialynas et al, 1986), suggesting that it was the elusive protein product of the TCRγ gene (Brenner et al, 1986). The 40 kDa species failed to react with anti-TCRγ sera and this appeared to represent an additional component of the TCRγ-CD3 complex, which was termed TCRδ (Brenner et al, 1986). Thus, the TCRγ gene encoded part of a second T cell receptor present on T lymphocytes that expressed CD3, but lacked TCRα/β.

I.2.3 Description of TCRγ/δ

While TCRα and β chains are always disulfide linked and of similar size on virtually all T lymphocytes examined, the protein subunit structure of the TCRγ/δ has proved to be complex. In man, TCRγ subunits are found in disulfide-linked (Borst et al, 1987; Brenner et al, 1987; Moingeon et al, 1987; Lanier et al, 1987; Van Dongen et al, 1987) and nondisulfide-linked forms (Brenner et al, 1986; 1987; Weiss et al, 1986; Loh et al, 1987; Van Dongen et al, 1987; Hochstenbach et al, 1988). Further, the disulfide-linked form of TCRγ is 36-42 kDa, whereas the nondisulfide-linked forms are either 55 kDa or 40-44 kDa. Characterization of cDNA clones reveals that the disulfide-linked TCRγ protein is encoded by the $C_\gamma 1$ gene, which contains a single CII exon that encodes a cysteine residue (Krangel et al, 1987). The large non disulfide-linked TCRγ protein is encoded by a $C_\gamma 2$ gene carrying three copies of the CII exon (copies a, b, and c), all lacking the cysteine residue (Pardoll et al, 1987; Krangel et al, 1987). The small nondisulfide-linked TCRγ protein is encoded by a $C_\gamma 2$ gene carrying two copies of the CII exon (copies b and c) (Hochstenbach et al. 1988). These three forms have been

denoted Form 1 (disulfide linked), Form 2abc (large, nondisulfide linked), and Form 2bc (small, nondisulfide linked) in accordance with $C_\gamma 1$ or $C_\gamma 2$ constant region and CII exon usage. Due to variation in the number and kind of CII exons, these forms vary in the size and disulfide linkage of the membrane proximal connector region (Hochstenbach et al, 1988).

The TCRδ polypeptide has a backbone size of 35 kDA and appears to carry two asparagine-linked glycans. However studies have not revealed important differences between TCRδ polypeptide of varied source (Band et al, 1987; Hochstenbach et al, 1988).

In some instances, the TCRγ subunit has been observed without detectable TCRδ species, and the existence of TCRγ-γ homodimers or γ-γ' heterodimers have been considered (Borst et al, 1987; Moingeon et al, 1987; Ang et al, 1987; Alarcon et al, 1987; Ioannides et al, 1987). In such cases, it is not clear whether TCRγ can be expressed without TCRδ, or whether TCRδ is not easily detected.

In man, the CD3 polypeptides associated with TCRγ/δ chains are different from the CD3 polypeptides associated with TCRα/β proteins (Brenner et al, 1986; Krangel et al, 1987). This variation results from differential processing of the oligosaccharides of the CD3δ chain.

I.2.3.1 Phenotypic Profile of TCRγ/δ

One of the phenotypic hallmarks of TCRγ/δ cells is the lack of reactivity with mAbs directed against TCRα/β (Brenner et al, 1986; Weiss et al, 1986; Moingeon et al, 1986). These mAbs include β Framework 1 (βF1), which detects a determinant on the TCR β chain and immunoprecipitates TCRα/β, but not TCRγ/δ (Brenner et al, 1987). However, mAb βF1 does not bind to the surface of living T cells. mAb WT31 displays a less clearly defined reactivity, but is nevertheless extremely useful (Tax et al, 1983).

This antibody binds strongly to the surface of TCRα/β cells and thus has been used to identify and to isolate brightly stained (TCRα/β) and unstained (TCRγ/δ) cells. However, under certain conditions (such as high antibody concentration or pretreatment of lymphocytes with neuraminidase), WT31 can bind at low levels to TCRγ/δ$^+$ cells (Van de Griend et al, 1988). Very weak binding can also be demonstrated on fresh peripheral blood lymphocytes of some individuals (Porcelli et al, 1991). Further, WT31 allows immunoprecipitation of either TCRα/β (Oettgen et al, 1984; Spits et al, 1985; Brenner et al, 1986; Littman et al, 1987) or TCRγ/δ$^+$CD3$^+$ cells (Van de Griend et al, 1988) with appropriate solubilisation conditions (using digitonin or low detergent concentrations). Thus, the epitope recognized by WT31 is unclear, but may be TCRα/β or a conformational determinant formed with CD3, with weak, but detectable, cross-reaction on TCRγ/δ$^+$CD3$^+$ cells. Although strong staining by WT31 suggests TCRα/β expression, corroboration as to which receptor is expressed should be obtained using either TCRγ/δ-specific mAb or biochemical analysis.

The WT31$^-$CD3$^+$ cells in human peripheral blood were found to constitute about 3% of peripheral blood T lymphocytes (range 0.5-10%) (Brenner et al, 1986; Weiss et al, 1986; Lanier and Weiss, 1986; Lanier et al, 1987). Recently, mAbs that react specifically with human TCRγ/δ cells were produced. Band et al (1987) characterized a mAb, anti-TCRδ-1, that reacts with the TCRδ chain common to all TCRγ/δ$^+$ cells. It stains from <0.5 to 12% of peripheral blood T cells and all WT31$^-$ CD3$^+$ T cells in the peripheral blood of several adults examined (Lanier et al, 1987).

Other mAbs directed against TCRγ/δ have been produced and used in different studies: TiγA is specific for Vγ9 (Vγ9 is expressed by the majority of the TCRγ/δ$^+$ cells) (Lanier et al, 1987). BB3 is directed against the Vγ2 and reacts with approximately two thirds of peripheral CD3$^+$WT31$^-$ lymphocytes (Triebel et al, 1988, Ciccone et al, 1988) and the δTCS-1 which is against the Vδ1 region present on a third

of the TCRγ/δ subset in human peripheral blood (Faure et al, 1988; Triebel and Hercend, 1989).

I.2.3.2 Cytolysis by TCRγ/δ

Studies on cell-mediated cytotoxicity suggest that TCRγ/δ lymphocytes may function as effector cytotoxic lymphocytes that recognize antigens on other cell surfaces (Bank et al, 1986; Borst et al, 1987; Brenner et al, 1987). *In vitro*, cultured TCRγ/δ cell lines lyse tumor targets, however it is not clear if tumor lysis is a T cell receptor-mediated, antigen-specific interaction (Borst et al, 1987; Brenner, 1987: Ferrini et al, 1987; Moretta et al, 1988). TCRγ/δ cells may recognize (or be restricted by) MHC-encoded molecules, although some data suggest that the molecules recognized may not be classical MHC class I and II proteins.

I.2.3.3 Activation of TCRγ/δ

As in TCRα/β lymphocytes, it appears that activation results from perturbation of the TCR-CD3 complex on the cell surface (Weiss and Stobo, 1984; Weiss et al, 1984; Imboden and Stobo, 1985; Oettgen et al, 1985; O'Flynn et al, 1985). Signal transduction would involve increases in cytoplasmic calcium and inositol phosphates in both TCR α/β and γ/δ subpopulations (Krangel et al, 1987; Pantaleo et al, 1988). Like TCRα/β$^+$ cells, once stimulated the TCRγ/δ$^+$ cell lines may secrete lymphokines such as IL-2 (Bank et al, 1986; Ferrini et al, 1987; Moretta et al, 1988).

Recent studies have convincingly shown that immune system activation plays an important role in the infectious and pathogenic processes of HIV infection, particularly for the CD4 lymphocyte subset (McDougal et al, 1985; Margolick et al, 1987; Folks et al, 1986; Gowda et al, 1989). The increase of CD8 in the peripheral blood

observed in HIV infection might also be due to immune system activation (Fahey et al, 1984). Evidence supporting a role for immune activation in HIV-induced infection and pathogenesis comes from studies demonstrating increased levels of soluble markers of immune activation in sera from HIV infected persons (Zolla-Pazner et al, 1984; Moss et al, 1988; Melmed et al, 1989; Honda et al, 1989; Agostini et al, 1989; Prince et al, 1990). Although the serologic activation markers appear to be useful in predicting disease progression in HIV, little is known about their relationship to each other or to CD4, CD8, TCRα/β and γ/δ lymphocyte subsets. These activated lymphocytes may be involved in the attempt of the immune system to control the HIV infection *in vivo*. The last part of this thesis focuses on some activation markers of importance in infectious diseases: HLA-DR, CD25, CD38, CD57.

I.2.4 TCRγ/δ^+ T cells and immunological disorders

TCRγ/δ lymphocytes displaying cytotoxic activity and lymphokine secretion suggest that they may have most, if not all, of the effector capabilities of TCRα/β lymphocytes. Furthermore, TCRγ/δ might fill certain distinct and selective roles in the immune system. Since the discovery of the TCRγ/δ^+ cells tremendous progress has been made in understanding genetic and protein chemistry. However, there remains lack of understanding as to how this receptor and the lymphocytes that bear it fit into the grand scheme of immunity.

Increases in TCRγ/δ^+ T cells have been reported in several immunodeficiency, autoimmune, and infectious states. Morio et al (1992) demonstrated that several immunodeficient patients, including Wiskott-Aldrich Syndrome, (a sex-linked recessive disorder with a defect in both T and B cell functions) (WAS), Severe Combined Immunodeficiencies (SCID), and Common Variable Immunodeficiency syndrome (CVID) possess a higher proportion of TCRγ/δ^+ cells in their peripheral blood.

In Epstein-Barr virus infection, the TCRγ/δ subset has also been shown to increase (Hassan et al, 1991, De Paoli et al, 1990). T cells coexpressing $V_\gamma 9$ and $V\delta 2$ proliferate in response to Daudi Burkitt's lymphoma cells (lymphoblastic lymphoma found in Africa and associated with Epstein-Barr virus infection) and to certain mycobacterial extracts. $V_\gamma 9^+/V\delta 2^+$ T cells are efficient killers of Daudi Burkitt's lymphoma cell line (Fisch et al, 1990). In hematologic malignancies, the TCR clonality has been used to evaluate the patient status (Reis et al, 1990). In ataxia-telangiectasia, a progressive multisystem disease, where B- and T-cell deficiencies are thought to be due to a defect of rearrangements of immunoglobulin and T-cell receptor genes, it was shown that patients had a relative increase of circulating T cells bearing the γ/δ receptors rather than α/β receptors (Carbonari et al, 1990). Polymyositis, a degenerative muscle disease, can be mediated by γ/δ T cells. In a patient with polymyositis, $CD4^-$ $CD8^-$ T cells expressing the γ/δ T-cell receptor surrounded and invaded nonnecrotic muscle fibers (Hohfeld et al, 1991). The statistically significant numbers of benign-appearing epidermal γ/δ cells found in mycosis fungoides (chronic lymphoma) may parallel these disorders functionally (Fiveson et al, 1991).

An increase in TCRγ/δ^+ cells can occur in certain systemic autoimmune diseases and is a persistent feature of systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS). This suggests that the increase of TCRγ/δ^+ cells in a subgroup of individuals with autoimmune diseases reflects a chronic change, rather than a short-lived perturbation, of the normal immune system homeostasis (Gerli et al, 1991).

I.3 Hypothesis

Since the TCRγ/δ^+ T cells are associated with a number of immunological disorders it could be that they play a role when the immune system is defective. Because HIV infection results in the impairment of the immune system, the

TCRγ/δ$^+$ T cells might increase as the disease progresses. Furthermore, if these TCRγ/δ$^+$ T cells becomes activated during HIV infection, it could suggest that they might play a role in the immune response against the disease.

I.4 Objectives

In summary, the general objective of this study was to analyze the TCRγ/δ T cell subset in HIV seropositive individuals. To achieve that objective, specific analyses had to be done:

1) Measurement of
   i) the frequency of major T cell subsets: CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ T cells, CD3$^+$CD4$^-$CD8$^-$ T cells, TCRα/β$^+$ T cells, TCRγ/δ$^+$ T cells in noninfected and HIV infected individuals.
   ii) the distribution of CD4 and CD8 markers in the TCRα/β and TCRγ/δ T cell subsets in noninfected and HIV infected individuals.
   iii) the surface CD8 expression, represented by CD8 fluorescence intensity, on TCRα/β$^+$ and TCRγ/δ$^+$ T cells. The surface CD8 expression will be correlated with disease progression.
   iv) the surface TCRα/β and TCRγ/δ expression on TCRα/β$^+$ and TCRγ/δ$^+$ T cells respectively. The surface TCRα/β and TCRγ/δ expression will be correlated with disease progression, CD4 antigen and treatment with zidovudine.
   v) the activational status of the TCRα/β$^+$ and TCRγ/δ$^+$ T cells, using five activational markers: HLA-DR, CD25, CD38, CD57 and CD5 in noninfected and in HIV infected individuals.

II MATERIALS and METHODS

II.1 Blood samples and total lymphocyte counts

HIV infected blood was obtained from informed consent patients at the Immunodeficiency clinic of Ottawa General Hospital. The patients were categorized clinically into stage II, III and IV as per the WHO criteria (1989) (see appendix I). The categories are characterized generally as follows: Stage I: Acute infection; Stage II: asymptomatic infection; Stage III: persistent generalized lymphadenopathy without any symptoms; Stage IV: a) constitutional disease, b) neurological disease, c) secondary infectious disease, d) secondary cancers, e) other conditions.

Venous blood from noninfected volunteers and HIV-infected individuals was collected in 5 ml vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) as anti-coagulant. Automated differential blood counts were performed by the Coulter Counter (Coulter Electronics) at the Hematology Department of Ottawa General Hospital (Ottawa, Ont.).

II.2 Immunophenotyping of subsets of mononuclear cells

Subset phenotyping of samples from noninfected and HIV infected whole blood was performed using flow cytometric analysis as follows: a 100µl volume of whole blood was distributed in 12 X 75 mm polystyrene round-bottom RIA tubes (Falcon, B-D, Mississauga, ONT). The appropriate volume of mAb -as determined by the company or by titration performed in our laboratory- was added (see Table 1 for the list of mAbs and Table 2 for mAb combinations used). Each sample was incubated for 10 min at room temperature (RT). Afterward, the samples were processed with a Q-Prep immunology

Table 1

List of monoclonal antibodies

A series of 20 mAbs was used for immunophenotyping. Eighteen mAbs were directly conjugated to the fluorochrome FITC or PE (B-D). Leu7 mAb (HNK-1 clone) was biotinylated (B-D). Anti-TCRδ1was FITC conjugated and was purchased from T Cell Sciences.

NAME	CD	COMPANY	ISOTYPE	FLUOROCHROME
Simulset Leucogate	45, 14	B-D*	IgG_1, IgG_{2b}	FITC , PE
Isotype control	---	B-D	IgG_1	FITC
Isotype control	---	B-D	IgG_1	PE
Isotype control	---	B-D	IgG_{2a}	FITC
Isotype control	---	B-D	IgG_{2a}	PE
Leu4	3	B-D	IgG_1	PE
Leu2a	8	B-D	IgG_1	PE
Leu3a	4	B-D	IgG_1	PE
Leu12	19	B-D	IgG_1	PE
Leu16	20	B-D	IgG_1	PE
Leu11c	16	B-D	IgG_1	PE
Leu19	56	B-D	IgG_1	PE
TCR-1	---	B-D	IgG_1	FITC
TCRδ1	---	T Cell Sciences	IgG_1	FITC
HLA-DR	---	B-D	IgG_{2a}	PE
IL-2R	25	B-D	IgG	PE
Leu17	38	B-D	IgG	PE
Leu7**	57	B-D	IgM	SA-PE
Leu1	5	B-D	IgG_{2a}	PE

*B-D = Becton- Dickinson

**HNK-1 was used in indirect staining with SA-PE

Table 2

Combinations of monoclonal antibodies

A battery of 20 combinations of mAbs was used for immunophenotyping. Combinations 1 to 6 are combinations used to perform a standard phenotypic profile. Combinations 7 to 20 are used to study the two TCR subsets from which combinations 11 to 20 are specifically used to study their activation state.

TUBE NUMBER	mAb COMBINATION (FITC/PE)
1	IgG_1/IgG_2
2	CD45/CD14
3	CD3/CD4
4	CD3/CD8
5	CD3/CD16 + CD56
6	CD3/CD19 or CD3/CD20
7	TCR-1/CD4
8	TCR-1/CD8
9	TCRδ1/CD4
10	TCRδ1/CD8
11	TCR-1/HLA-DR
12	TCR-1/CD25
13	TCR-1/CD38
14	TCR-1/CD57
15	TCR-1/CD5
16	TCRδ1/HLA-DR
17	TCRδ1/CD25
18	TCRδ1/CD38
19	TCRδ1/CD57
20	TCRδ1/CD5

work station (Coulter, Hialeah, FL) using the 35 second cycle. This work station automatically lysed, stabilized and fixed the samples. The lysing reagent (Immunoprep A) which consisted of a solution of formic acid, was added to the test tube to rapidly lyse the erythrocytes while leaving the leukocytes intact. The stabilizing reagent (Immunoprep B), which is PBS, preserved the form and structure of the leukocytes. The fixing reagent (Immunoprep C) which consisted of 2% paraformaldehyde, was added to fix the cell membranes of the leukocytes. The samples, which contained Leu 7-biotin mAb, prior to the Q Prep processing, were washed with PBS and the secondary step reagent, streptavidin-phycoerythrin (SA-PE), was added and incubated for 10 min at RT. Following this processing, the samples were washed twice with PBS and resuspended in 2% paraformaldehyde (BDH Inc, Toronto, Ont). In flow cytometry the data are obtained using a two-step procedure: first the sample is "acquired" with the flow cytometer, second the listmode file generated is analysed. The sample could be acquired after an incubation of 30 min at 4°C or stored at 4°C up to 48 hours before acquisition with the flow cytometer. The flow cytometer used for this study was a FACScan from Becton-Dickinson (Mountain-View, CA); an air-cooled 15mWatt argon-ion laser emitting at 488nm served as the light source (Figure 1). The optical filters were factory-set standard configuration, with green fluorescence through a 530 ± 30nm bandpass, orange fluorescence through 585 ± 42 nm bandpass, and red through a 650 nm longpass filter to collect signals from FITC, PE and PerCP (not used) respectively.

II.2.1 Flow cytometer calibration

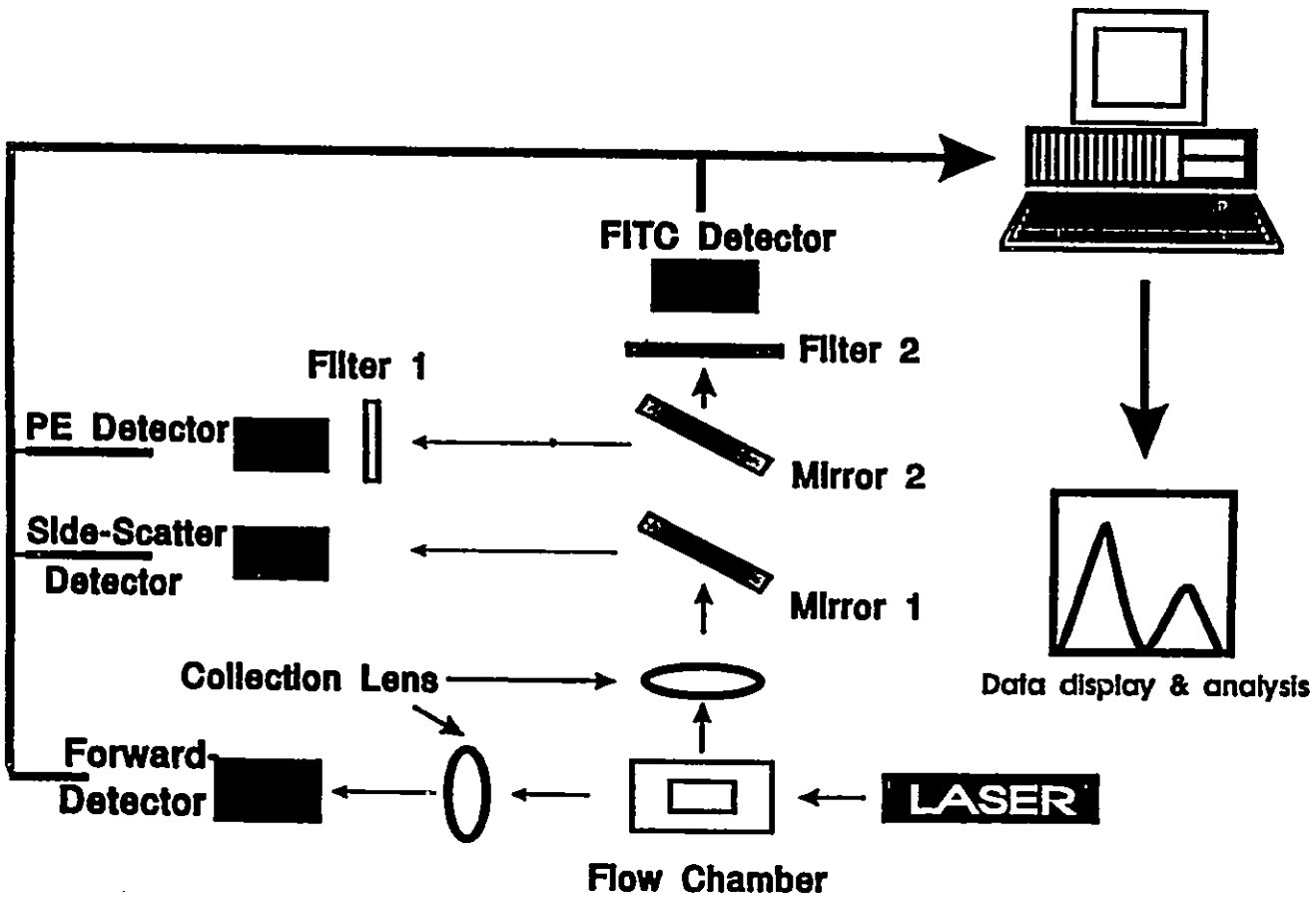
The cytometer was calibrated daily to insure that the instrument was operating properly. This calibration was achieved in three different steps employing AutoCOMP, Quickcal and QC3 beads. AutoCOMP is a menu-driven program that adjusts the

Figure 1

Diagrammatic representation of a single laser flow cytometer

(adapted from N.P. Carter and E. W. Meyer In Flow Cytometry, A Practical Approach. Ed M. G. Ormerod, IRL Press)

Cytometer description: A flow cytometer operates by causing cells in a fluid stream to pass single file through a beam of light, usually generated by a laser. The photons of light that are scattered and emitted by the cells following their interaction with the laser beam are separated into constituent wavelengths by a series of filters and mirrors. This separated light falls on individual detectors that generate electrical impulses, or analog signal, proportional to the amount of incident light striking the detectors. Each analog signals is converted to a digital signal. These individual numbers are accumulated in a frequency distribution, or histogram. The numbers are proportional to the amount of light emitted from, or scattered by individual cells.


FITC Detector
Filter 2
Filter 1
PE Detector
Mirror 2
Side-Scatter
Detector
Mirror 1
Collection Lens
Data display & analysis
Forward
Detector
LASER
Flow Chamber

photoelectric components (gain settings and fluorescence compensation) of the FACScan flow cytometer to known standards using CaliBRITE beads (Becton-Dickinson) as its reference standard. The AutoComp program performs 3 FACScan adjustments: gating single events, adjusting photomultiplier tube (PMT) gain, and fluorescence compensation. The QuickCal program also uses plastic beads of which one population is unstained and five have differential levels of FITC (from 10, 000 to 500, 000 MESF). The MESF (Molecule of Equivalent Soluble Fluorescence) value indicates the level of fluorescence found on each bead. Besides gating singlet events this program performs statistical analysis on inter-measurement variance (CV) and allows the creation of history files on the analytical performance of the instrument. Finally, the QC3 beads are exclusively used for channel targeting which permits comparison of fluorescence intensity from day-to-day data.

II.3 Statistical analysis

Group means were compared using the Student t-test. Significance was defined as $p<0.05$. Correlation between lymphocyte subsets was determined using a Pearson correlation coefficient.

III. RESULTS

III.1 Phenotypic subset analysis of mononuclear cells

A variety of peripheral blood mononuclear cells (PBMNCs) are identified with mAbs that react with cell surface antigens. Almost one hundred such antigens have been described at the IVth International Human Leukocyte Differentiation Antigen Workshop in 1989, and the functional activity of about one-third of these molecules has been determined. At this workshop, 78 of the human leukocyte antigens were officially recognized and given cluster designation numbers 'CD1' through 'CD78' (Knapp et al. 1989). The leukocytes enumerated in routine immunophenotyping are the various types of lymphocytes, i.e., T cells and T-cell subsets, B cells and natural killer (NK) cells. T lymphocytes are characterized by mAbs against CD3 (Leu4), CD8 (Leu2a) and CD4 (Leu3a), while B lymphocytes are identified with a mAb against CD20 (Leu16); all mAbs are directly conjugated to FITC or PE. Monoclonal Abs against CD16 (Leu11c) and CD56 (Leu19) are used to identify NK cells. T lymphocytes are grouped into subpopulations based on the presence of specific TCR (α/β or γ/δ). TCR-1 mAb is used to characterize TCRα/β bearing lymphocytes while TCRδ1 mAb are used to investigate the expression of TCRγ/δ molecules. mAbs against HLA-DR, CD25, CD38, CD57 and CD5 are used to study the activational state of the TCRα/β and the TCRγ/δ lymphocyte populations (Table 1). Most of the cell surface antigens are not lineage-specific and antibodies against them are reactive with more than one type of leukocyte, thus making multiparametric flow analysis essential even for routine phenotyping.

Table 2 lists the mAb combinations used in this study. Using two-colour staining, it is possible to determine whether CD4$^+$ (helper/inducer) and CD8$^+$ (suppressor/cytotoxic) cells are T cells. Monocytes which are CD4$^+$CD3$^-$ and NK cells which are CD8$^+$CD3$^-$ (Lanier et al, 1986b, c) can be excluded and are not counted as

they would be by using single-colour staining. T cells and NK cells can be enumerated simultaneously to make sure that the T cells that are $CD56^+$ (Lanier et al, 1986b) are not included in the NK cell count as well as in the T cell count.

The mAbs in the first two samples of Table 2 are controls. The immunoglobulin G (IgG) isotype control indicates whether non-specific binding of IgG_1 or IgG_2 occurred and determines where the integration cursors are set (boundaries that determine the percentage of positively stained cells). For all mAb combinations, the cursor was placed just to the right of the negative cluster. Sample 2 was run first to determine where the lymphocytes fall on the forward scatter versus right-angle scatter plot (Loken et al, 1990). The mAbs in this sample allowed the determination of the percentage of lymphocytes ($CD45^{bright}CD14^-$) in the gated light scatter analysis region (see page 33 for explanation on lymphocyte gate determination). The mAb combinations 3-5 utilize an anti-CD3 mAb. As mentioned earlier, the anti-CD3 mAb in combination with the anti-CD4 mAb is used to enumerate $CD4^+$ T cells. This procedure ensures that no monocytes are included in the enumeration. Monocytes appear as $CD4^{dim}$ staining cells and this reactivity can be excluded from the enumeration of the cells by resetting the cursor to count only the $CD4^{bright}$ cells. In mAb combination 4 , the anti-CD3 mAb together with the anti-CD8 mAb enumerated the $CD8^+$ T cells and excluded the $CD8^+$ NK cells. In combination 5, the anti CD3 mAb together with the anti-CD16 and the anti-CD56 mAbs permitted accurate enumeration of all NK cells and T cells. The combination 6 of mAbs provided the B cell number. The mAb combinations 7-10 were used to study in more detail the phenotypic characteristics of the two subsets TCRα/β and TCRγ/δ. The combinations 11-20 were used to study the activational state of these two TCR subsets.

III.2 Methods of analysis of data generated by flow cytometry

Cell surface markers on peripheral blood mononuclear cells (PBMNCs) in normal and HIV patients were studied using two-colour immunofluorescence (Table 1, mAb list and Table 2, combinations of mAbs used). Fresh whole blood was stained and processed with a Q-Prep immunology workstation which automatically lyses the erythrocytes and fixes the leukocytes. The samples were acquired with a FACScan (Becton-Dickinson) flow cytometer. Analyses were performed with the Hewlett Packard computer assigned to the cytometer. The softwares used for data collection and analyses were: Simulset, Facscan Research, and Lysis (I and II). All programs allowed the storage of data in listmode which could be retrieved at any given time for analysis. The Simulset program was used for acquisition and simultaneous analysis.

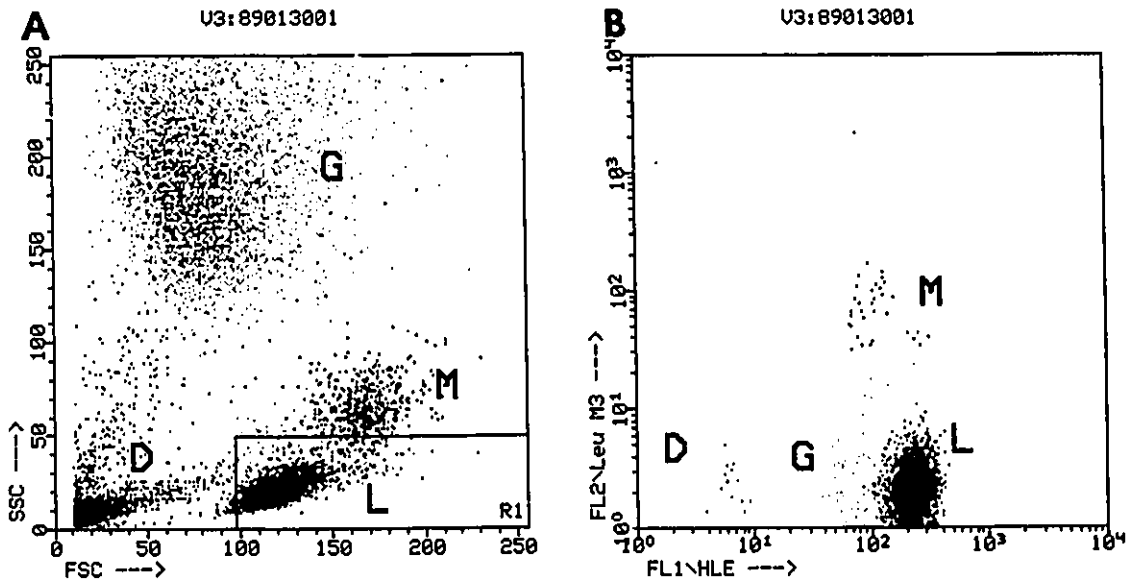
The data were first presented on a bivariate dot plot: forward scatter (FSC) measurement (representing the size of the cell) versus side scatter (SSC) measurement (representing the granularity of the cell), on a linear scale (Figure 2a). The resulting dot plot allowed the three major leukocyte types in peripheral blood to be differentiated from each other and from debris. Counts were performed on the population of interest only: the lymphocytes. The lymphocyte population was isolated by electronic gating by drawing a barrier around the population that appeared at the bottom and middle position of the FSC versus SSC dot plot. This position corresponds to middle sized (6-10 μm) non-granular cells.

The lymphocyte cluster is easily differentiated from the monocyte cluster on a scatter plot. However, small monocytes are often found inside the lymphocyte gate while small and large lymphocytes are found outside of the lymphocyte gate. To minimize this technical difficulty, the Simulset program electronically defines a gate around the lymphocyte cluster aiming at an optimal number of lymphocytes in the gate

Figure 2

Automatic lymphocyte gating using the Simulset program

This figure represent data obtained using a non-infected sample. The samples were prepared as described in the Methods section.
a) In the side scatter versus forward scatter plot, the leukocytes segregate in well defined populations: the lymphocytes (L), monocytes (M), granulocytes (G), and debris (D). Using its fluorescence characteristic -brightly stained with CD45 and non-stained with CD14- the program Simulset automatically sets up a gate to encompass the lymphocyte population and exclude the monocytes, granulocytes, and debris.
b) The program computes and gives the differential count, i.e. lymphocytes (L), monocytes(M), granulocytes(G), and debris(D), of the gate and the proportion of lymphocytes gated.
c) The gated events were analyzed and presented in a fluorescence dot plot where the X-axis is the fluorescence intensity of FITC ($CD45^+$ cells) and the Y-axis is the fluorescence intensity of PE ($CD14^+$ cells). This plot illustrates the population of lymphocytes and the contamination by monocytes, granulocytes, and debris.



	%Lymphs	%Monos	%Grans	%Debris	%Lymphs total
Gate description:	97	1	1	1	100

with a minimal number of contaminants (monocytes, granulocytes and debris) (Figure 2b).

The combination of mAbs used for the determination of contaminating events in the lymphocyte gate is called Leucogate Simulset and consists of an anti-CD45-FITC and an anti-CD14-PE. The anti-CD45 stains lymphocytes brightly and stains granulocytes dimly. The anti-CD14 stains only the monocytes (Figure 2b). Thus, by using the Leucogate Simulset mAbs, the proportions of lymphocytes (L), monocytes (M), granulocytes (G) and debris (D) included in the gate were computed as well as the proportion of gated lymphocytes compared to the total number of lymphocytes in the sample (Figure 2).

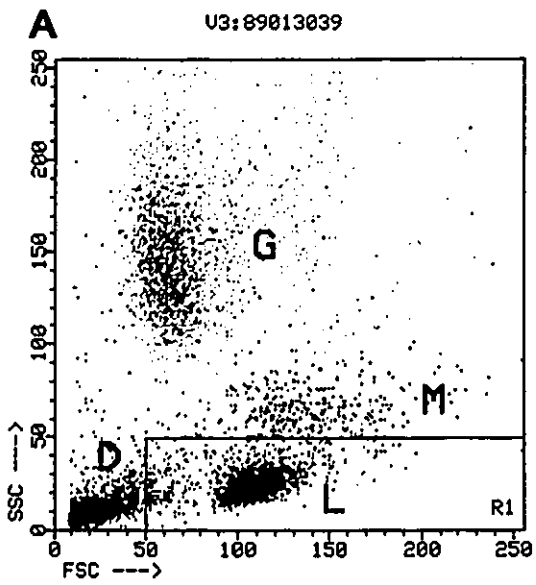
The program also allows manual drawing of the lymphocyte gate when needed.This feature became important when analysing abnormal samples such as blood from patients with advanced HIV infection (Figure 3). In the case of a sample from an AIDS patient, the semi-automatically defined gate included 97% of the total lymphocytes, but it included 9% contaminant events (Figure 3a, 3b). When the gate was manually drawn, the proportion of contaminant events was lowered to 2% (below the 5% permitted) (Figure 3c, 3d). By tightening the gate around the lymphocyte population, some of the bigger and smaller lymphocytes were lost and only 85% of the lymphocytes were analyzed as opposed to 97% in the automatically drawn gate. This was a compromise I made in order to avoid elevated backgrounds in fluorescence analysis. On the fluorescence dot plot, orange versus green intensity, the negative integration cursors were set up by the program automatically using isotype control mAbs. These cursors were used to analyse the other mAb combinations (Figure 4).

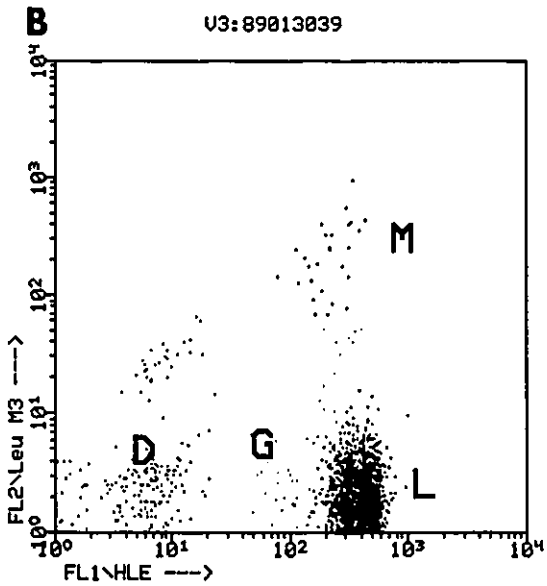
The Simulset software was an excellent program to use for rapid and efficient analysis of lymphocyte subpopulations. However, the study of a relatively new marker, TCR$\gamma\delta$, in "abnormal" conditions (HIV infection) demanded more flexibility in

Figure 3

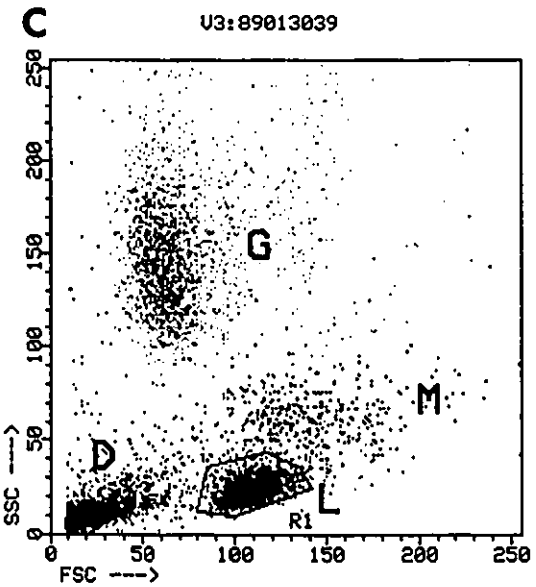
Manual lymphocyte gating using the Simulset program

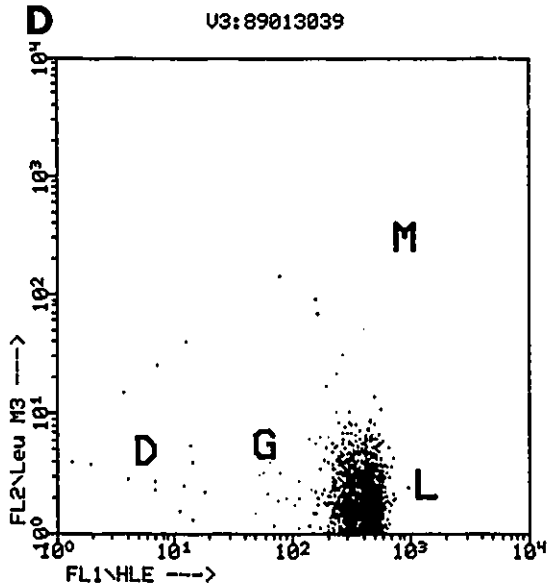
This sample, from an HIV infected individual, was processed as described in Figure 2 and was analysed with manual gating.
a) In the side scatter versus forward scatter plot, the lymphocyte gate is drawn to encompass the maximum lymphocyte events and the minimum contamination from monocytes, granulocytes or debris. Repeatedly with patient samples the automatically drawn lymphocyte gate would contain the maximum number of lymphocytes but it would also contain a high proportion of contaminant events. Each contaminant population was electonically identified and computed by the program e.g. monocytes = 3%, granulocytes = 2% and debris = 4%, for a total of 9% contaminant events.
b) The gated events were then distributed in a fluorescence 1 (FL1) ($CD45\text{-}FITC^+$ cells) versus fluorescence 2 (FL2) ($CD14\text{-}PE^+$ cells) plot. The lymphocyte population (L) appears brightly positive for FL1 ($CD45^+$ cells) and negative for FL2 ($CD14^-$ cells). The contaminants could be identified based on their staining properties for CD45 and CD14. The $CD45^{dim+}CD14^-$ cells are granulocyte (G) contaminants, while the $CD45^{bright+}$ $CD14^{bright+}$ cells are monocytes (M) and the $CD45^-CD14^-$ events are debris (D).
c) Whenever the proportion of lymphocytes into the gate was less than 95%, a manual gate was drawn to exclude the contaminant events with the minimal loss of lymphocytes. Only 2% of the lymphocyte gate were contaminants. However, this manual gating was usually associated with a certain loss of lymphocytes. In this case only 85% of the lymphocytes were included in the gate.
d) The fluorescence dot plot shows that with manual gating the contaminant populations were almost totally eliminated.





	%Lymphs	%Monos	%Grans	%Debris	%Lymphs total
Gate description:	85	3	2	4	97



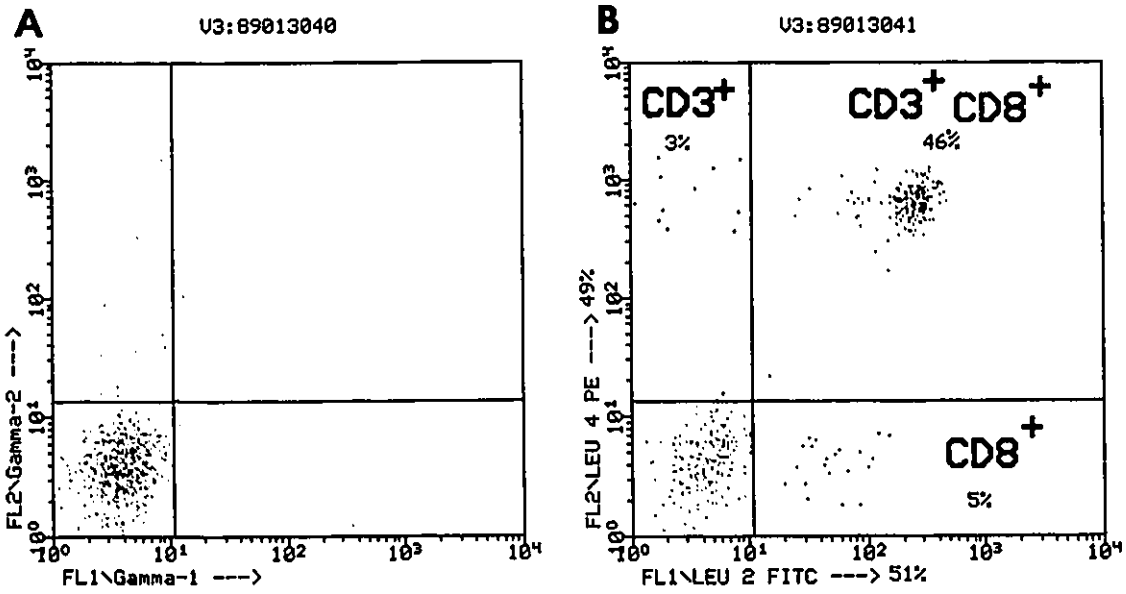


	%Lymphs	%Monos	%Grans	%Debris	%Lymphs total
Gate description:	98	1	1	0	85

Figure 4

Determination of integration cursors position using isotypic controls

As described in the Methods section:
a) In the third tube of each sample, the cells were marked with isotype control antibodies FITC and PE conjugated. Using this tube, the integration cursors for the fluorescence plot were automatically determined by the program.
b) This negative control cursor was used for the analysis of the other tubes from the same sample. Cells found in the lower left quadrant (quadrant number 3) are negative cells whereas cells found in the upper left (1), upper right (2) and lower right (4) are positive.


A
V3:89013040
FL2\Gamma-2 --->
FL1\Gamma-1 --->
B
V3:89013041
CD3+
3%
CD3+ CD8+
46%
CD8+
5%
FL2\LEU 4 PE ---> 49%
FL1\LEU 2 FITC ---> 51%

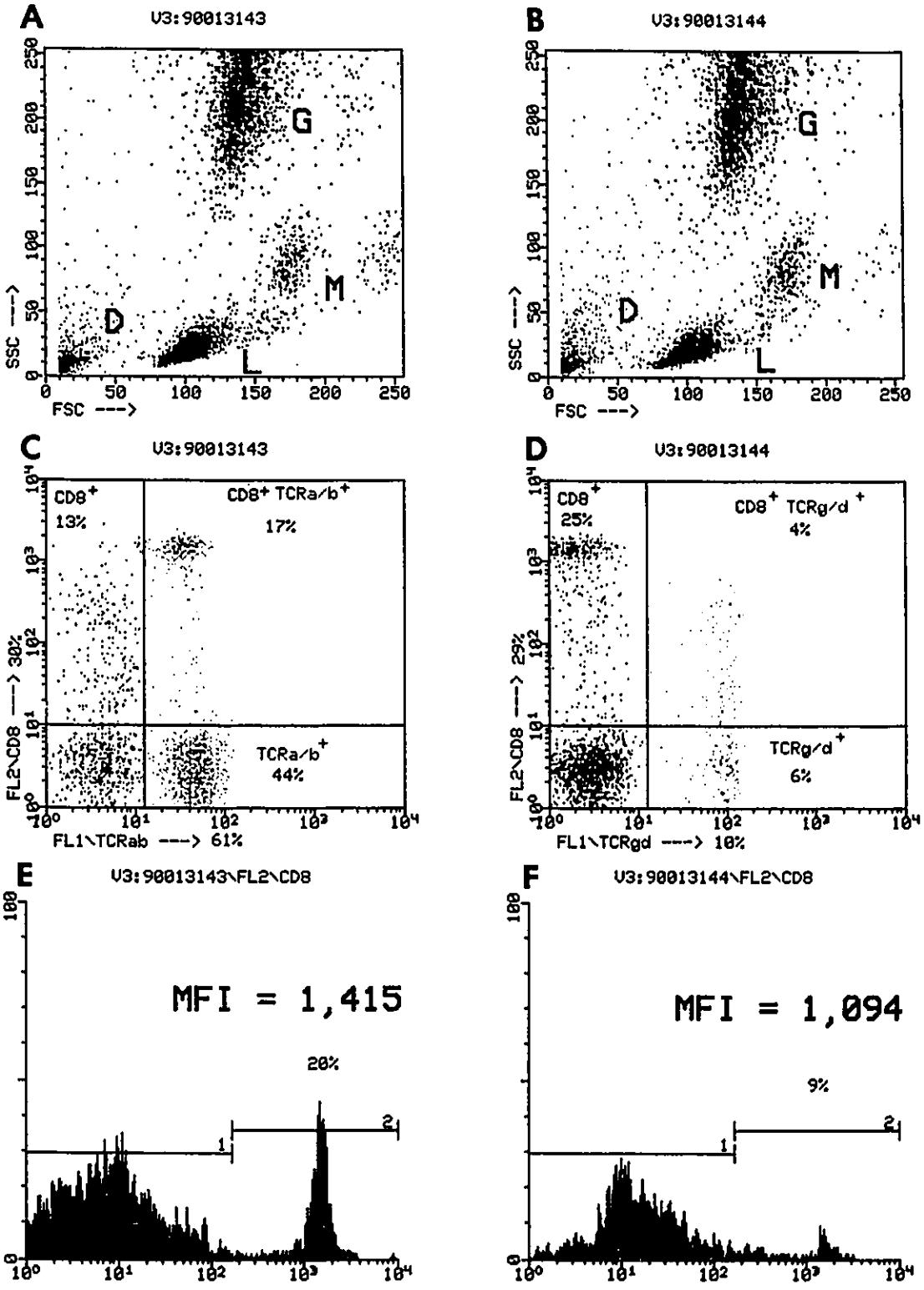
the analysis of the data than what the Simulset program offered. Therefore two additional softwares were used for data collection and list mode analysis: FACScan Research and Lysis (I and II). These programs computed statistics such as number of events, proportion of different subsets, and most importantly, the mean fluorescence intensity (MFI) of the peak of a given fluorescence. The MFI of a peak corresponds to the number of fluorescent molecules found on each positive cell i.e. the number of molecules of the marker of interest.

Figure 5 illustrates the analysis done to obtain the MFI and the frequency of a marker in a specific subset of lymphocytes. The cells were immunophenotyped with anti-TCRα/β-FITC mAb or anti-TCRγ/δ-FITC mAb and anti-CD8-PE mAb. The sample was processed with a Q-Prep immunology workstation and then acquired using a FACScan flow cytometer. The data were stored in list mode. First a scatter plot was produced to identify the three different populations of leukocytes: the lymphocytes (L), the monocytes (M) and the granulocytes (G) (Figure 5a, 5b). The lymphocyte gate was defined using the scatter plot (green population, Figure 5a, 5b). The gated events were analysed by plotting the green fluorescence intensity (representing the TCRα/β or TCRγ/δ marker) versus the orange fluorescence intensity (representing the CD8 marker) (Figure 5c, 5d). A new gate was defined on that fluorescence plot to encompass the lymphocytes that were positive for TCRα/β or TCRγ/δ (i.e. quadrants 2 and 4); this manipulation is called backgating (light blue populations) (Figure 5c, 5d). The backgated events were illustrated on a single parameter graph: the fluorescence histogram. The fluorescence histogram shows the proportion of $CD8^+$ cells among the TCR lymphocyte subsets. The histogram also shows the MFI of the CD8 marker at the surface of the TCR lymphocyte subsets. In the TCRα/β subset, the CD8 marker is expressed at the surface of the cell with a MFI of 1,415 (Figure 5e), while on the TCRγ/δ subset this marker is expressed with a MFI of 1,094 (Figure 5f).

Figure 5

Bivariate and single parameter graph using the Lysis program

Every sample tested for CD8, TCRα/β and TCRγ/δ as described in the Methods section, was analysed as follows:
a) The events were first plotted on a scatter plot, the X-axis being the forward scatter determining the size of the cell and the Y-axis being the side scatter determining the granulosity of the cells. This bivariate histogram allowed gating around the population of lymphocytes (low on the granularity scale and mid-way on the size scale) (green population). This sample from a noninfected volunteer, was stained with TCR-1-FITC mAb in combination with anti-CD8-PE mAb. The scatter plot represents the TCRα/β^+ cells.
b) Scatter plot of sample from a non infected volunteer, stained with anti-TCRδ1-FITC in combination with anti-CD8-PE mAb, represents the TCRγ/δ^+ cells.
c) d) The gated lymphocytes were then illustrated on a bivariate fluorescence dot plot. A new gate was drawn on that plot to include only the cells which are positive for the CD8 marker (i.e. quadrants 2 and 4). This manipulation is called "backgating".
e) f) The backgated events were then illustrated on a fluorescence histogram showing the distribution of the CD8 marker. Statistics were obtained from the histogram such as mean fluorescence intensity of the peak (MFI). The MFI of the CD8 marker at the surface of the TCRα/β^+ cells was higher than on TCRγ/δ^+ cells.


A
V3:90013143
G
M
D
L
SSC --->
FSC --->
B
V3:90013144
C
V3:90013143
CD8+ 13%
CD8+ TCRa/b+ 17%
TCRa/b+ 44%
FL2\CD8 ---> 30%
FL1\TCRab ---> 61%
D
V3:90013144
CD8+ 25%
CD8+ TCRg/d+ 4%
TCRg/d+ 6%
FL2\CD8 ---> 29%
FL1\TCRgd ---> 10%
E
V3:90013143\FL2\CD8
MFI = 1,415
20%
F
V3:90013144\FL2\CD8
MFI = 1,094
9%

The analysis of the activational state of the TCRγ/δ$^+$ cells brought an additional problem. To compute valid statistics, a relatively high number of TCRγ/δ$^+$ cells had to be acquired; this number was established at 5, 000 events. Because TCRγ/δ$^+$ cells were found in low numbers in peripheral blood, up to 100,000 events had to be acquired in order to obtain 5,000 TCRγ/δ$^+$ cells. The Facscan research program was the program of choice for acquisition of such a high number of events.

III.3 CD3$^+$, CD4$^+$ and CD8$^+$ cells: noninfected vs HIV infected individuals

III.3.1 Cell surface marker values in noninfected individuals

Cell surface marker values in noninfected donors were evaluated by immunophenotyping the major lymphocyte subsets. This experiment was performed so that a comparison could be made with the clinical samples. The pan-T cell population (CD3$^+$ cells) and its two major subsets, T helper/inducer cells (CD3$^+$CD4$^+$) and T cytotoxic/suppressor cells (CD3$^+$CD8$^+$) were stained using two-colour immunophenotyping with anti-CD3-FITC conjugated mAb and anti-CD4 or anti-CD8 - PE conjugated mAbs. As previously explained (page 30) these combinations of mAbs were necessary to make accurate counts of T cells. The samples were lysed using the Q-Prep immunology workstation and were acquired with a FACScan flow cytometer. Twenty eight noninfected blood samples were studied to establish normal values and range for relative and absolute concentration of CD3$^+$ T cells, CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ T cells. The results are presented in Table 3. The CD3$^+$ cells ranged from 57 to 82% of the lymphocytes which represented 868 to 2,072 cells/mm^3 while the CD3$^+$CD4$^+$ and the CD3$^+$CD8$^+$ T cells ranged from 34 to 58% and 14 to 47% respectively which represented 540 to 1,232 cells/mm^3 and 270 to 952 cells/mm^3. The absolute lymphocyte counts ranged from 1,300 to 2,900 cells/mm^3.

Table 3

Major T cell subsets in noninfected individuals

CD3, CD4, CD8, TCRα/β and TCRγ/δ subsets were analysed as outlined in Figure 2 and the Methods section: anti-CD3-FITC in combination with anti-CD4- and anti-CD8-PE mAbs; anti-TCRα/β- and anti-TCRγ/δ-FITC in combination with anti-CD4- and anti-CD8-PE mAbs. Twenty-eight samples, from noninfected volunteers, were tested.

T CELL SUBSET	RANGE	MEAN	S.E.
$CD3^+$:			
relative*	57-82	70	1
absolute**	868-2,072	1,333	60
$CD3^+CD4^+$:			
relative	34-58	45	1
absolute	540-1,232	870	42
$CD3^+CD8^+$:			
relative	14-47	30	1
absolute	270-952	580	35
Ratio CD4/CD8	0.9-3.2	1.6	0.5
Absolute Lymphocyte Count:	1,300-2,900	1,900	100

*relative = % of positive cells over all lymphocytes

**absolute = cells/mm^3

III.3.2 T cell surface marker analysis in blood samples from HIV infected individuals

T cell marker analysis of blood samples from HIV infected individuals was also performed. Each sample received from the Infectious Diseases Clinic of Ottawa General Hospital was taken from a patient infected with HIV. A general phenotypic profile (CD3, CD4, and CD8 counts) for each patient was performed to determine the levels of each T cell population in the circulation at the time of sampling. These data were critical in the analysis of TCR expression in these patients. Each sample was stained using two-colour immunophenotyping and erythrocytes were lysed with a Q-Prep immunology workstation. The FACScan flow cytometer was used to acquire the samples. Fifty-two patients from stage II to stage IV of the disease were studied: 14 patients in stage II, 24 patients in stage III, and 14 patients in stage IV (Tables 4, 5, and 6). Although the $CD3^+$ T cell counts remained stable through stages II and III, they dropped significantly at stage IV: from 82% and 83% at stage II and III respectively to 71% of the lymphocytes at stage IV ($p<0.01$). The CD4 subset showed a drastic decrease. At stage II of the disease only 46% (50% at stage III) of the $CD4^+$ cells found in noninfected individuals, remained. At stage IV of the disease, the drop in the CD4 subset became even greater, and only 10% of the initial CD4 count remained. This decrease from 25% at stage II to 23% at stage III and to 9% at stage IV ($p<0.01$) generated an inversion in the CD4/CD8 ratio. In noninfected individuals, the CD4/CD8 ratio observed ranged from 0.9 to 3.2. In HIV infected individuals, it could be as low as <0.01: the CD4/CD8 ratio ranged from 0.1 to 0.9, 0.1 to 0.8, and from 0 to 0.4 at stages II, III and IV of the disease respectively.

The $CD8^+$ sub-population showed an increase in HIV infected individuals. The inversion of the CD4/CD8 ratio might not have been only due to a drop in the CD4 counts but also to a concomitant increase of $CD8^+$ cells which remained high throughout

Table 4

Major T cell subsets in HIV infected individuals at stage II

The experiment was performed as in Table 3. Fourteen samples, from patients at stage II of the disease, were tested.

T CELL SUBSET	RANGE	MEAN	S.E.
$CD3^+$:			
relative	62-94	82	8
absolute	900-2,210	1,504	60
$CD3^+CD4^+$:			
relative	11-37	25	1
absolute	176-858	466	42
$CD3^+CD8^+$:			
relative	40-80	59	2
absolute	648-1,482	1,060	35
Ratio CD4/CD8	0.1-0.9	0.5	0.2
Absolute Lymphocyte Count:	1,200-2,600	1 800	100

Table 5

Major T cell subsets in HIV infected individuals at stage III

The experiment was performed as in Table 3. Twenty-five samples, from patients at stage III of the disease, were tested.

T CELL SUBSET	RANGE	MEAN	S.E.
$CD3^+$:			
relative	60-94	83	1
absolute	178-2,816	1,493	60
$CD3^+CD4^+$:			
relative	6-33	23	1
absolute	26-1,110	427	49
$CD3^+CD8^+$:			
relative	36-87	62	1
absolute	160-2,496	1,094	35
Ratio CD4/CD8	0.1-0.8	0.4	0.2
Absolute Lymphocyte Count:	200-3,700	1 800	100

Table 6

Major T cell subsets in HIV infected individuals at stage IV

The experiment was performed as in Table 3. Seventeen samples, from patients at stage IV of the disease, were tested.

T CELL SUBSET	RANGE	MEAN	S.E.
$CD3^+$:			
relative	20-92	71	1
absolute	144-2,150	650	60
$CD3^+CD4^+$:			
relative	0-25	9	1
absolute	0-475	90	42
$CD3^+CD8^+$:			
relative	29-84	64	1
absolute	138-1,875	563	35
Ratio CD4/CD8	0-0.4	0.1	0.1
Absolute Lymphocyte Count:	300-2,500	800	100

the disease with 59% of the lymphocytes (1,060 cells/mm^3) and 62% (1,094 cells/mm^3) at stages II and III respectively ($p<0.01$). At stage IV, the CD8 counts decreased and the absolute number of $CD8^+$ cells was reduced to 563 cells/mm^3, however, the proportion of $CD8^+$ lymphocytes remained high (64%). This decreased number of $CD8^+$ T lymphocytes combined with the drop of the CD4 subset, resulted in a decrease of $CD3^+$ cells. At stages II and III of the disease the $CD3^+$ cells were 82% and 83% of the lymphocytes respectively (1,504 and 1,493 cells/mm^3) while in samples from noninfected individuals, 70% of the lymphocytes (1,333 cells/mm^3) were CD3 cells. At stage IV of the disease the proportion of CD3 cells remained similar to normal values (71%). However, 71% represented only 650 cells/mm^3 ($p<0.01$). In general, the total absolute lymphocyte count was shown to decrease only at stage IV: in samples from noninfected individuals, 1,900 lymphocytes/mm^3 were counted; at stages II and III of the disease this number did not vary much (1,800) but at stage IV of the disease, the lymphocyte count decreased drastically to 800 cells/mm^3 ($p<0.01$). When CD4 counts were compared to CD4 percentages a strong correlation was observed in HIV infected individuals ($r=0.86$), however, no correlation was found in noninfected individuals ($r=0.43$) (Figure 6).

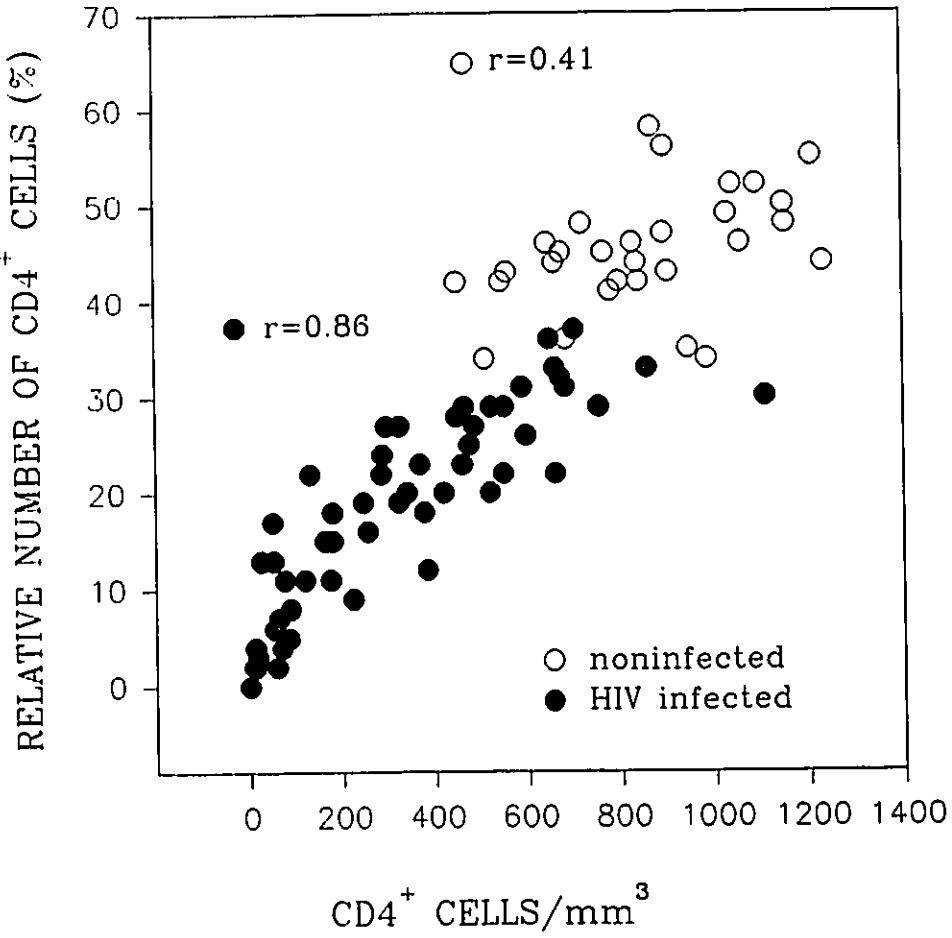
III.4 T cell receptor for antigen

The objective of this study was to determine if differences in the TCR subsets existed in HIV infected individuals. For this purpose, the data obtained from AIDS patients were compared to the data obtained from noninfected individuals. As reported by several researchers, the $TCR\alpha/\beta^+$ cells belong to the $CD3^+CD4^+CD8^-$ subset and to the $CD3^+CD4^-CD8^+$ subset (Acuto et al, 1985; Brenner et al, 1986). On the other hand, the majority of $TCR\gamma/\delta^+$ cells belong to the $CD3^+CD4^-CD8^-$ subset (Brenner et al, 1986; Bank et al, 1986; Moingeon et al, 1986; Lanier et al, 1986d). A

Figure 6

Correlation between the frequency and absolute number of $CD4^+$ T cell subsets in noninfected and HIV infected individuals

The experiment was performed as in Table 3. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III; and n=17, stage IV of the disease). The Pearson test was used to measure the correlation between the frequency and the absolute numbers of $CD4^+$ T cells.


70
60
50
40
30
20
10
0
RELATIVE NUMBER OF CD4+ CELLS (%)
r=0.41
r=0.86
noninfected
HIV infected
0
200
400
600
800
1000
1200
1400
CD4+ CELLS/mm3

few $CD3^+CD4^-CD8^+$ cells also bear the TCRγ/δ (Scott et al, 1990; Groh et al, 1989; Kozbor et al, 1989; Borst et al,1988; Jitsukawa et al, 1987) and even fewer $CD3^+CD4^+CD8^-$ cells are also positive for TCRγ/δ (Jitsukawa et al, 1987; Borst et al, 1988; Groh et al, 1989). The presence of the TCRα/β^+ cells and TCRγ/δ^+ cells was extensively studied among the three major subsets of T cells: $CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$, and $CD3^+CD4^-CD8^-$. The samples were immunophenotyped using a two-colour staining technique. Anti-CD3, CD4, and CD8 were PE conjugated and anti-TCRα/β (TCR-1) and TCRγ/δ (anti-TCRδ1) were FITC conjugated. Each sample was lysed with a Q-Prep immunology workstation and acquired with a FACScan flow cytometer. Figure 7 represents a typical distribution of the TCRα/β^+ cells and the TCRγ/δ^+ cells in the major T cell subsets ($CD3^+CD4^+$ and $CD3^+CD8^+$). A more extensive analysis of the distribution of the TCR in the $CD3^+CD8^+$ T cell subset is given at page 50 to 54. Tables 7 and 8 report the proportion and absolute values of TCRα/β^+ and TCRγ/δ^+ cells found in noninfected individuals and HIV infected individuals. The majority of T lymphocytes were TCRα/β^+ cells: 64% of lymphocytes or 1,220 cells/mm^3 in noninfected individuals. At stages II and III of the disease these numbers increased to reach 76% and 78% or 1,394 and 1,322 cells/mm^3 respectively. At stage IV of the disease 68% of the lymphocytes or 621 cells/mm^3 were found to be TCRα/β^+ cells. Very few PBMNCs were TCRγ/δ^+ cells; in noninfected individuals only 5% of lymphocytes or 100 cells/mm^3 bear the TCRγ/δ marker. At stages II, III and IV of the disease, the percentage of TCRγ/δ^+ lymphocytes decreased to 4% or 81 cells/mm^3, 72 cells/mm^3, and 38 cells/mm^3 respectively (Table 8).

III.4.1 Correlation between the TCRγ/δ subset and $CD4^+$ lymphocytes

Since $CD4^+$ lymphocyte counts are used as a reference for the progression of AIDS, my findings were correlated to the CD4 counts to evaluate their significance in

Figure 7

Distribution of TCR markers in major T cell subsets

This Figure illustrates the typical distribution of TCR subsets in the CD3, CD4 and CD8 T lymphocyte subsets in a sample from a non infected individual. The samples were tested as described in the Methods section and the TCR subsets were differentiated using mAbs as outlined in Table 2. Eighty-four samples were tested (n=28, normal; n=14, stage II of HIV infection; n=25, stage III of HIV infection; n=17, stage IV of HIV infection).

a) A large subset of $TCR\alpha/\beta^{+}CD4^{+}$ cells is observed while
b) no $TCR\gamma/\delta^{+}CD4^{+}$ were detected.
c) Some CD8 cells are $TCR\alpha/\beta^{+}$ while
d) fewer are $TCR\gamma/\delta^{+}CD8^{+}$.

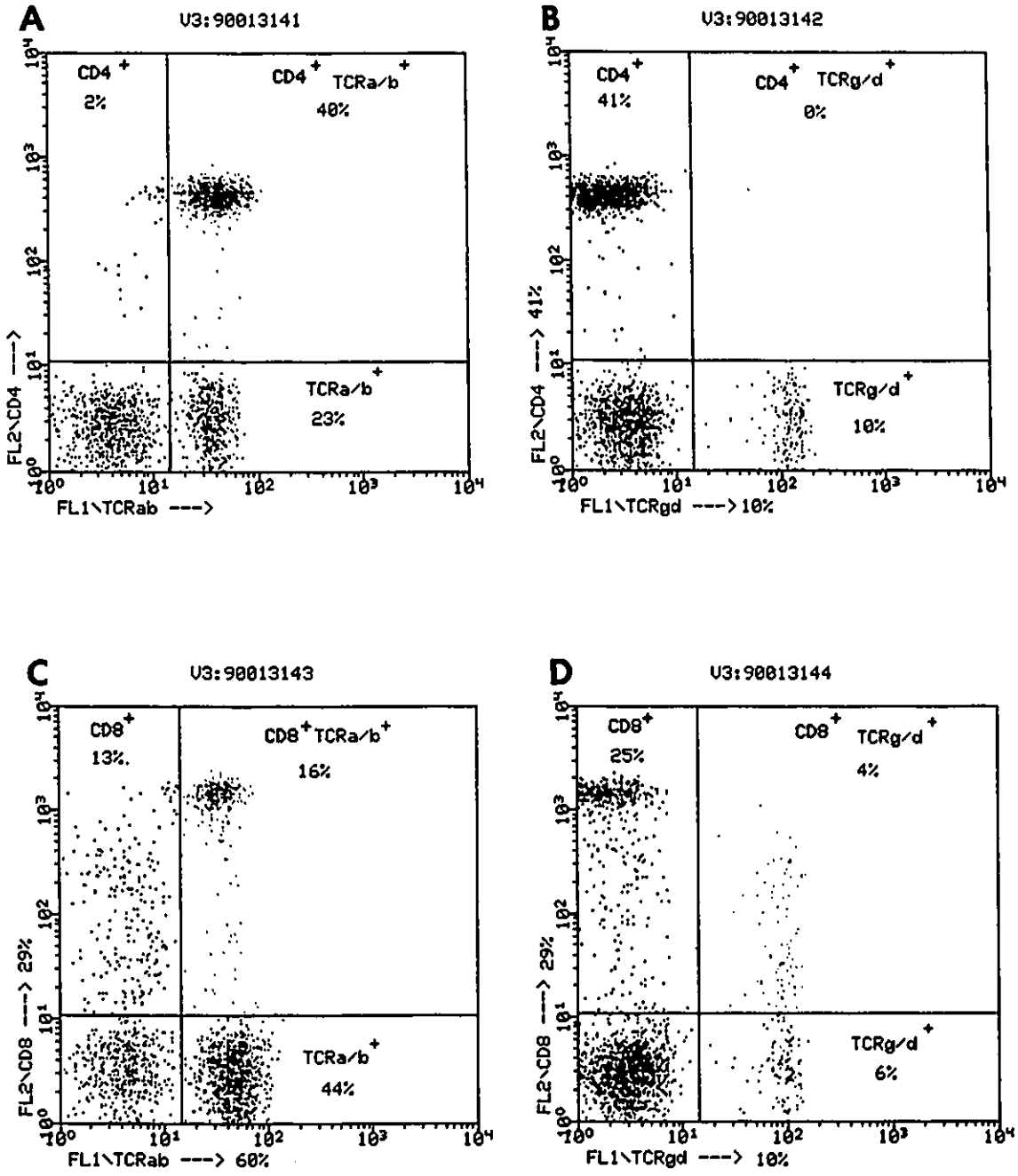

A
V3:90013141
CD4+ 2%
CD4+ TCRa/b+ 40%
TCRa/b+ 23%
FL2\CD4 --->
FL1\TCRab --->
B
V3:90013142
CD4+ 41%
CD4+ TCRg/d+ 0%
TCRg/d+ 10%
FL2\CD4 ---> 41%
FL1\TCRgd ---> 10%
C
V3:90013143
CD8+ 13%
CD8+TCRa/b+ 16%
TCRa/b+ 44%
FL2\CD8 ---> 29%
FL1\TCRab ---> 60%
D
V3:90013144
CD8+ 25%
CD8+ TCRg/d+ 4%
TCRg/d+ 6%
FL2\CD8 ---> 29%
FL1\TCRgd ---> 10%

Table 7

Cell surface phenotyping of TCRα/β subsets

The TCRα/β subset was tested as outlined in the Methods section. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III and n=17, stage IV of the disease).

T CELL SUBSET: TCRα/β	n	RANGE	MEAN	S.E.
relative*		46-75	64	2
normal	28			
absolute**		735-2,025	1,220	57
relative		60-89	76	2
stage II	14			
absolute		840-2,106	1,394	96
relative		46-90	74	2
stage III	25			
absolute		152-2,368	1,322	126
relative		43-84	68	4
stage IV	17			
absolute		150-1,800	621	200

*relative = % of positive cells over all lymphocytes

**absolute = cells/mm^3

Table 8

Cell surface phenotyping of TCRγ/δ subsets

The TCRγ/δ subset was tested as outlined in the Methods section. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III and n=17, stage IV of the disease).

T CELL SUBSET: TCRγ/δ	n	RANGE	MEAN	S.E.
relative*		1-13	5	1
normal	28			
absolute**		22- 264	100	12
relative		1-8	4	1
stage II	14			
absolute		19- 147	81	11
relative		1-12	4	1
stage III	25			
absolute		0- 224	72	12
relative		1-10	4	1
stage IV	17			
absolute		6- 150	38	14

*relative = % of positive cells over all lymphocytes

**absolute = cells/mm^3

the progression of the disease. When the relationship between the TCRγ/δ and CD4 subsets was examined I found that no particular relationship existed between the relative numbers of TCRγ/δ$^+$ cells and CD4$^+$ cells in normal and in HIV seropositive samples (r= 0.2 and r=0.16 respectively) (Figure 8a, b). No correlation was found between the TCRγ/δ subset and the CD4 subset whether the patients were treated with drugs such as zidovudine (AZT) or not (Figure 8c,d).

III.4.2 The TCR marker in the CD8 subset

Although, in early studies, TCRγ/δ$^+$ cells were identified according to negative criteria (e.g. CD3$^+$CD4$^-$CD8$^-$) (Lanier et al, 1986d; Brenner et al, 1986; Borst et al, 1986), more recent data have shown that a subset of TCRγ/δ$^+$ lymphocytes ranging from 1 to 70% of the TCRγ/δ$^+$ cells were CD3$^+$CD8$^+$ cells (Scott et al, 1990; Groh et al, 1989, Borst et al, 1988). Thus, I investigated the presence of the CD8 antigen at the surface of the TCR subsets both in noninfected and in HIV infected individuals. Attempts to correlate TCR antigens with T cell subsets revealed that all TCRγ/δ$^+$ cells were CD3$^+$, some were also CD8$^+$ but none were found to be CD4$^+$ (Figure 7). In blood samples from noninfected individuals, 65% of the CD8 lymphocyte subset were TCRα/β$^+$. These CD8$^+$TCRα/β$^+$ cells represented 19.5 ± 5.7% (37.1 X 10^4 ± 12.6 cells/ml) of the total lymphocytes in normal peripheral blood. Only 7.1% of the CD8$^+$ lymphocytes which represents 2% of the total lymphocyte population or 4.1 X 10^4 ± 3.2 cells/ml, in normal peripheral blood samples, were found to be also positive for TCRγ/δ.

At stage II of the disease, the proportion of lymphocytes simultaneously bearing the CD8 and the TCRα/β markers increased to 52 ± 3% (947 cells/mm^3) while 89% of the CD8$^+$ cells were TCRα/β$^+$. At stage III and at stage IV these numbers remained similar: 81.6% of the CD8$^+$ cells were TCRα/β$^+$ (51% of the lymphocytes were CD8$^+$TCRα/β$^+$ cells or 877 cells/mm^3) and 89% of the CD8$^+$ cells were TCRα/β$^+$

Figure 8

Correlation between the CD4 and TCRγ/δ T cell subsets in noninfected and HIV infected individuals

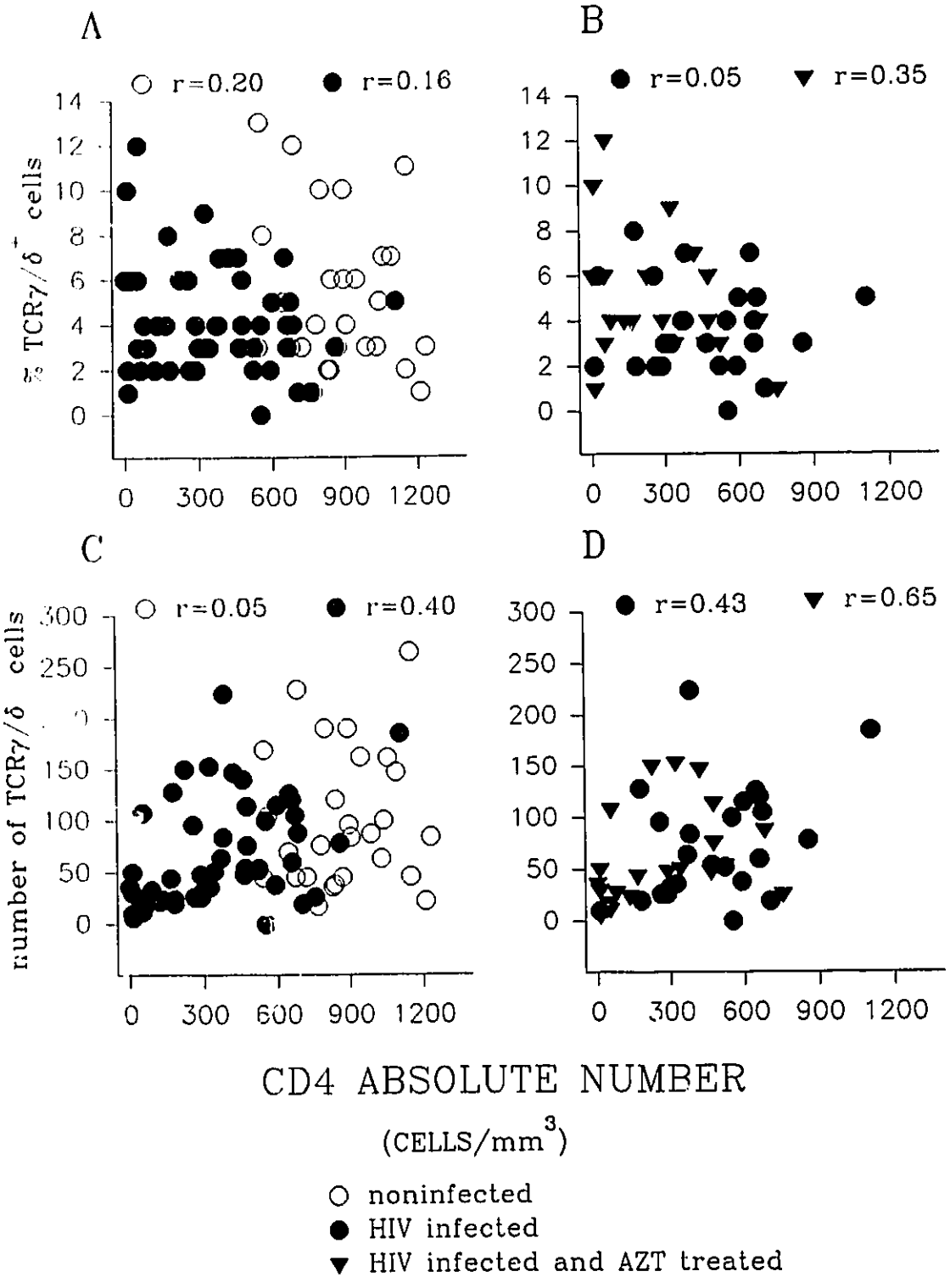
Experiment was performed as described in the Methods section. The $CD4^{+}$ cells were stained using anti-CD4-PE mAb in combination with anti-TCRα/β-, anti-TCRγ/δ- and anti-CD3-FITC. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III; and n=17, stage IV of the disease).

a) correlation between absolute counts of $CD4^{+}$ cells and relative numbers of $TCR\gamma/\delta^{+}$ T cells in samples from noninfected and HIV infected individuals.

b) correlation between absolute counts of $CD4^{+}$ cells and absolute counts of $TCR\gamma/\delta^{+}$ T cells in noninfected and HIV infected individuals.

c) correlation between absolute counts of $CD4^{+}$ cells and relative numbers of $TCR\gamma/\delta^{+}$ T cells in samples from HIV infected individuals treated with zidovudine and non treated with zidovudine.

d) correlation between absolute counts of $CD4^{+}$ cells and absolute counts of $TCR\gamma/\delta^{+}$ T cells in HIV infected individuals treated with zidovudine and non-treated with zidovudine.


A
B
C
D
r=0.20
r=0.16
r=0.05
r=0.35
r=0.05
r=0.40
r=0.43
r=0.65
% TCRγ/δ+ cells
number of TCRγ/δ+ cells
0 300 600 900 1200
CD4 ABSOLUTE NUMBER
(CELLS/mm3)
noninfected
HIV infected
HIV infected and AZT treated

(57% of the lymphocytes were $CD8^+TCR\alpha/\beta^+$ cells). However at stage IV, there was a clear decrease in absolute count: 506 cells/mm^3 (Table 9, 10).

The $TCR\gamma/\delta$ marker was found only on 2% of the $CD8^+$ lymphocytes or 23 ± 6 $CD8^+TCR\gamma/\delta^+$ cells/mm^3 in samples from patients at stage II of the disease. These counts did not vary much at stage III and IV of the disease: 3% of $CD8^+$ lymphocytes were $TCR\gamma/\delta^+$ cells or 28 ± 6 $CD8^+TCR\gamma/\delta^+$ cells/mm^3 and 3% of $CD8^+$ lymphocytes were $TCR\gamma/\delta^+$ cells or 16 ± 5 $CD8^+TCR\gamma/\delta^+$ cells/mm^3 respectively (Tables 9 and 10).

III.4.3 Mean Fluorescence Intensity (MFI) of $TCR\alpha/\beta^+$ and $TCR\gamma/\delta^+$ cells

To draw a more detailed phenotypic characterization of the samples analysed, the MFI of $TCR\alpha/\beta^+$ and γ/δ^+cells was determined using the TCR-1 and anti-TCRδ1 mAbs. The MFI is a semi-quantitative value that represents the brightness of a stained cell. When the cell appears bright on the fluorescence scale, it indicates a strongly expressed marker at the surface of the cell. Figure 9 illustrates the data analysis for MFI measurements. The MFI was measured in log values but converted to relative channel numbers (Appendix II). This transformation allowed comparison of data from this study with other studies. The side scatter parameter was plotted on the "y" axis while the fluorescence 1 ($TCR\gamma/\delta$ subset) was plotted on the "x" axis. This procedure allowed gating around all and only $TCR\gamma/\delta^+$ cells in one step and was called $TCR\gamma/\delta$ gating technique (Figures 9a, c, e). The fluorescence 1 histogram was drawn from the gated events (Figures 9b, d, f). The MFI was computed by the program Lysis.

Figure 10 shows the distribution of the MFI for $TCR\gamma/\delta$ molecules in noninfected and HIV infected individuals. No significant difference between the MFI of $TCR\alpha/\beta^+$ cells in noninfected and in HIV infected samples was found (not shown). However, the MFI of the $TCR\gamma/\delta$ receptor showed interesting changes. In noninfected individuals

Table 9

Presence of CD8 marker in TCRα/β subsets

Experiment was performed as described in the Methods section. The TCRα/β$^+$ cells were stained in combination with anti-CD8-PE mAb. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III and n=17, stage IV of the disease).

T CELL SUBSET: $CD8^+/TCR\alpha/\beta^+$	n	RANGE	MEAN	S.E.
relative*		9-33	20	1
normal	28			
absolute**		189-783	371	2
relative		29-73	52	3
stage II	14			
absolute		551-1,365	947	7
relative		35-76	51	4
stage III	25			
absolute		48-2,144	877	105
relative		34-78	57	3
stage IV	17			
absolute		102-1,750	506	120

*relative = % of positive cells over all lymphocytes

**absolute = cells/mm^3

Table 10

Presence of CD8 marker in TCRγ/δ subsets

The TCRγ/δ$^+$ cells were tested as in Table 9. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III and n=17, stage IV of the disease).

T CELL SUBSET: $CD8^+TCR\gamma/\delta^+$	n	RANGE	MEAN	S.E.
relative*		0-6	2	0.3
normal	28			
absolute**		0- 136	41	6
relative		0-3	2	0.3
stage II	14			
absolute		0-48	23	6
relative		0-9	3	1
stage III	25			
absolute		0-96	28	6
relative		0-4	3	0.3
stage IV	17			
absolute		0-75	16	5

*relative = % of positive cells over all lymphocytes

**absolute = cells/mm^3

Figure 9

Mean Fluorescence Intensity of TCRγ/δ$^+$ cells in noninfected and HIV infected individuals

The TCR subset was tested as outlined in the Methods section. The MFI was obtained using TCR gating and histogram statistics. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease).
a) and b) illustrate a typical example obtained from a noninfected sample. The side scatter versus fluorescence 1 plot was used to gate TCRγ/δ$^+$ cells and the histogram analysis of the TCRγ/δ$^+$ population allowed comparison between noninfected and infected samples.
c) and d) illustrate a sample obtained from a HIV infected patient at stage III of the disease.
e) and f) iluustrate a sample obtained from a HIV infected patient at stage IV of the disease.

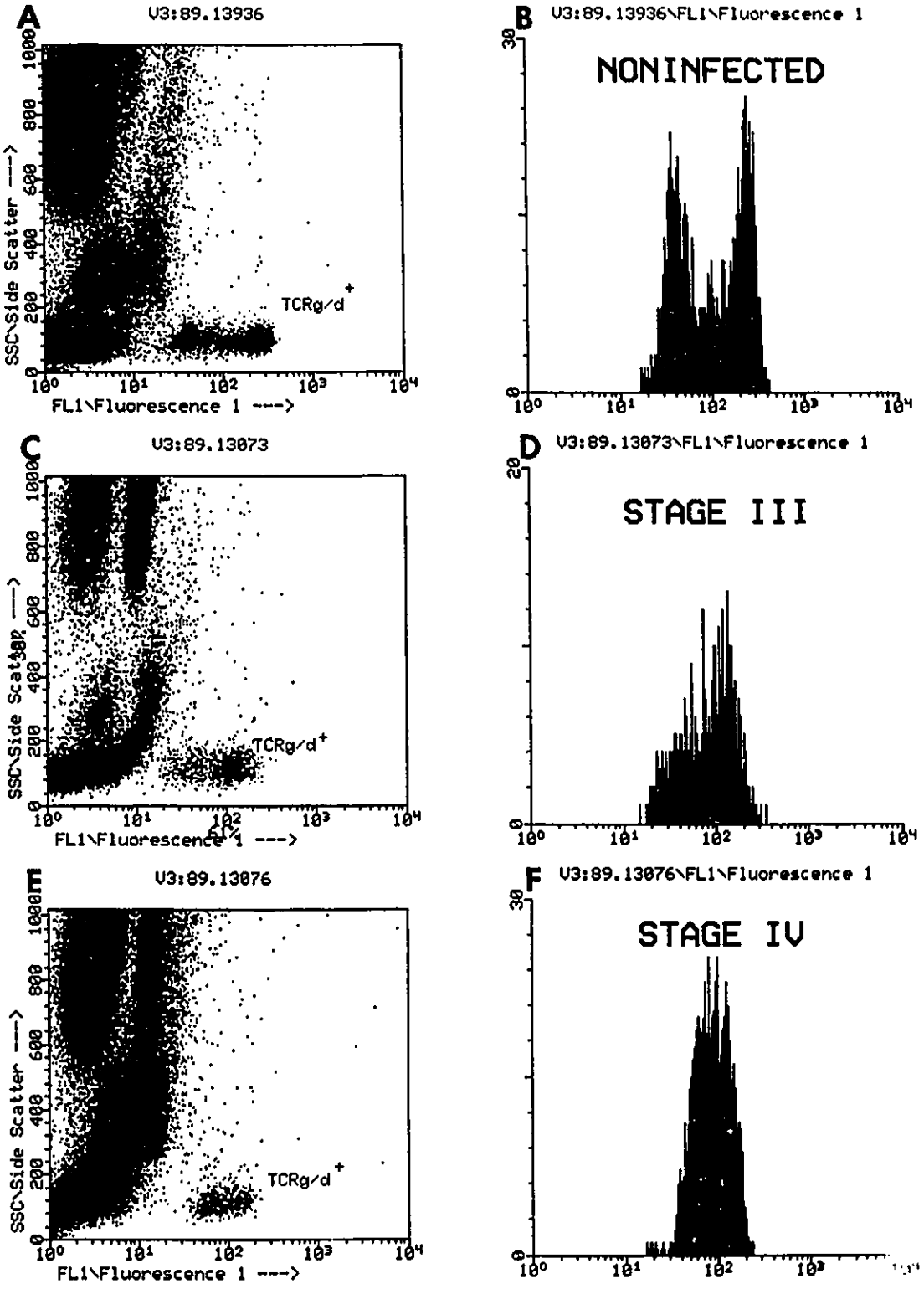
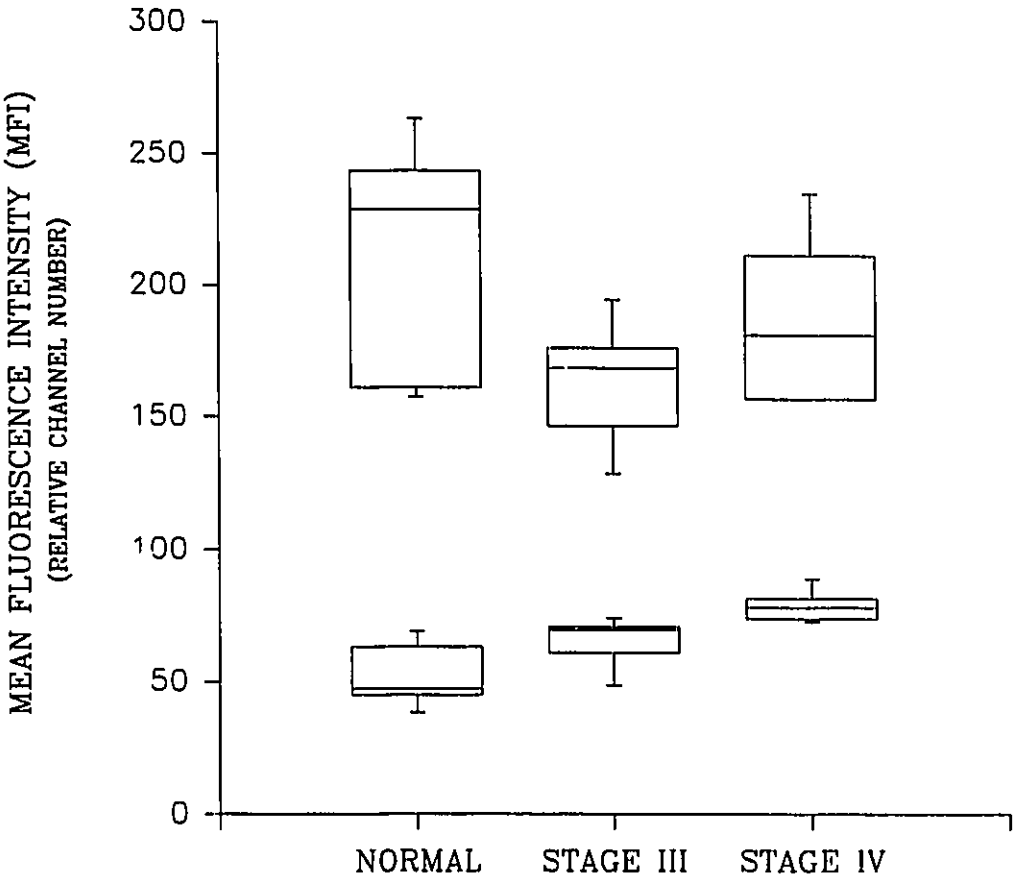

A
V3:89.13936
SSC\Side Scatter --->
FL1\Fluorescence 1 --->
TCRg/d+
B
V3:89.13936\FL1\Fluorescence 1
NONINFECTED
C
V3:89.13073
SSC\Side Scatter --->
FL1\Fluorescence 1 --->
TCRg/d+
D
V3:89.13073\FL1\Fluorescence 1
STAGE III
E
V3:89.13076
SSC\Side Scatter --->
FL1\Fluorescence 1 --->
TCRg/d+
F
V3:89.13076\FL1\Fluorescence 1
STAGE IV

Figure 10

Distribution of MFI of TCRγ/δ^{+} cells in noninfected and HIV infected individuals

The TCRγ/δ subset was tested as outlined in the Methods section. It was stained in combination with different activation markers (CD25, HLA-DR, CD38, CD57 and CD5). Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The samples were analyzed as in Figure 9.
The boxes are divided into two parts by a line representing the median value. The bars in the upper and lower part of each box represent the standard deviation.


MEAN FLUORESCENCE INTENSITY (MFI)
(RELATIVE CHANNEL NUMBER)
300
250
200
150
100
50
0
NORMAL
STAGE III
STAGE IV

there were two levels of fluorescence for this marker: one peak ranged from 38 to 75 and another from 155 to 279 (relative channel number) in noninfected samples. In samples from HIV infected individuals these two peaks of fluorescence intensity seemed to draw closer. At stage III of the disease, one peak of fluorescence ranged from 49 to 74 and the other from 129 to 195. And at stage IV, the lowest relative mean fluorescence was from channel 73 to 89 and the highest from channel 157 to 235; on a log scale, such difference between the two peaks of fluorescence is hardly visible. Furthermore, the narrowing of the gap between the two populations of cells appeared to be the result of a concomitant decrease of cells showing very low fluorescence intensity, a decrease in cells showing a very high level of fluorescence intensity and an increase of cells showing medium fluorescence intensity levels (Figures 9 and 10).

The MFI of the TCR markers expressed at the surface of $TCR\alpha/\beta^+$ cells and $TCR\gamma/\delta^+$ cells were obtained analyzing ten samples from HIV infected individuals. Two of these samples did not show clear limits between low and high density of $TCR\gamma/\delta$ molecule but showed an irregularly shaped histogram. In these cases the markers that separated the two levels of fluorescence were drawn to separate the histogram in two equal parts (Figure 9).

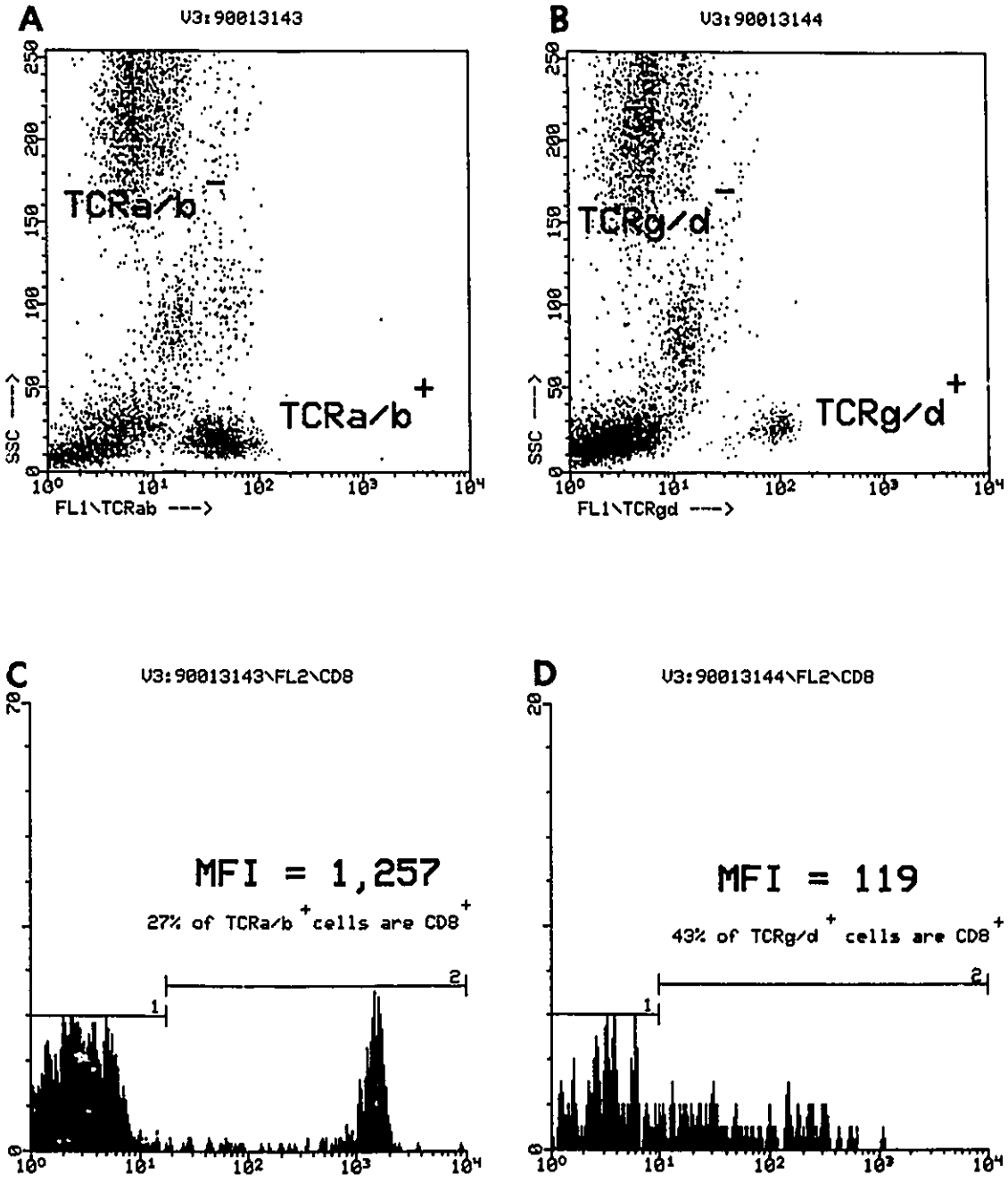
As previously mentioned, some of the $TCR\gamma/\delta^+$ cells are also $CD8^+$ cells. The MFI for the CD8 marker at the surface of the two TCR subsets was also analysed. At the surface of the $TCR\alpha/\beta^+CD8^+$ cells, the CD8 marker was bright and peaked at channel 5 ± 1 (relative channel number). At the surface of the $TCR\gamma/\delta^+CD8^+$ cells the CD8 marker was dimmer with a MFI of 3 ± 1 (relative channel number), in samples from noninfected and HIV infected individuals (Figure 11).

Figure 11

Fluorescence intensity of $CD8^+$ cells in TCRα/β and TCRγ/δ subsets

Experiment was performed as outlined in the Methods section, both TCR subsets were tested for the frequency of the CD8 marker. Eighty-four samples were tested (n=28, normal; n=14, stage II; n=25, stage III and n=17, stage IV of the disease). The samples were analysed as in Figure 9. The MFI of the CD8 marker is clearly higher in the TCRα/β subset than in the TCRγ/δ subset.

a) Side scatter versus fluorescence 1 dot plot allowed gating around TCRα/β cells
b) or around TCRγ/δ cells.
c) Using the histogram, the MFI is computed for the CD8 marker within the TCRα/β subset
d) or within the TCRγ/δ subset.


A
V3:90013143
TCRa/b⁻
TCRa/b⁺
SSC --->
FL1\TCRab --->
B
V3:90013144
TCRg/d⁻
TCRg/d⁺
SSC --->
FL1\TCRgd --->
C
V3:90013143\FL2\CD8
MFI = 1,257
27% of TCRa/b⁺ cells are CD8⁺
D
V3:90013144\FL2\CD8
MFI = 119
43% of TCRg/d⁺ cells are CD8⁺

III.5 Activation markers in the TCRα/β and TCRγ/δ subsets

Products of immune activation, such as serum levels of α-interferon, soluble CD8 (sCD8) and soluble interleukin-2 receptor (sIL-2R), have been associated with HIV disease progression (Lang et al, 1989; Fernandez-Cruz et al, 1990). Engagement of the T cell receptor represents the prime signal for T cell activation. Thus, I investigated the possible correlation between activation markers found at the surface of TCRα/β$^+$ and TCRγ/δ$^+$ cells and the progression of the disease. The TCRα/β and the TCRγ/δ subsets were immunophenotyped using the TCR-1 and anti-TCRδ1 mAbs in combination with different anti-activation marker mAbs: HLA-DR, CD25, CD38, CD57 and CD5. HLA-DR is a major histocompatibility complex component which is produced when the cell is activated. The CD25 marker is also known as the Tac antigen or IL-2 receptor. Expression of the CD25 marker can be induced by exposure of T lymphocytes to ligands that cross-link antigen-specific receptors on the cell surface. This marker is produced in large amount at the surface of activated cells (Hemler et al, 1984; Cantrell et al, 1983; Meuer et al, 1984). The CD38 molecule is a key marker in lymphocyte regulation; it appears at the surface of T cells in both early and late stages of maturation but not in intermediate stages (Jackson and Bell, 1990). The CD57, a cell adhesion molecule, has been associated with non-antigen-specific inhibitory activity on a broad spectrum of lymphocyte functions (Sadat-Sowti et al, 1991).

In samples from noninfected individuals, CD38 was found on 44 ± 5% of TCRα/β$^+$ cells (Table 11 and Figure 12) while the other activation markers studied, HLA-DR, CD25, and CD57 were found on less than 20% of the TCRα/β$^+$ cells (Tables 12, 13, 14 and Figure 12). In contrast, in samples from HIV infected individuals, the activation markers at the surface of TCRα/β$^+$ cells increased drastically except for the CD25 marker (Tables 11-14, Figure 12). HLA-DR increased from 14% to 53% at stages III and IV of the disease, CD38 marker increased from 44% to 73% and 81% at stage III

Table 11

HLA-DR expression on TCRα/β T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRα/β$^+$ cells were stained with TCR-1-FITC and anti-HLA-DR-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values were obtained using the Facscan Research program and were computed as follows:

Absolute count of HLA-DR$^+$TCRα/β$^+$ X 100 / Absolute count of TCRα/β$^+$ cells

T CELL SUBSET: HLA-DR	n	RANGE	MEAN	S.E.
relative*		5-34	14	3
normal	10			
absolute**		60-359	178	32
relative		30-70	53	7
stage III	5			
absolute		296-725	568	9
relative		40-73	53	6
stage IV	5			
absolute		112-798	358	119

*relative = % of HLA-DR$^+$ cells among TCRα/β^+ cells

**absolute = cells/mm^3

Table 12

CD25 expression on TCRα/β T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRα/β$^+$ cells were stained with TCR-1-FITC and anti-CD25-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: CD25	n	RANGE	MEAN	S.E.
relative*		2-18	8	2
normal	10			
absolute**		41-228	97	20
relative		1-10	5	1
stage III	5			
absolute		19-71	49	10
relative		1-11	4	2
stage IV	5			
absolute		4-39	20	7

*relative = % of $CD25^+$ cells among $TCR\alpha/\beta^+$ cells

**absolute = cells/mm^3

Table 13

CD38 expression on TCRα/β T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRα/β$^+$ cells were stained with TCR-1-FITC and anti-CD38-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: CD38	n	RANGE	MEAN	S.E.
relative*		19-68	44	5
normal	10			
absolute**		172-997	605	84
relative		33-91	73	11
stage III	5			
absolute		320-1, 177	790	146
relative		46-97	81	9
stage IV	5			
absolute		199-1, 422	562	228

*relative = % of $CD38^+$ cells among $TCR\alpha/\beta^+$ cells

**absolute = cells/mm^3

Table 14

CD57 expression on TCRα/β T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRα/β$^+$ cells were stained with TCR-1-FITC and anti-CD57-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: CD57	n	RANGE	MEAN	S.E.
relative*		3-18	6	1
normal	10			
absolute**		33-160	87	23
relative		44-50	47	3
stage III	5			
absolute		504-537	520	16
relative		29-62	62	29
stage IV	5			
absolute		60-526	257	100

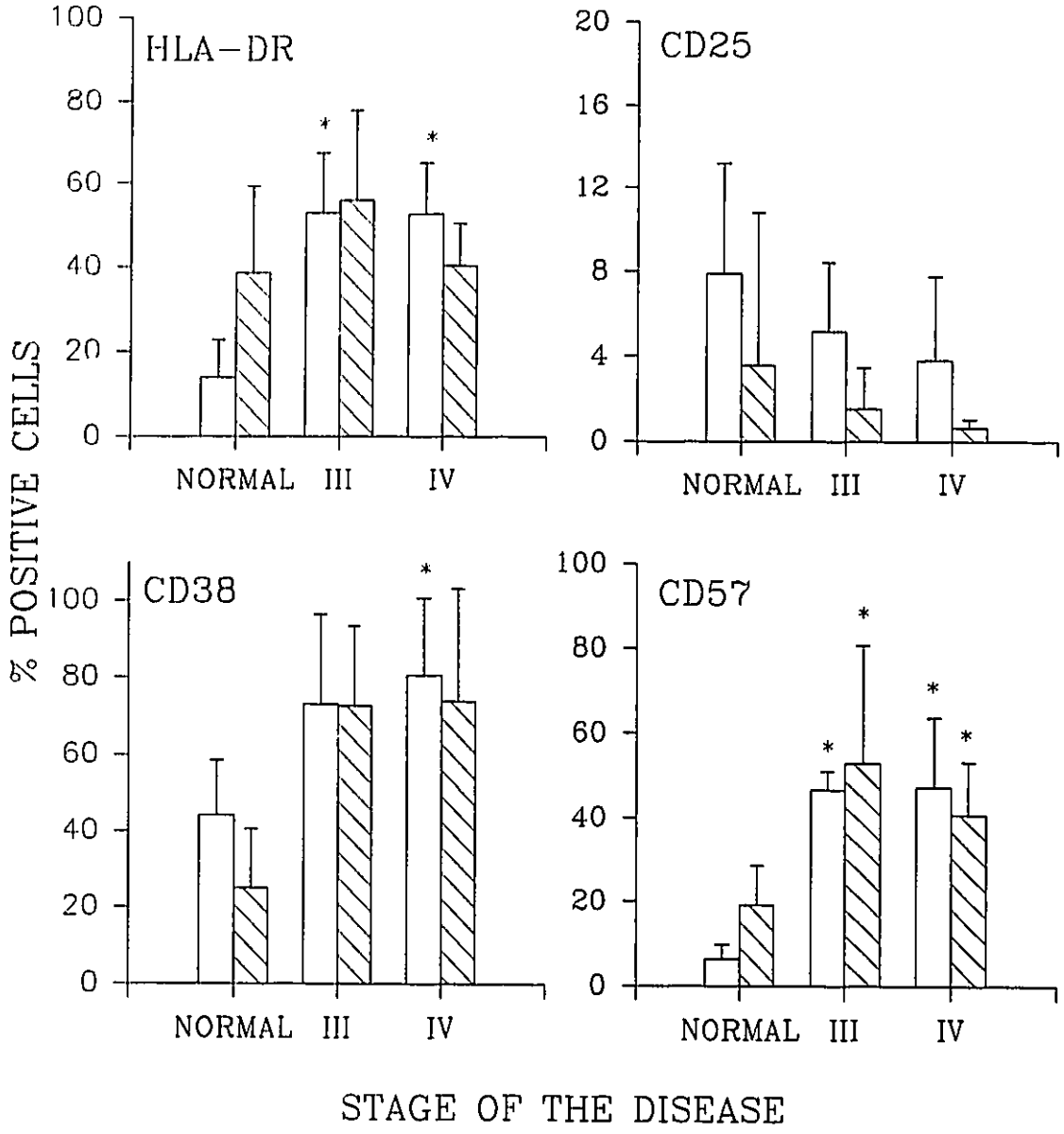
*relative = % of $CD57^+$ cells among $TCR\alpha/\beta^+$ cells

**absolute = cells/mm^3

Figure 12

Relative counts of activated T cell subsets in noninfected and HIV infected individuals

This figure illustrates the changes observed in activated TCRα/β and TCRγ/δ subsets when samples from noninfected and infected individuals were compared. Experiment was performed as outlined in the Methods section. A series of activation markers were tested for each TCR subset, as described in Table 2. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.


HLA-DR
CD25
CD38
CD57
% POSITIVE CELLS
NORMAL
III
IV
STAGE OF THE DISEASE
TCRα/β+ T CELLS
TCRγ/δ+ T CELLS
n = 10 normals
n = 5 stage III
n = 5 stage IV
* P<0.05

and stage IV of the disease respectively, CD57 marker increased from 6% to 47% at stage III and to 62% at stage IV of the disease.

In samples from noninfected individuals, the most commonly seen activation marker on TCRγ/δ$^+$ cells was HLA-DR: 39% of the TCRγ/δ$^+$ cells were positive for HLA-DR (Table 15, Figure 12). CD25, was present at the surface of very few TCRγ/δ$^+$ cells in samples from noninfected individuals, 4%, the least seen marker (Table 16, Figure 12). CD38 appeared on 25% of the TCRγ/δ$^+$ cells while 19% of the TCRγ/δ$^+$ cells were positive for CD57 in samples from non infected individuals (Tables 17 and 18, Figure 12).

In samples from HIV infected individuals, HLA-DR was present on 56% of TCRγ/δ$^+$ cells at stage III and 40% at stage IV (Table 15, Figure 12). The CD25 marker decreased to 2% and 1% at stage III and stage IV of the disease respectively (Table 16, Figure 12). The activation marker most commonly found on TCRγ/δ$^+$ cells was CD38: 73% and 74% at stages III and IV of the disease respectively (Table 17, Figure 12). CD57 was increased significantly ($p<0.01$) from 19% of the TCRγ/δ$^+$ cells to 53% at stage III and to 41% at stage IV of the disease (Table 18, Figure 12).

III.6 CD5 marker on TCRα/β$^+$ and TCRγ/δ$^+$ cells

Since some activation markers appeared affected by the progression of the disease I decided to investigate a surface markers that transduce antigenic signal to result in T cell activation, theCD3 molecule and a surface marker that enhance activation of the T cells, the CD5 molecule. The CD3 surface marker was included in the first part of the study (Tables 3, 4, 5, and 6). The results in Table 19 and Figure 13 show that in HIV infection the presence of CD5 was very stable; 99%, 99% and 97% of the TCRα/β$^+$ cells in noninfected individuals, stage III, and stage IV of the disease respectively were CD5$^+$ cells. Interestingly, in the TCRγ/δ subset, a significant decrease ($p<0.01$) of CD5$^+$ cells

Table 15

HLA-DR expression on TCRγ/δ T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRγ/δ$^+$ cells were stained with TCR-δ-1-FITC and anti-HLA-DR-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: HLA-DR	n	RANGE	MEAN	S.E.
relative*		7-74	39	7
normal	10			
absolute**		12-99	32	8
relative		21-79	56	10
stage III	5			
absolute		21-44	32	5
relative		28-51	40	5
stage IV	5			
absolute		12-40	23	6

*relative = % of HLA-DR$^+$ cells among TCRγ/δ^+ cells

**absolute = cells/mm^3

Table 16

CD25 expression on TCRγ/δ T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRγ/δ$^+$ cells were stained with TCR-δ-1-FITC and anti-CD25-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: CD25	n	RANGE	MEAN	S.E.
relative*		0-24	4	2
normal	10			
absolute**		0-13	2	1
relative		4-8	2	1
stage III	5			
absolute		0-21	5	4
relative		0-1	1	0
stage IV	5			
absolute		0-1	0	0

*relative = % of $CD25^+$ cells among $TCR\gamma/\delta^+$ cells

**absolute = cells/mm^3

Table 17

CD38 expression on TCRγ/δ T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRγ/δ$^+$ cells were stained with TCR-δ-1-FITC and anti-CD38-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: CD38	n	RANGE	MEAN	S.E.
relative*		5-51	25	5
normal	10			
absolute**		6-48	20	4
relative		38-90	73	9
stage III	5			
absolute		21-60	43	7
relative		30-100	74	13
stage IV	5			
absolute		16-83	46	14

*relative = % of $CD38^+$ cells among $TCR\gamma/\delta^+$ cells

**absolute = $cells/mm^3$

Table 18

CD57 expression on TCRγ/δ T cell subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRγ/δ$^+$ cells were stained with TCR-δ-1-FITC and anti-CD57-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values obtained using the Facscan Research program were computed as in Table 11.

T CELL SUBSET: CD57	n	RANGE	MEAN	S.E.
relative*		8-35	19	4
normal	10			
absolute**		3-45	18	6
relative		33-72	53	20
stage III	5			
absolute		17-144	81	64
relative		34-59	41	6
stage IV	5			
absolute		1-31	21	5

*relative = % of $CD57^+$ cells among $TCR\gamma/\delta^+$ cells

**absolute = cells/mm^3

Table 19

Cell surface phenotyping of TCRα/β$^+$CD5$^+$ subset in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRα/β$^+$ cells were stained with TCR-1-FITC and anti-CD5-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values were computed as follows:

Absolute count of leukocytes X % lymphocytes X % TCRα/β$^+$CD5$^+$ cells

T CELL SUBSET: CD5	n	RANGE	MEAN	S.E.
relative*		99-100	99	0
normal	10			
absolute**		902-2, 036	1, 316	116
relative		97-100	99	1
stage III	5			
absolute		729-1, 346	1, 056	114
relative		95-98	97	1
stage IV	5			
absolute		260-1, 543	663	234

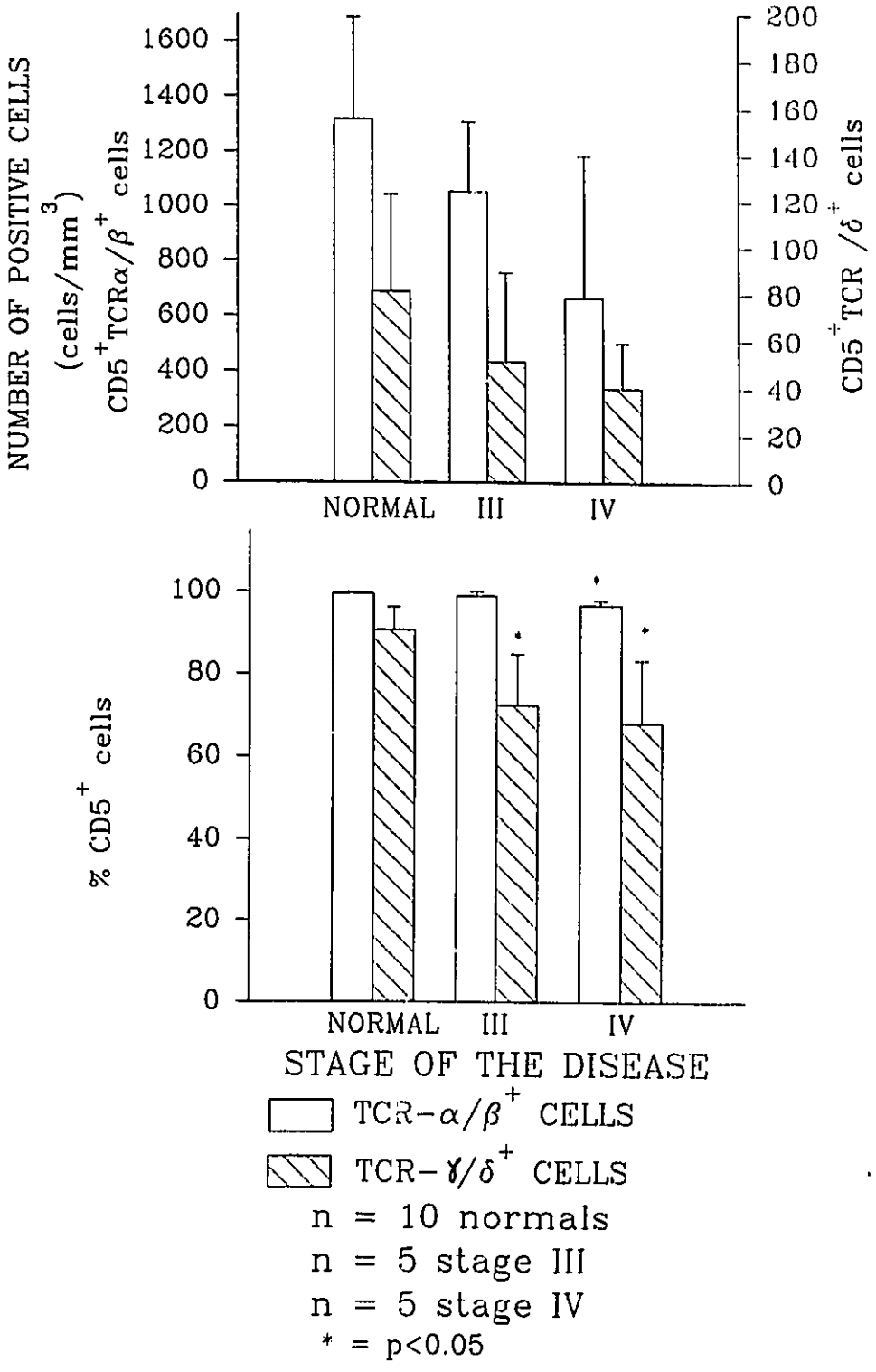
*relative = % of $CD5^+$ cells among $TCR\alpha/\beta^+$ cells

**absolute = cells/mm^3

Figure 13

Absolute and relative counts of TCRα/β^+CD5^+ and TCRγ/δ^+CD5^+ subsets in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, both TCR subsets were stained with anti-CD5-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values were computed as in Table 19.


NUMBER OF POSITIVE CELLS
(cells/mm3)
CD5+TCRα/β+ cells
CD5+TCR /δ+ cells
1600
1400
1200
1000
800
600
400
200
0
200
180
160
140
120
100
80
60
40
20
0
NORMAL
III
IV
% CD5+ cells
100
80
60
40
20
0
NORMAL
III
IV
STAGE OF THE DISEASE
TCR-α/β+ CELLS
TCR-γ/δ+ CELLS
n = 10 normals
n = 5 stage III
n = 5 stage IV
* = p<0.05

was observed: 91% of the TCRγ/δ^+ cells in noninfected individuals were positive for CD5, 72% at stage III of the disease and 68% at stage IV (Table 20 and Figure 13).

Table 20

Cell surface phenotyping of TCRγ/δ$^+$CD5$^+$ subset in noninfected and HIV infected individuals

Experiment was performed as outlined in the Methods section. As described in Table 2, the TCRγ/δ$^+$ cells were stained with TCR-δ-1-FITC and anti-CD5-PE mAbs. Twenty samples were tested (n=10, normal; n=5, stage III; n=5, stage IV of the disease). The percentage values were computed as in Table 19.

T CELL SUBSET: CD5	n	RANGE	MEAN	S.E.
relative*		81-98	91	2
normal	10			
absolute**		34-154	81	13
relative		57-83	72	6
stage III	5			
absolute		25-117	51	17
relative		51-90	68	7
stage IV	5			
absolute		19-58	40	8

*relative = % of $CD5^+$ cells among $TCR\gamma/\delta^+$ cells

**absolute = $cells/mm^3$

IV DISCUSSION

IV.1 Quality control assessment of flow cytometric analysis

The cell surface marker profile of white cells in the peripheral blood, such as the various functional subsets of lymphocytes, reflects the immune status of an individual (Giorgi, 1986; Ault, 1983; Coon et al, 1987; Lovett et al, 1984; Hudson et al, 1985). In the present study, I investigated the effect of HIV on the distribution of TCRα/β^+ and TCRγ/δ^+ cells. The cell surface marker analysis was done using immunophenotyping and flow cytometry technology. Because of the complexity of this technology, it may be helpful to discuss the experimental parameters and variables which need to be monitored for meaningful and reproducible data.

As quality control is a critical aspect with this technology, it was very important to make sure that every criterion for reliable and reproducible data was met. Once reliable and reproducible data were assured, the objective of this study could be achieved.

The electronic and optical machinery of the flow cytometer contributes to the variability in how the instrument sees and interprets a particular sample. To ensure good, reliable, and reproducible data, appropriate standards and good controls must be utilized and become a routine protocol. The AIDS Clinical Trial Group (ACTG) Immunology Committee established a subcommittee for the standardization of flow cytometry performance and set up a quality control program which led to several recommendations as to how to collect, handle, and prepare a sample for immunophenotyping, as well as to standardize the use of the flow cytometer, antibody panel and data analysis (Paxton et al, 1989). Immunocheck fluorescent beads for optical alignment (Coulter Corp.) or equivalent should be run daily. The coefficient of variation (CV) and mean peak channel fluorescence of the standard particle should be

recorded. Log fluorescence rather than linear fluorescence should be used in analysis. A standard subtraction or compensation protocol should be established for dual analysis using FITC/PE bead standards and/or appropriate mutually exclusive dual antibody stained samples. A daily log must be maintained on the flow cytometer; standards are commercially available and their use assures the proper functioning of the flow cytometer. A good control is one that is similar to the sample to be analysed and will reveal whether the sample preparation has been done properly, if the result is reproducible and if it is what was expected.

It is important to know if what is being measured is real fluorescence or not. Each part of the optical component of the flow cytometer (laser, filters, lenses) and the electronic components (photomultiplier tubes (PMTs), amplifiers) can lead to poor signal discrimination and a baseline fluorescence signal which is known as instrument noise. To determine the noise level of the instrument, we use blank microbeads (part of the Quickcal Calibration System, see Materials and Methods). They are non-fluorescent plastic beads; the flow cytometer should record them as negative fluorescence. The flow cytometer detectors should be sensitive enough to detect the inherent fluorescence of the unstained cells and those non-specifically stained by isotypic control mAbs. Quantitatively, calibration plots can be obtained to evaluate the instrument's fluorescence threshold or sensitivity. Using the Quickcal beads, a calibration graph is obtained and the Molecules of Equivalent Soluble Fluorochrome (MESF) value can be measured to indicate the fluorescence threshold or the noise level of the instrument. Our instrument was calibrated daily using the Quickcal system and the sensitivity was in the range of 500 MESF which is well below autofluorescence and is considered excellent sensitivity. Present commercial flow cytometers have sensitivities of 1,000-3,000 MESF.

IV.1.1 Cell staining and acquisition of data

The procedure for staining whole blood samples from noninfected and HIV infected individuals was standardized with special emphasis on TCRγ/δ phenotypic analysis. A detailed protocol is included in the Methods. Because the TCRγ/δ$^+$ cells are present in very low proportion in peripheral blood (0-12%), the most critical aspect of the technique was the acquisition of the sample. To obtain useful statistics on the sub-population TCRγ/δ we had to acquire enough events to finally obtain at least 500 TCRγ/δ$^+$ events per sample. The program Simulset was used to acquire the data. With this program the Leucogate mixture of mAbs, which consists of anti-CD45/CD14 mAbs, was used. This mixture of mAbs allowed the setting of the lymphocyte analysis region to include all the lymphocytes. As shown in Figures 2 and 3 in the Results section, the gating can be automatically defined by the program or modified by the operator. The percentage of events in the selected lymphocyte region which are CD45^{bright}CD14$^-$ ranged from 95% to 100%. When this proportion was not obtained with automatic gating, the gate was changed manually to obtain the desired proportion. In three cases, even with manual gating, this proportion was lower than 95%. Based on the recommendation by the committee for the standardization of flow cytometry performance (ACTG), I have decided to report such a sample as "assay not reliable" and exclude it from the study. A sample presenting less than 95% of lymphocytes (CD45^{bright}CD14$^-$) in the lymphocyte gate would generate false data. This artefact would be due to an estimation of lymphocyte subpopulation desired over not only the lymphocytes but over the lymphocytes plus some undesired cells. The nucleated red cells (which are not sensitive to the lytic reagent) can cause such problems especially in patients on zidovudine therapy. Once the lymphocyte gating was satisfactory, the data were analysed to evaluate the proportion of each subset of cells using a two parameter

dot plot. Using this two parameter dot plot, the $CD8^+$ cells were then analysed to obtain mean fluorescence intensity through histogram analysis.

IV.2 Immunological profile

IV.2.1 CD3, CD4, and CD8 subsets in noninfected individuals

As expected I have found the pan T cell subset ($CD3^+$ subset) constituting the major sub-population of the peripheral lymphocytes ranging between 868 and 2, 072 cells/mm^3 or 57 to 82% of the lymphocyte population. The $CD3^+CD4^+$ cells were found to constitute the major sub-population of T cells with 540 to 1, 232 cells/mm^3 or 34 to 58% of the lymphocytes, while the $CD3^+CD8^+$ T cell subset was estimated at 270-952 cells/mm^3 or 14 to 47% of lymphocytes.

IV.2.2 CD3, CD4, and CD8 subsets in HIV infected individuals

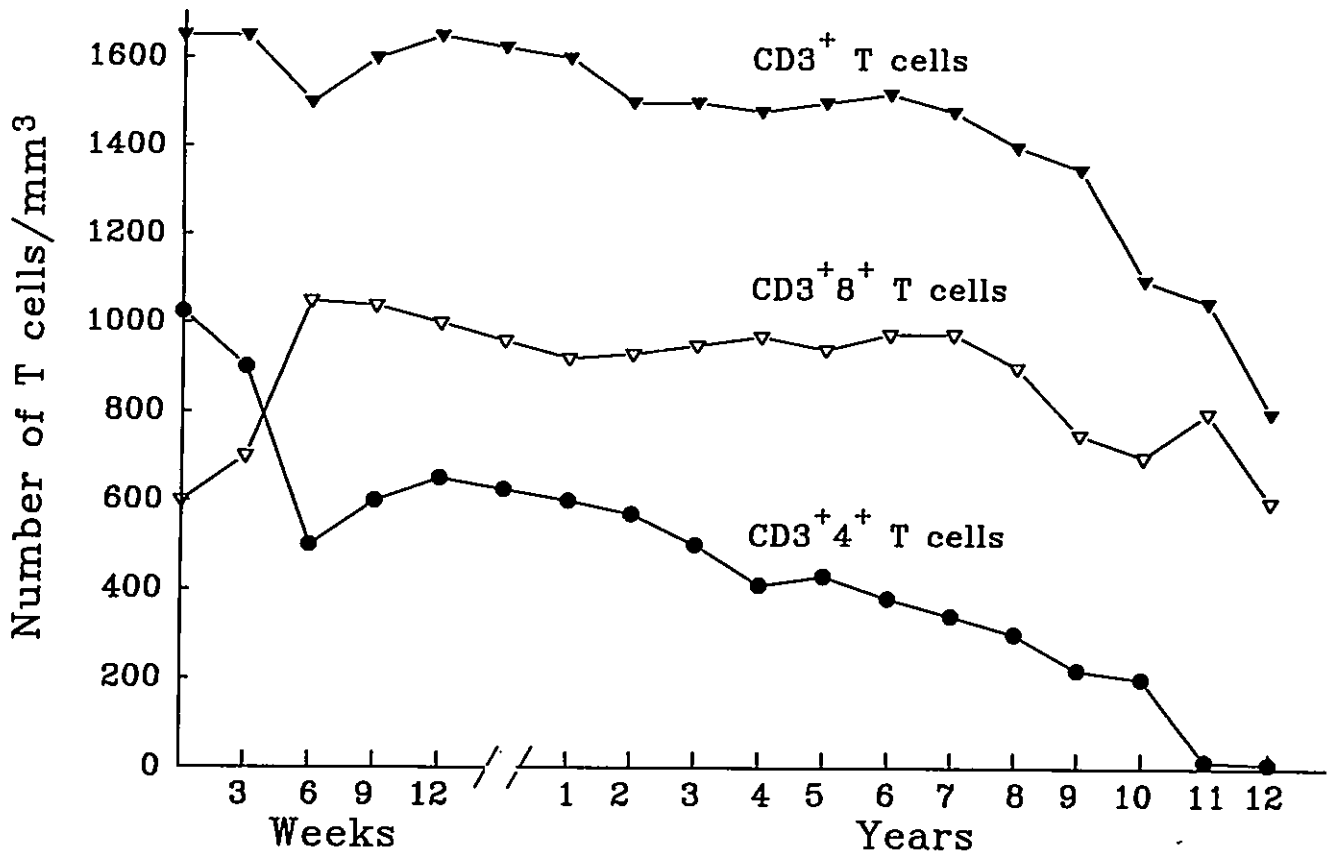
In HIV infected individuals, a consistently elevated proportion of pan T cells was observed. However at Stage IV, although the proportion of pan-T cells was still high, the absolute counts revealed an overall cytopenia. Such an observation suggests a correlation between the number of lymphocytes in circulation and the progression of the disease and is in agreement with existing literature (Landay et al, 1990). Figure 14 represent an idiogram of the variation of the major T cell subsets throughout the disease and agrees with my results.

More remarkably, the CD4 counts showed a significant decrease; counts as low as 8% of the lymphocytes or 85 cells/mm^3 were observed. It has been reported that all HIV-1 infected individuals have low CD4 counts and that $CD4^+$ cells are depleted as early as at seroconversion (Moss, 1990). The loss of $CD4^+$ lymphocytes is in fact important in the pathology of the disease. It has been reported that low or declining

Figure 14

Natural history of T cell subsets in HIV infection

This idiogram correlates with the results I obtained for the $CD3^+$ T cells, the $CD3^+CD4^+CD8^-$ and the $CD3^+CD4^-CD8^+$ subsets at different stages of HIV infection. The $CD3^+CD4^+CD8^-$ subset decreases as soon as at stage II and continue to drop while the $CD3^+CD4^-CD8^+$ subset increases at stage II and the total $CD3^+$ T cells remain stable until AIDS develops.


Number of T cells/mm3
1600
1400
1200
1000
800
600
400
200
0
CD3+ T cells
CD3+8+ T cells
CD3+4+ T cells
3
6
9
12
Weeks
1
2
3
4
5
6
7
8
9
10
11
12
Years

CD4 count is very clearly associated with deleterious effects of the disease. The lowest value of the CD4 count during therapy (rather than the change in the CD4 count during therapy) was shown to predict clinical outcome (Moss, 1990).

The $CD4^+$ cell loss during HIV disease occurs in four stages (Giorgi et al, 1987). I observed an important decrease at the onset of the disease from 45% to 25% or 870 cells/mm^3 to 466 cells/mm^3. Such a fast depletion at that stage of the disease has been reported extensively (Giorgi et al, 1987). There appears to be a correlation between the extent of the loss of CD4 and disease stage in HIV infection. During the second stage, the asymptomatic period, the CD4 levels have been reported to remain relatively stable or to show a small but steady fall (Lang et al, 1989). During the third stage, a further rapid $CD4^+$ cell loss leads to the development of frank AIDS. In the fourth stage, from AIDS diagnosis to death, $CD4^+$ cell levels fall even further. I have also observed an abrupt decline of the CD4 count at stage IV of the disease with CD4 counts of 90 cells/mm^3. The decline of the $CD4^+$ cells probably results at least in part from failure to suppress HIV replication *in vivo*. $CD4^+$ cell levels are highly prognostic for survival, especially during the last two stages; death occurs within 1 year in about 80% of untreated people whose level has fallen to 10% $CD4^+$ cells. $CD4^+$ cell percentages were once considered slightly more predictive for AIDS-free survival than $CD4^+$ cell absolute counts (Taylor et al, 1989). It is true that relative numbers of $CD4^+$ cells show less variability than absolute counts, but the latter is currently used by clinicians to evaluate the patient condition (Sanford et al, 1992). When the relative and absolute numbers of CD4 cells were correlated, no relationship was found in noninfected individuals ($r=0.41$) but a strong relationaship ($r=0.86$) was found in HIV infected individuals. In fact the absolute numbers of $CD4^+$ cells vary more in the noninfected group than in the HIV infected individuals. It may be that the absolute number is responsible for the lack of relationship between CD4 counts and percentages in noninfected individuals.

Before AIDS diagnosis, the total T-cell levels in HIV infected blood remained fairly constant in spite of the fall in the $CD4^+$ cell number (Table 3). This can be explained by the concomitant CD8 lymphocytosis (Landay et al, 1990). I have observed that the increase of CD8 cells is as important as the decrease of CD4 cells. Such observation has also been reported by Landay's group (Landay et al, 1990). This specific augmentation in the CD8 subset might result from an activation of the immune system by pathogens and will be discussed later.

IV.3 T cell receptors

The study of the T cell receptor for antigen was focused on three T cell subsets: a) $CD3^+CD4^+CD8^-$, b) $CD3^+CD4^-CD8^+$, c) $CD3^+CD4^-CD8^-$. Attempts to correlate TCR antigens with T cell subsets revealed that all $TCR\alpha/\beta^+$ and $TCR\gamma/\delta^+$ cells were $CD3^+$. First reports on $TCR\gamma/\delta^+$ cells referred to these as $CD4^-CD8^-$ (double negative) cells (Saito et al, 1984a; Brenner et al, 1986; Bank et al, 1986; Borst et al, 1987; Lew et al, 1986). It is now clear that a subset of $TCR\gamma/\delta^+$ cells (<10%) are also $CD8^+$ (Jitsukawa et al, 1987; Borst et al, 1988; Groh et al, 1989; Moretta et al, 1988; Kozbor et al, 1989). Furthermore, expression of the CD4 molecule on a small fraction of the $TCR\gamma/\delta^+$ cells has been inferred from flow cytometry studies of peripheral blood lymphocytes, although the frequency of such cells in PBMNCs appears to be <5% of total $TCR\gamma/\delta^+$ cells (Aparicio et al, 1989; Jitsukawa et al, 1987; Borst et al, 1988; Groh et al, 1989). In my study, the $TCR\alpha/\beta$ molecule was found on both CD4 and CD8 T cell subsets; some $TCR\gamma/\delta^+$ cells were also $CD8^+$ (16 to 41 cells/mm^3) but none were found to be $CD4^+$ (see Figure 5).

As there was an increase in the $CD8^+$ T cell population, there was also an increase in the $TCR\alpha/\beta$ subset at stages II and III of the disease. Since my main objective was to investigate TCR markers I analyzed the presence of TCR within both

major subsets: $CD3^+CD8^+$ and $CD3^+CD4^+$. The proportion and absolute count of $TCR\gamma/\delta^+CD8^+$ cells was quite stable throughout the different stages of the disease. On the contrary, the $TCR\alpha/\beta^+CD8^+$ cells were significantly ($p<0.01$) increased as early as stage II of the disease. Conclusively, the majority of the newly generated $CD8^+$ cells seem to bear the TCRα/β molecule. This observation would lead me to think that it is the newly produced T cell that bears the TCRα/β marker at its surface. In fact, at stage II of the disease, the increase in the TCRα/β subset is equivalent to the increase in T cells. At stage III of the disease, they could account for 63% of the increase of $CD8^+$ T cells. Consequently, at stage III, the remaining portion of newly synthesized $CD8^+$ T cells should bear the other TCR: TCRγ/δ. However, as the patient deteriorates and the CD4 count decreases, the $TCR\gamma/\delta^+$ cells are also depleted. This observation coincides with the findings that newly generated cells ($CD8^+$) were not of the TCRγ/δ subset at stage II, but does not explain the 37% of newly produced $CD3^+$ cells which should bear the TCRγ/δ marker. It could be that in early stages of the disease the production of $TCR\alpha/\beta^+$ cells and $TCR\gamma/\delta^+$ cells is somehow stimulated by the HIV virus or the pathogenesis of the disease, while at a later stage, the TCRα/β subset would still be "stimulated" to proliferate but not the TCRγ/δ subset, at least not the TCRγ/δ subset that was recognized by the δTCR-1 mAb. These findings do not exclude the possibility that the newly produced $CD3^+$ cells at stage III could bear the TCRγ/δ marker. The δTCR-1 mAb binds specifically to the Cδ chain of the TCRγ/δ complex. Other mAbs such as δTCS-1 for example, which is directed against a minor sub-population of $TCR\gamma/\delta^+$ cells, could show an increase in HIV infected individuals that would correspond to the 37% increase of $CD3^+$ cells. However, it remains to be proven and we can not ignore the possibility that the newly produced $CD3^+$ cells might be altered to such an extent that they would not bear any TCR, which would add to the immunodeficiency status of the HIV infected patient.

CD4 counts have been considered as very important in the clinical follow-up of the disease progression in HIV infected patients. Hence, CD4 counts are considered a reference with which any other parameters have been correlated to investigate their importance in HIV infection. Therefore, it was in my interest to study the correlation between the CD4 counts and the TCRγ/δ^+ cells. This analysis revealed that the number of TCRγ/δ^+ cells had no relationship (r=0.09) to the CD4 count in noninfected individuals or in HIV infected individuals (r=0.45) whether they received AZT treatment or not.

IV.3.1 Mean fluorescence intensity

Considering that TCRγ/δ^+ cells did not seem to increase in HIV infected patients, I decided to investigate if the remaining TCRγ/δ^+ cells in the peripheral blood showed any peculiarity when compared to TCRγ/δ^+ cells in noninfected individuals. Using flow cytometry technology, I investigated the mean fluorescence intensity (MFI) of the marker TCRγ/δ at the surface of the lymphocyte. The MFI for TCRγ/δ represents an estimation of the relative quantity of TCRγ/δ molecules found at the surface of the cell. As explained earlier, histograms were obtained from the dual-colour dot plot to analyze the mean fluorescence intensity (MFI) of the TCRα/β^+CD8$^+$ and the TCRγ/δ^+CD8$^+$. I consistently found that the MFI of CD8 marker at the surface of the TCRγ/δ^+ cells was lower than that at the surface of the TCRα/β^+ cells. While the MFI of the TCRα/β molecules on peripheral blood lymphocytes from noninfected individuals and infected individuals did not show any peculiarity, the MFI of the TCRγ/δ subset showed a specific pattern. The MFI of this molecule shows two well separated peaks in noninfected individuals (MFI = 23 and 77) representing two levels of density of the TCRγ/δ at the surface of the peripheral blood lymphocyte (Figures 9 and 10). In HIV patients, at stage III there were still two populations but much closer to each other and difficult to discern

in some cases. At stage IV of the disease, only one population remained (MFI = 21). These findings indicated that not only were the TCRγ/δ$^+$ cells not selectively generated in HIV infection, but the few TCRγ/δ$^+$ cells that were produced were altered. More investigation on this subset of cells is needed to understand the relevance of this observation. I would suggest that the cell could be impaired in such a way that the mRNA coding for the Cδ molecule is neither synthesized nor translated, or simply not allowed to be expressed at the surface of the cell. Although the functional role of the TCRγ/δ$^+$ cells is still unclear, it has been proposed that this subset would be a primary barrier for the immune system against invasion of pathogens. Therefore, the lack or the impairment of this subset of cells and the induction of a different or "altered" δ chain synthesis for the TCRγ/δ molecule could be a defence mechanism in response to the HIV invasion and might play a role in the battle against the immunodeficient status of the HIV infected patient.

IV.4 Activation markers

My second objective was to investigate the activation markers within the TCRα/β$^+$ and TCRγ/δ$^+$ cell populations. Four markers, namely, HLA-DR, CD25, CD38, and CD57 were investigated. My results demonstrated that HLA-DR$^+$ TCRα/β$^+$ cells and CD57$^+$ cells in both TCR subsets increased during the HIV-seropositive period before AIDS developed, and decreased at stage IV (Tables 11, 15 and 18). High levels of HLA-DR$^+$ TCRγ/δ$^+$ cells in the noninfected control group and high variability between individuals in all groups masked the increase of this subset in HIV infected patients. These observations support reports published by other groups on the CD8 subset (Giorgi et al, 1989; Ziegler-Heitbrock et al, 1985; Lewis, 1985; Gupta, 1986). In this study the proportion and number of HLA-DR$^+$ TCRα/β$^+$ were higher in seropositive patients than in AIDS patients. It could be possible that at least some HLA-DR$^+$

TCRα/β^+ cells represent a protective component of the specific anti-HIV immune response. I also observed an increase in the CD57 subset in both TCR sub-populations. It has been reported that this subset was equally increased in HIV-seronegative homosexuals in comparison to the heterosexual control group (Giorgi, 1987; Prince et al, 1985; Ziegler-Heitbrock et al, 1985; Lewis, 1985). There is no overall selective elevation in the CD57 subsets; both TCRα/β^+ and TCRγ/δ^+ cells seem to respond well to proliferative stimuli. This marker, being associated with *in vitro* cytotoxicity, might be an indication that the body responds to the HIV infection with all possible means, even during the later stage of the disease when the impairment of the immune system is at its worst.

Analysis of the $CD38^+$ cells showed a progressive increase throughout the course of the disease, including during the AIDS phase. In acute Epstein-Barr virus and cytomegalovirus infections, the $CD38^+CD8^+$ cell number also rises quickly during active infection and then diminishes at about the time that the herpes virus infections are cleared by the host (Landay et al, 1990). In HIV infection, the high level of $CD38^+CD8^+$ cells throughout the disease may reflect persistent immune stimulation by HIV. At least some of the $CD38^+$ cells may be anti-HIV T-cytotoxic cells.

It has been reported that selective increases in $CD8^+$ cell subsets occur in HIV infections (Landay et al, 1990). The $CD38^+$ and HLA-DR^+ $CD8^+$ cell increases occur at seroconversion. The number of HLA-DR^+CD8^+ cells increases during the HIV-seropositive period before AIDS develops, while the $CD38^+CD8^+$ cells increase at all stages of HIV disease including during AIDS. High levels of $CD38^+CD8^+$ cells are characteristic of late HIV disease and are highly prognostic for HIV disease progression (Giorgi, 1987; Landay et al, 1990).

The literature is not clear about the CD25 (IL-2R) marker. The CD25 represents the IL-2R (Tac antigen) component of the high-affinity IL-2R found on activated, but not resting, T cells (Rubin et al, 1985). Some researchers reported no

changes in the CD25 counts while others reported a decrease of the marker (Zola et al, 1991). My results showed a decrease of the CD25 counts at all stages of the disease, including during AIDS. Interestingly, this decrease is more remarkable in the TCRγ/δ subset than in the TCRα/β subset. It was reported that the level of soluble IL-2R (sIL-2R) found in circulation doubles in HIV infection (Prince et al, 1990). The mechanism for conversion of cell-associated IL-2R to sIL-2R probably involves cleavage by a cell-surface protease (Robb and Kutny, 1987). It could happen that in the stimulation process, the production of this enzyme would also be stimulated and lead to an increased dissociation of the IL-2R molecule from the surface of activated cells. Therefore, this decrease in IL-2R expression at the surface of the T cells might not represent a lack of activation of the IL-2 pathway but simply a secondary effect of the HIV infection. Furthermore, cytokines produced by activated CD4 cells prior to their destruction were reported to play an accessory role in such immune activation (Nossal, 1987).

Increases observed on the CD8 subset might reflect stimulation through various pathways. Imboden et al (1990) reported that stimulation of the CD5 molecule enhances signal transduction by the T cell antigen receptor. Therefore, it was in my interest to include this marker in the activation marker study. The lymphocyte cell surface glycoprotein CD5 is expressed on a majority of human thymocytes and peripheral T cells, as well as on a subpopulation of B cells (Reinherz et al, 1979; Wang et al, 1980; Bierer et al, 1988). A role for CD5 in T-cell activation has been suggested by functional studies using monoclonal antibodies directed against CD5. The mAbs have been shown to augment proliferation of human purified T cells stimulated with anti-CD3 mAb or with suboptimal concentrations of mitogen (Ledbetter et al, 1985; Ceuppens and Baroja, 1986). It has been shown that IL-2-induced proliferation of human thymocytes requires the presence of CD3 and CD5 positive cells (Lanier et al, 1986a; Hayward et al, 1981) Hence, it has been suggested that CD5 may provide an accessory signal for T cell immune responses. It has been reported that the $CD3^{+}CD5^{-}$ cell subset has a higher

frequency of cytotoxic cells than the $CD3^+CD5^+$ T cell subset (Srour et al, 1988; Bierer et al, 1988). On the other hand it has been shown that $TCR\gamma/\delta^+$ cells display different types of cytotoxicity (Borst et al, 1987; Moretta et al, 1987; Janeway et al, 1988). These properties of the $TCR\gamma/\delta^+$ cells coupled with the functional characteristics of $CD3^+CD5^-$ cells prompted me to investigate the status of the $TCR\gamma/\delta^+$ cells regarding the CD5 marker. Tables 19 and 20 show the distribution of CD5 in the two TCR subsets. In the $TCR\alpha/\beta$ subset the proportion of CD5 markers is very stable in both noninfected and HIV infected individuals. However, in the $TCR\gamma/\delta$ subset, the proportion of CD5 decreases before AIDS developed and during AIDS. The absolute counts of $CD5^+$ cells show a progressive depletion throughout the disease. A lower frequency and number of $CD5^+$ cells might reflect a selective production of cytotoxic cells. This phenomenon does not seem to be specific to HIV infection since it has also been reported in other immunological disorders such as Sjögren's syndrome (Dauphinée et al, 1989).

In conclusion, I found, as has been extensively published, that the CD4 sub-population of T cells was depleted in HIV infected patients and the CD8 subset was increased. However, the postulated hypothesis was shown to be false, and no increase of $TCR\gamma/\delta^+$ cells was observed like in other immunological disorders. Unlike Autran, Paxton and De Paoli's groups who reported an increase of the $TCR\gamma/\delta$ subset in HIV infection, my results showed that the $TCR\gamma/\delta$ subset was depleted in HIV infected individuals (Autran et al, 1989; Paxton et al, 1989; De Paoli et al, 1990). The method of preparation of the sample could be responsible for such a difference. These three groups did not use the whole blood lysis technique, that I used, but rather separated leukocytes on a Ficoll density gradient or buffy coat. Their techniques lead to a loss of $CD8^+$ cells that could generate a greater proportion of $TCR\gamma/\delta^+$ cells. This difference could explain the significant differences between my data and theirs. I found that the $TCR\gamma/\delta$ subset was distributed in $CD3^+CD4^-CD8^+$ and $CD3^+CD4^-CD8^-$ populations. I also found a new $CD3^+TCR\alpha/\beta^-TCR\gamma/\delta^-$ population in the HIV infected individuals. No correlation

between the $CD4^+$ T cell subset and the TCRγ/δ subset was observed even when the zidovudine treatment was taken into consideration. I found that the fluorescence intensity of the CD8 marker at the surface of the TCRγ/δ^+cells was not as bright as on the TCRα/β^+cells. Also, the fluorescence intensity of the TCRα/β marker remained unchanged throughout the disease while the TCRγ/δ marker was affected with the progression of the disease.

It was suggested in the hypothesis that if the TCRγ/δ^+ T cells becomes activated during HIV infection, they might play a role in the defense against AIDS. The detection of less $CD25^+$ T cells during HIV infection does not necessarily lead to the conclusion that the T cells are not activated during HIV infection. The concomitant increase of HLA-DR, CD57 and CD38 in both TCR sub-populations, indicate an involvment of the T cells in the defense against HIV infection or, reflect an effect of the viral infection on the immune cells. In fact, increases of CD57 and CD38 suggest an increase of cytotoxic T cell activity. Finally, the loss of CD5 molecules -observed to a greater extand in the TCRγ/δ subset- has also been associated with increased cytotoxic T cell activity (). Taking these phenotypic changes into consideration with the fact that TCRγ/δ^+ cells are recognized to be cytotoxic cells *in vitro*, it is tempting to speculate that those cells could play a role in the host defense against HIV infection by destroying HIV infected cells and delaying the viral replication.

From this study as well as others, it is clear that either the immune cells do not function in patients with HIV infection the same way they would in noninfected individuals or that the functions they perform are not sufficient to control the effects of HIV infection.

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APPENDICES

Appendix I

Classification of HIV infected patients as per WHO criteria (1989)

Group I: Acute infection

Group II: Asymptomatic infection.
No symptoms or signs of HIV disease (constitutional symptoms i.e. fever, fatigue, sweats, may be present but for no more than a total of three weeks during twelve months prior to enrollment); may be subgrouped on laboratory evaluation.

A. No laboratory abnormalities
B. Laboratory abnormalities

Group III Persistent generalized lymphadenopathy without any symptoms or signs as above. May be subgrouped on laboratory evaluation.

Group IV Other diseases:

A. Constitutional disease (if any of the following symptoms persist for more than three months: fever, night sweats, fatigue, dyspnea, diarrhea).
HIV wasting syndrome (reduction of 10% of baseline bodyweight).
ITP (persistent platelet counts < 100,000/mm^3 during or for longer than three months.

B. Neurological diseases

HIV encephalopathy (dementia)
Peripheral neuropathy
Other neurologic disorders

C. Secondary infectious diseases

1. Specified secondary infections of AIDS
Pneumocystis carinii pneumonia
Extraintestinal strongyloidiasis
Candidiasis

Appendix I

(continued)

Toxoplasmosis
Cytomegalovirus infection
Mycobacterial infection with M. avium or M. kansasii
Chronic mucocutaneous or disseminated herpes simplex virus infection
Progressive multifocal leukoencephalopathy
Chronic cryptosporidiosis
Isosporiasis
Cryptococcosis
Histoplasmosis
Coccidioidomycosis

2. Other specified secondary infectious diseases

Oral hairy leukoplasia
Recurrent Salmonella bacteremia
Multidermal herpes zoster
Nocardiosis
Tuberculosis
Oral candidiasis

D. Secondary cancers

Kaposi's sarcoma
Non-Hodgkin's lymphoma

Small-noncleaved-cell or immunoblastic primary lymphoma of brain

Appendix II

Mean channel fluorescence intensity conversion:

In order to correctly plot the mean channel number (a log value) on a linear scale, the numbers were converted into linear values using the following equation:

$$10^{\text{channel number}/256}$$